**ROLE OF THE DUOX1/LPO ANTIMICROBIAL SYSTEM AGAINST INFLUENZA VIRUS AND *STREPTOCOCCUS PNEUMONIAE***

by

AARON DAVID GINGERICH

(Under the Direction of Balázs Rada)

ABSTRACT

Viral and bacterial respiratory infections are among the most common illnesses in human and animal populations globally. Influenza virus and *Streptococcus pneumoniae* infections affect millions of people worldwide, causing serious mortality and morbidity that result in a major economic burden. The first line of defense against respiratory pathogens is the respiratory epithelium. One of the innate defense mechanisms respiratory epithelial cells use to prevent infections with pathogens such as influenza virus and *Streptococcus pneumoniae* is the extracellular oxidative antimicrobial ion hypothiocyanite ($OSCN^-$). $OSCN^-$ is produced at the airway surface by lactoperoxidase (LPO), thiocyanate anion ($SCN^-$), and hydrogen peroxide ($H_2O_2$). Our focus was to determine if $OSCN^-$ has an antimicrobial effect against influenza virus and *Streptococcus pneumoniae.*

Our results demonstrated that *in vitro* $OSCN^-$ is capable of reducing viral titers of influenza A (H1N1, H1N2, H3N2), and B (Yamagata and Victoria lineages) by 2-6 logs. To elucidate the mechanism, we performed neuraminidase (NA) activity assays that showed no reduction in viral NA activity when exposed to

hypothiocyanite. Additionally, pre/co-incubation of $OSCN^-$ with influenza prevents infection of MDCK cells and primary human tracheobronchial epithelial cells by influenza virus. The ability of influenza to attach to host receptors is decreased by up to 65% when exposed to $OSCN^-$. By utilizing a *Duox1*-deficient mouse, we determined that Duox1 deficiency plays a negative role, leading to increased mortality and morbidity in a murine influenza infection model. Additionally, we showed that both encapsulated and nonencapsulated *Spn* strains are susceptible to $OSCN^-$ *in vitro.* Taken together the DUOX1/LPO system represents a novel innate immune mechanism capable of inactivating influenza virus and killing *Spn* using $OSCN^-$.

INDEX WORDS: Influenza, DUOX1, LPO, *Streptococcus*, $OSCN^-$, $SCN^-$

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## DEDICATION

This work is dedicated to my parents for pushing me to always chase my dreams and to my partner Cristina for keeping me sane through this process.

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# CHAPTER 1

# INTRODUCTION

Airborne microbes that target the respiratory system are responsible for epidemics and periodic pandemics that affect millions of people worldwide. Two of the most import microbes are influenza virus and the bacterium *Streptococcus pneumoniae*. Influenza is a single-stranded, negative-sense, segmented RNA virus in the Orthomyxovirus family. Influenza has four genera, A, B, C and D that allow it to have unmatched zoonotic potential as it is able to transmit inter- and intra-species. Studies have shown that influenza readily modifies the host immune response and airways in order to evade the host. For humans, this modification poses an even greater threat. It has been found that the majority of deaths associated with influenza pandemics are due to secondary bacterial infections, *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Staphylococcus aureus* have been reported as the most common causes[1]. The virus creates an environment where the structure of the epithelium is compromised, full of proinflammatory chemokines and an upregulation in adherence receptors perfect for the normally asymptomatic *Streptococcus pneumoniae (Spn)* to become invasive and pathogenic[2]. *Spn* is a Gram-positive extracellular bacterium that is the leading cause of bacterial infections such as otitis media, pneumonia, septicemia and meningitis[2, 3]. Currently, both microbes have prophylactic options

in the forms of vaccines, the pneumococcal conjugate vaccines (PCV) which is effective against 13 types of *Spn,* and the quadrivalent influenza vaccine that is adjusted seasonally. Additionally, there are antibiotics and antiviral drugs currently being used to combat these microbes, but with both microbes' ability to rapidly evade and develop resistance to current treatment options, we need to identify novel mechanisms to combat *Spn* and influenza.

The best place to look for novel mechanisms is likely our first line of defense, the innate immune system. While not normally thought of in the same breath as neutrophils and macrophages, airway epithelial cells also employ their own antimicrobial defense systems[4]. Epithelial cells are able to orchestrate an antimicrobial oxidative system present in the airway surface liquid consisting of lactoperoxidase (LPO), its substrate, the anion thiocyanate ($SCN^-$) and hydrogen peroxide ($H_2O_2$), creating the antimicrobial ion hypothiocyanite ($OSCN^-$)[5]. In the human airways, $H_2O_2$ is mainly supplied by the NADPH oxidase Dual oxidase 1, DUOX1[6, 7, 8]. The LPO-based system has previously been shown to be an effective *in vitro* neutralizer of a wide variety of viruses[9, 10, 11] and bacteria[5, 12]. Interestingly, the effectiveness of this system had only been tested against one strain[11] of influenza and had not been looked at against *Spn*. We first set out to determine if this system is an effective antimicrobial against influenza virus and *Spn*. We additionally wanted to develop a better understanding of the interactions between the LPO-based system and these two widespread pathogens to determine if a novel mechanism could be discovered.

## Specific Aims

The *central hypothesis* of the proposed research is that DUOX1 supplies the $H_2O_2$ for the LPO/$SCN^-$ antimicrobial defense system producing hypothiocyanite in the host bronchial epithelium, which is capable of inactivating extracellular influenza virus and killing *Spn,* ultimately altering the course of respiratory infections.

**Specific Aim 1**. Identify the mechanism of influenza inactivation by the DUOX1/ $H_2O_2$/LPO/$SCN^-$ system.

**Specific Aim 2**. Identify and evaluate the role of Duox1 in survival and lung viral titers in a murine influenza infection model.

**Specific Aim 3.** Determine if the $H_2O_2$/LPO/$SCN^-$ system is effective at killing *Streptococcus pneumoniae*.

# CHAPTER 2

# LITERATURE REVIEW

## AIRWAYS:

Worldwide viral and bacterial respiratory tract infections are the most common illnesses affecting people today that range from acute and asymptomatic to chronic and life threatening[13]. At the most basic level the immune systems main function is to defend against foreign infectious microbes, but noninfectious material can also lead to an immune response. The respiratory epithelium is one of the body's first defenders against invading microbes. Not only does the bronchial epithelium orchestrate the innate immune defense, but it also plays a vital role in the downstream adaptive immune response. While it is one of the first tissues exposed to airway pathogens, it must also protect the host from other environmental hazards and maintain normal airway function from the upper respiratory tract (URT) of the trachea all the way down to the alveoli. To accomplish all of these feats, this dynamic tissue is composed of a variety of cell types with different roles including ciliated, goblet, clara, and basal cells[14]. These cells are connected to each other by cell-cell junctions which help form a tight physical barrier[15].

Basal cells are highly abundant in both the large and small airways where they are relatively undifferentiated and express Trp-63 and cytokeratin's 5 and 14[14]. Basal cells have also shown that they can self-renew and give rise to

secretory and ciliated epithelial cells in response to injury[16]. Clara cells contain electron-dense granules and are found predominately in the small airways [17]. Clara cells have been shown to produce bronchoalveolar surfactants and anti-proteases to aid in the regulation of bronchiolar epithelial integrity and immunity[18]. Goblet cells are defined by their electron-lucent acidic granules and constantly secrete mucous into the airways to trap pathogens and other harmful particles[14]. In an average human trachea it is estimated that there are around 6800 mucous secreting cells/mm^2 on the surface of the epithelium[19]. An interesting feature of goblet cells is that they have been shown to self-renew as well as trans differentiate into ciliated epithelial cells[20] The mucous layer found in the airways is a mixture of highly glycosylated mucin proteins that are tightly regulated in healthy individuals[14]. However, in the case of a chronic airway disease such as asthma, excessive mucous is produced and it cannot properly be cleared. Most respiratory viruses and bacteria induce mucin production which allows for trapping and clearance of these viruses[21]. Ciliated cells are a vital portion of the airway epithelium, because they are responsible for clearing mucous out of the airways that has trapped pathogens or harmful particles. Ciliated epithelial cells make up approximately 50 percent of all epithelial cells in the airways of humans. These cells usually contain 300 or more cilia per cell, and because of this, they also require a large number of mitochondria to power the cells[14]. One of the most critical features that the cells mentioned above share is their ability to undergo de-differentiation, proliferation and re-differentiation into goblet or ciliated cells after being damaged[14].

The airway epithelium represents one of the body's largest physical barriers against invading pathogens, but it also acts as an immune barrier. It accomplishes this feat via Fas and Fas-ligand interactions[22]. It has been shown that FasL expression in the airway epithelium protects against tissue injury by inducing apoptosis of infiltrating immune cells that have the Fas receptor present during infections[23]. The airways require a tight regulation of incoming immune cells in the airways; two of the main players in the innate immune response are alveolar macrophages (AMs) and neutrophils. These immune cells are usually tipped off to a foreign invader by pattern recognition receptors (PRRs) which identify pathogen-associated molecular patterns (PAMPS) resulting in the release of cytokines. Pathogens can also be detected by the toll-like receptors (TLRs), which are microbial sensing proteins. For example, to detect viruses the immune system utilizes TLR3 and TLR7/8 to recognize double-stranded RNA and ssRNA [24] [25] respectively, all of which are located in the endosome of the cell. In order to detect gram-positive bacteria such as *Spn* the immune system utilizes a different repertoire of TLRs. Lipoteichoic acid (LTA) and lipoproteins, which are components of the bacterial cell wall, are recognized by TLR2[26]. The bacterial toxin pneumolysin activates TLR4[27], and TLR9 detects bacterial DNA[28]. These TLRs are able to induce the cytokines type I and III interferons (IFNs), which are able to mediate antiviral states in surrounding cells preventing the further spread of infection[29]. Once induced, type I IFNs (IFNα and IFNβ) bind to their receptors IFNAR1 and IFNAR2. IFNAR then activates the receptor-associated protein

tyrosine kinases Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2)[30]. Once activated, JAK1 and TYK2 phosphorylate signal transducer and activator of transcription 1 (STAT1) and STAT2 molecules that are present in the cytosol[30], ultimately leading to expression of interferon-stimulated genes (ISGs). IFNα/β can also signal through STAT1 homodimers, mitogen-activated protein kinases (MAPKs) and phosphoinositide 3-kinase (PI3K)[30]. The diversity of signaling pathways helps explain type I IFNs wide variety of downstream expression of several hundred ISGs, a large number of which function to induce an antiviral state within the cell[30]. While usually thought to be a response to viral microbes, it has been shown that *Spn* also stimulates IFNα/β production leading to an increased expression of ISGs[31]. Type I IFNs were shown to protect mice against bacterial migration across epithelial and endothelial cell barriers due to increased expression of tight junction proteins, enhancing barrier function, and reducing surface expression levels of platelet activating factor receptor, a host receptor known to be commandeered by bacteria for migration across blood barriers[32]. While Type I IFNs have a protective role against microbes, they can also be harmful to the host. Type 1 IFNs are capable of inducing immunosuppressive effects leading to impaired clearance of microbes[33] and also eliciting tissue damage and inflammation that aggravate diseases[34]. Type III IFNs are most abundantly expressed in the airways[35]. Recently it was shown that type III IFNs are the front line of antiviral defense in the respiratory tract, preceding type I IFNs[36]. Type III IFNs are produced by epithelial cells, where they act locally at the epithelial surface where they inhibit viral spread, without having to utilize an

inflammatory response as type I IFNs do[36]. However it was also shown that type III IFNs not only act on epithelial cells but on neutrophils as well[36]. Neutrophils at the epithelial barrier clear infected cells and inactivate infectious virions by secreting granule contents, generating ROS or even undergoing NETosis and trapping and killing the invaders[37, 38, 39, 40]. Another important cell type found in the airways are alveolar macrophages (AMs). Once AMs are alerted, they play an important role in defense against harmful microbes and pollutants as they contain a large compartment of enzymes, which they use to destroy foreign invaders[14]. They are also able to utilize reactive oxygen species (ROS) which are able to mediate bacterial killing. With such great antimicrobial potential, these means of defense are particularly effective against invaders, but they do come at a cost to the host if not properly regulated. ROS show no specificity in what they damage, which is why this innate immune defense presents a double-edged sword. Another mechanism of the innate immune system is the use of surfactant proteins A and D bind to PAMPS on the surface of numerous pathogens[41]. These surfactants are involved in viral neutralization and clearance of microbes from the airways. Antimicrobial compounds such as defensins, which are small cationic peptides produced by epithelial cells, and leukocytes are important tools of the innate immune system[42]. Defensins can employ deadly toxic effects against microbes by inserting and causing loss of integrity of their outer membrane[42]. The innate system also contains blood proteins such as the complement system. The complement system is composed of a series of proteins that undergo a proteolytic cascade where the first protein binds to surface structures that it recognizes such as

bacterial LPS. Once the binding and the cascade occurs, it ends in the assembly of the membrane attack complex (MAC), which causes lysis of the cells it attaches to. While not a new player to the game, as it was first discovered in 1966, hypothiocyanite ($OSCN^-$) is an antimicrobial compound found in the airway surface liquid (ASL) with broad antimicrobial activity[43]. All of these cells, proteins, and compounds are not simply freely residing in the airways, but rather they are found within the airway surface liquid (ASL). This thin layer protects the epithelium from harm utilizing some of the tools mentioned above as well as many more.

***DUOX*:**

Our respiratory system is one of the most important systems for survival, it is vital for respiration and it is also one of the first systems exposed to foreign pathogens. In addition to the pressure received from pathogens, it is susceptible to various forms of reactive oxygen species (ROS). It is particularly interesting that the airways actually utilize ROS even though it comes with potentially negative consequences. The enzymes responsible for the production of ROS are known as NADPH oxidases[44]. This family is made up of five different NOX1-5 proteins and 2 dual oxidases, DUOX1 and 2. This family of enzymes are all transmembrane proteins that are characterized by a COOH-terminal NADPH oxidase catalytic core responsible for ROS production[45]. The NOX enzymes have a well-known function in producing superoxide by catalyzing the transfer of one electron from NADPH to molecular oxygen[46]. Under normal conditions, most NOX isoforms have very low or no constitutive activity but their expression can be high in disease states. In

these disease conditions, the activation of NOX isoforms generates high levels of ROS that can overwhelm the antioxidant system, leading to increased oxidative stress[5]. The Dual oxidases were originally described as thyroid oxidases because they were first found in the thyroid gland[47]. DUOX enzymes have been implicated in a vast array of biological processes including hormone synthesis, fertilization, cell differentiation, cell proliferation, cell death, extracellular matrix formation, vascular regulation, angiogenesis, and host defense mechanisms [5, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58]

The *DUOX* genes are located on chromosome 15, with *DUOX1* being approximately 36kb long with 35 exons and *DUOX2* approximately 22kb with 34 exons[46]. In humans, *DUOX1* and *2* show an 83 percent sequence homology[46]. The DUOX enzymes contain the catalytic core mentioned previously and also a $NH_2$-terminal extended by an extracellular peroxidase-like domain that is trailed by a membrane spanning segment and an intracellular domain containing two canonical EF-hand motifs[59]. Unlike the other members of the NADPH oxidase family, the DUOX ROS produced is $H_2O_2$ and not superoxide. The DUOX enzymes are the only proteins that directly generate $H_2O_2$ outside of the cell[45]. DUOX isoforms do require a maturation process to reach their active conformation at the apical surface of cells[60]. DUOX requires co-expression with its maturation factors known as DUOX1 Activator 1 and 2, DUOXA1 and DUOXA2[61]. Without DUOXA co-expression, DUOX is not functionally active[61]. The DUOX maturation factors function is to allow the properly folded DUOX enzymes to exit the endoplasmic reticulum (ER) and reach the apical surface of the cell[62]. Both DUOX1 and 2

contain EF-hand motifs, which are involved in the main regulation step, carried out by calcium[60, 63]. Rigutto et al, determined that the EF-hand motif's sequences needed to remain intact for Duox to be functional. They also discovered that serine 955 is the residue responsible for upregulating DUOX1 through PKA-mediated phosphorylation[45]. Since DUOX1 is responsible for $H_2O_2$ production in the airways, its activity or expression must be tightly regulated, as excessive production would damage host cells. It was shown that serine 1217 is responsible for limiting $H_2O_2$ production, but once serine 955 is phosphorylated, DUOX1 begins to produce $H_2O_2$[45]. DUOX2 has been shown to play a vital role in thyroid hormone biosynthesis where hydrogen peroxide is utilized to oxidize iodine to allow hormone biosynthesis[45, 46, 60]. Defects in the *DUOX2* gene lead to permanent congenital hypothyroidism in both humans and mice[59, 60, 63, 64]. DUOX1 and 2 are both present in the thyroid gland but DUOX2 is the dominant isoform[65]. It has been proposed that when thyroid hormone levels decrease thyrotropin hormone triggers a release of calcium, which activates the DUOX system[45].

There are two DUOX isoforms. Why would the body keep both of them when only one is required for normal thyroid biosynthesis? It has been proposed that in the thyroid gland, DUOX1 plays the role of an emergency switch in the case of a defect in DUOX2, but it has been shown that DUOX1 alone cannot fully handle the role of sole $H_2O_2$ producer in the thyroid[45]. Therefore, the role of DUOX1 in the thyroid gland remains controversial. In the airway epithelium, DUOX1 and 2 can be upregulated under pathophysiological conditions by IL-4, IL-13 and IFN-γ secreted by inflammatory cells in response to airway pathogens[66]. Former

smokers with COPD or patients with cystic fibrosis show significant down regulation of DUOX1 mRNA expression[67, 68, 69] which leads us to believe that a down regulation of DUOX1 mRNA has negative consequences.

The DUOX isoforms are not restricted to just the thyroid gland as originally believed; they are also expressed at different levels of the apical surface of the epithelium throughout the body and are conserved amongst higher organisms[6, 46, 66]. DUOX1 is most heavily expressed in airway epithelium, while DUOX2 is expressed in the thyroid, salivary glands, stomach, and bladder[66]. The role of DUOX1 is the focus of this project. In the airways there are a variety of possible roles that have been hypothesized, but never definitively proven, especially in a mammalian *in vivo* system. The presence of DUOX1 at the apical surface of the airway epithelium provides it the unique opportunity to be one of the first responders to foreign microbes[5]. DUOX1 has been shown to play a role in wound healing[59, 70], pathogen killing [7, 9, 71, 72], oxidative damage[73] and even a role in cancer biology[74] mainly based on *in vitro* experimental data. DUOX1 has a wide variety of proposed roles, which can have both positive and negative consequences, but we focus on the potential role of DUOX1 in microbial defense in the airway epithelium.

The airways use a combination of lactoperoxidase (LPO), thiocyanate ($SCN^-$), and $H_2O_2$ to create the antimicrobial compound $OSCN^-$[75]. LPO and $SCN^-$ are transported and secreted into the ASL of the airways, but where the $H_2O_2$ came from was a mystery until 2003. It was discovered that the apical enzyme DUOX was responsible for the $H_2O_2$ produced.[6] Several groups have demonstrated the

antimicrobial effect of the DUOX system against a variety of bacterial and viral pathogens *in vitro*. There have been numerous studies demonstrating this system's *in vitro* antimicrobial potential against several bacterial species: *E. coli*, *Streptococcus mutans, Pseudomonas fluorescens, Aeromonas hydrophila*, *Citrobacter freundi*, *Klebsiella pneumonia*, *Proteus mirablis*, *Salmonella enteritidis*, *Salmonella schottmuelleri*, *Serratia marcescens*, *Shigella dysenteriae*, *Shigella sonnei*, *Staphylococcus aureus*, *Staphylococcus citreus* and *Vibrio cholera.* A complete list of all the bacteria, viruses, fungi and even parasites killed/inactivated by the LPO-based system can be found in a recent review of our group[5].

The DUOX system is not restricted to only killing bacteria, it has antimicrobial activity against viruses such as RSV[76], HIV[10] and influenza[9, 72]. The DUOX system is not impossible to overcome, as *Pseudomonas aeruginosa* is able to inhibit the DUOX system by one of its toxins, pyocyanin[8]. This is accomplished via the redox toxin pyocyanin by imposing oxidative stress in the form of intracellular superoxide and blocking the DUOX1 expression-enhancing effects of IL-4 and IL-13[8]. While this system is effective against bacteria, its mechanism of action remains largely unknown. While viral inactivation has been shown, its mechanism of action remains also elusive. Most of the studies focusing on the antimicrobial role of the DUOX/LPO-based system utilize *in vitro* experimental systems including human primary cells. These primary cells are normal human bronchial epithelial cells and are capable of fully differentiating into polarized cells that have cilia, produce mucus and $H_2O_2$[9]. This system is advantageous as it

accurately mimics the airways *in vitro,* and the cells express DUOX, which current epithelial cell lines do not[71, 77, 78].

DUOX1 plays not only a role in infections, but it also has an important role in allergic asthma. While DUOX1 seems to be beneficial during the course of infection, it can also play a negative role in immune-mediated damage. Chronic inflammation is a hallmark of allergic asthma and has been associated with enhanced expression and activation of EGFR signaling[73]. It was shown via an *in vivo* murine knockout model that DUOX1 causes persistent oxidant dependent epithelial activation of EGFR, leading to an increase in type 2 cytokines, which ultimately leads to immune mediated damage to the host. In *Duox1*-deficient mice the enhanced EGFR phenotype was no longer seen[73]. This study clearly demonstrated the negative role of Duox1 in allergies due to enhanced activation of EGFR leading to deleterious consequences downstream. This also suggests that DUOX1 could be a potential therapeutic target for allergic asthma cases. It is also important to note that this study used only *Duox1*-deficient mice, so Duox2 was still present allowing a clear role for Duox1 to be elucidated. While this study revealed a disadvantageous role of Duox1 in an airway disease pathogenesis[73], studies are awaited to prove the beneficial role of DUOX1 in any mammalian organism.

As discussed, DUOX1 was proposed to play different roles in the body and more specifically in the airways where it could act as a double-edged sword. While DUOX2 has been heavily studied due to its clear role in thyroid hormone production, DUOX1 has not been studied that well until recently. To date, most of

the work on the antimicrobial role of DUOX1 has been performed *in vitro*, which has its limitations. The issue with most of the *in vivo* mouse models used thus far is that the complete Duox-deficient mice are missing both Duox1 and 2 and have the unavoidable, broad consequences of thyroid hormone deficiency[63, 64]. While DUOX2 mutations have been well-described in humans with a clear, hypothyroid, not infectious, phenotype[79, 80], interestingly no such mutations have been described so far for DUOX1 in humans. *Duox1*-deficient mice show no noticeable phenotype when unchallenged and thyroid function in *Duox1*-deficient mice is normal (unpublished data).

**LPO:**

Lactoperoxidase (LPO) is a member of the mammalian heme peroxidase family that are best known for their antimicrobial activity[81]. LPO is a major antimicrobial protein found in milk, saliva, tears and airway secretions[82, 83, 84, 85]. Other members of this family are the commonly known myeloperoxidase, which is found in neutrophils and macrophages, and eosinophil peroxidase found in eosinophils, both of which are known for their potent antimicrobial activity[86, 87].

LPO is a calcium- and iron-containing glycoprotein in a single polypeptide chain with a molecular weight of around 80 kDa, and is found in the secretions of the exocrine glands[81]. LPO's *in vitro* biocidal activity has been well documented against viruses[10, 88], bacteria[89] and fungi[90]. LPO is synthesized and secreted by the airway epithelium and it comprises one percent of the total protein in the airway

mucus[91]. Until recently, LPO had only been detected in human and sheep airways, but it was recently discovered that it is also present in the murine airways[5]. While LPO can consume $H_2O_2$ in the airways, it needs an additional substrate to function properly. These substrates include the halides $I^-$, $Br^-$, $F^-$ and the pseudohalide $SCN^-$. Of these 4 substrates, $SCN^-$ shows the highest affinity for LPO based on the second-order rate constants of the reaction between LPO Compound I and its (pseudo)halide substrate, followed by $I^-$, $Br^-$ and $F^-$ [81].

Due to its known biocidal activity, it was determined that LPO was vital for airway sterility[91]. Sheep were exposed to *Pasteurella hemolytica* (a natural bacterial pathogen), and LPO was inhibited leading to a 100-fold increase in lung bacterial burden compared to controls after 1 hour[91]. One major advantage of LPO as a biocidal agent in the airways is that bacteria can be catalase-positive, which protects them from the oxidizing effects of $H_2O_2$[92]. LPO utilizes $H_2O_2$ (before it encounters the bacterial catalases) to create antimicrobial compounds that are not affected by catalase.

**$SCN^-$:**

Thiocyanate ($SCN^-$) is a pseudohalide thiolate that is universally found in extracellular fluids including saliva, plasma, airway surface liquid (ASL), milk, tears, and gastric juices of mammals[93]. The concentration at which $SCN^-$ is present is variable and can range from 0.01-3 mM, but in the airways and ASL it is estimated to be around 0.4mM[93]. $SCN^-$ enters the body through dietary uptake or can also be synthesized from cyanide by sulfurtransferase enzymes[93]. Thiocyanate is

eliminated in the kidneys and has a half-life of approximately 3 days[93]. $SCN^-$ is transported into the airways after it is first incorporated from the basal side through the Na+/$I^-$ symporter into the airway epithelium[93]. It is then transported into the apical pulmonary lumens through anion transporters, Cystic fibrosis transmembrane conductance regulator (CFTR) and pendrin/SLC26A4[94]. Once transported into the ASL and in the presence of extracellular $H_2O_2$ and LPO, thiocyanate is oxidized into the antimicrobial compound hypothiocyanite[7, 93].

Due to $SCN^-$ being transported by the CFTR and its role in antimicrobial defense it was believed that cystic fibrosis patients were having trouble clearing bacteria because of the lack of $SCN^-$ being transported to their airways, leading to a decrease in the amount of downstream $OSCN^-$ and compromised microbial clearance[7]. However, it was determined that while levels of $SCN^-$ in humans varied, it was not statistically different in CF and non-CF patients meaning that there could be a backup transport mechanism for $OSCN^-$ if CFTR is deficient[95]. Additionally, it was shown that higher levels of $SCN^-$ in the airways correlated with better lung function in CF patients leading to the belief that $SCN^-$ has a beneficial role[95]. This correlation obtained in human patients presents very encouraging data for the potential antimicrobial and anti-inflammatory effects of $SCN^-$ administration in the airways.

$SCN^-$ has been of pharmacologic interest since the early-to-mid 20th century when it was used by physicians as an oral treatment for hypertension[93]. Treatment doses of $SCN^-$ as potassium salt were given in doses of 400-1000 mg a day and therapies could last from weeks to years and $SCN^-$ levels were

recommended to stay between 1400-200μM to have a therapeutic effect but avoid toxicity[96]. The results of such therapies had variable outcomes against placebo groups as some studies showed a reduction in blood pressure while others did not. $SCN^-$ toxicity did occur but it took plasma levels above 1mM[96]. Symptoms were related to the nervous system, thyroid, kidneys and the skin[97, 98, 99]. Eventually $SCN^-$ as a treatment fell out of favor for drugs that were more effective and as such, $SCN^-$ fell off the radar for nearly 50 years. $SCN^-$ was briefly looked at as a metabolite of drug exposure but those studies were inconclusive[100]. One of the major advantages of targeting $SCN^-$ as a therapeutic approach is that increased concentrations needed to achieve antimicrobial effects have yet to demonstrate host cell toxicity *in vitro*[101]. Since animal cells do not synthesize it, increased levels of $SCN^-$ come from the dietary uptake of cruciferous vegetables such as broccoli and cabbage[95]. During bacterial challenge in the respiratory tract, neutrophils produce hypochlorous acid (HOCl) which is more toxic to host cells than $OSCN^-$[95]. $SCN^-$ helps alleviate the overabundance of HOCl because it outcompetes Cl- for Myeloperoxidase (MPO) and $H_2O_2$, and it also reacts with HOCl directly which leads to the production of more $OSCN^-$[95]. Recent and ongoing research with mouse models supported the hypothesis that $SCN^-$ may be a useful intervention for resolving infection and inflammation at the same time. Nebulized $SCN^-$ was given to *pseudomonas*-infected mice 24hrs post-infection, leading to dramatic improvements in body weight, reduced bacterial titers and a decrease in the virulence marker pyocyanin[102]. $SCN^-$ presents a unique opportunity for use as a therapy because of its unique dual nature. It not only leads to a downstream

antimicrobial product that is effective against pathogens as well as sustained antioxidant features[102], but also has low toxicity against host cells unlike other currently used antimicrobial compounds which are effective and yet toxic at higher concentrations.

**Influenza Virus:**

In 1918 a virus swept across the globe killing an estimated 40 million people making it the single most fatal event in human history; the virus responsible was Influenza A/H1N1[103]. Humans are not the only species susceptible to influenza. From 2003-2005 influenza, A/H5N1 killed over 100 million birds in Asia and Africa[104]. It is believed that influenza dates back 2400 years ago to the time of Hippocrates, but the most convincing case of an outbreak was in 1580 in Russia[105].

Influenza is a single stranded, negative sense, segmented RNA virus belonging to the orthomyxovirus family[106]. Influenza virus is one of the most researched viruses due to its zoonotic properties and ability to transmit via intra- and inter-species routes. There are currently four different genera of influenza virus: A, B, C, and D[106]. These different species have different host species preferences [107]. Influenza A (IAV) virus is the most common and pathogenic subtype and resides in avian reservoirs but can also affect pigs, humans, ferrets, guinea pigs, bats, horses, dogs, and cats[107]. Influenza B has a narrower range of hosts (primarily humans), but can also affect seals and ferrets. Influenza A virus is the main cause of human seasonal cases, influenza B represents 25% of human

seasonal cases[108]. Influenza B infections are as severe as influenza A infections, more so in children who are over-proportionally affected by influenza B[109]. Influenza C virus can infect dogs, pigs, and humans. Meanwhile, influenza D affects cattle and pigs but has recently begun to appear in human hosts who are in close contact with livestock[106].

Influenza A virus is made up of eight negative-strand RNA segments, which encode 9 proteins PB1, PB2, HA, NA, NP, PA, M1, NS1-2[107]. Influenza B has two additional proteins NB and BM2[110]. Influenza A virus is further subdivided based on its surface proteins hemagglutinin (HA) and neuraminidase (NA) which have 18 HA and 11 NA variants that can be assembled to create unique viruses that behave very differently than other Influenza A viruses[111]. Influenza B is split into two distinct lineages, Victoria and Yamagata lineages[112]. The HA protein is responsible for binding to the sialic acid (SA) residues that are present on the surface of the host cells (epithelial cells)[113]. The NA protein is responsible for cleaving the sialic acid residues on the surface of the infected cells which allows the virus to detach from the infected host cell[113]. The variations and different combinations of HA and NA allow the virus to mutate through two processes known as antigenic shift and drift[114]. In influenza A virus drift occurs due to *de novo* mutations that arise during infection[115]. The molecular mechanism behind this is influenza has a low fidelity RNA polymerase that does not have a function for error proofreading and with each replication cycle *de novo* mutations can occur[115]. These mutations however are random and do not necessarily confer a positive or negative benefit, they could be neutral. Positive mutations in the genes that encode for the surface proteins HA

and NA alter antibody recognition by the host. This allows the mutated virus to spread unrecognized due to the immune system not being able to properly identify the virus. Antigenic shift occurs when two or more different subtypes have infected the same cell and create a new subtype by having a mixture of the gene segments from the different subtypes[115]. Either of these processes can cause significant effects on viral pathogenicity and transmission allowing the virus to become much more deadly and harder to contain. Due to these rapid mutations, influenza A virus has become one of the most prevalent respiratory viruses in the world.

Influenza infection occurs when aerosolized particles containing influenza virions enter the host's respiratory tract via the nasal cavity or bronchial epithelium, or via direct inoculation into other susceptible areas including the eyes, nose, or mouth where the virus can then be inhaled or directly inoculated onto an epithelial surface where it can replicate[116]. Once inside the host, influenza binds to sialic acid (SA) residues, which are present on epithelial cells. They utilize their surface protein HA to bind to these residues. The species origin of the virus determines which residue it specifically binds to, SAα2,6 is the target for human virus and SAα2,3 is the preferred for avian viruses[117]. Interestingly, it is possible for human cells to have both forms of the residues, which allows the avian viruses to infect human hosts, as well[117]. Once attached to a host cell, the virus utilizes one of 4 possible mechanisms: clatherin-coated pits, non clatherin-coated pits, caveolae, or macropinocytosis to be endocytosed into the host cell[118]. Once the virus is in the endosome, the low pH facilitates viral fusion with the endosomal membrane[113]. The HA in the viral membrane and the fusion peptide in the endosomal membrane

open a pore which then releases the viral RNPs into the cytoplasm of the cell[119]. The viral RNPs are then transported to the nucleus where they hijack the host machinery to produce viral mRNA (vRNA)[113]. They also create a positive sense copy of the vRNA which serves as the template to produce more vRNA[113]. Once the new RNPs are created, they are exported into the cytoplasm by the M1 and nuclear export protein NEP/NS2[120]. Once in the cytoplasm new virions converge and assemble at the apical plasma membrane of polarized cells where they bud off into the extracellular space resulting in the death of the host cell[121]. This is not a passive process because both HA and NA bind to the SA containing residues that are present on the surface of the cell. In order to break free, the NA protein destroys sialic acid receptors. If the NA is overactive, it can inhibit viral propagation leading to sialic acid receptors being eliminated from uninfected cells thereby decreasing the infective potential of the virus[113].

In order to properly respond to an influenza infection, the immune system must orchestrate an innate and adaptive response to the virus. The first line of defense against airborne pathogens are the epithelial cells, which form a physical barrier. One of the first mechanisms used by the host is the mucosal lining that can prevent binding and aid in clearance through ciliary action. Once in the ASL the innate immune system employs antimicrobial peptides as a frontline defense. Collectins have been shown to inhibit HA activity[122], while defensins have been shown to directly decrease infection *in vitro*[123]. Professional phagocytes also play a role in internalizing the virus and presenting it to naïve T cells leading to an IFN response[124]. Importantly, influenza is able to suppress type I IFN via NS1[124]. NS1

accomplishes this by regulating RIG-I activity through blocking RIG-Is interaction with dsRNA preventing IFN expression[125]. During infection, neutrophils are among the first innate cells recruited at high levels into the airways and are critical to mounting an effective response to the virus[126]. Secreted chemotactic factors CXCL-1 and CXCL-2 act as chemotactic signals for neutrophil recruitment[127]. Being the first leukocyte to arrive at the site of infection, they utilize an assortment of antimicrobial functions; phagocytosis, release of granular contents and production of cytokines[37, 126]. One of the antimicrobial peptides that neutrophils utilize is cathelicidin LL-37, which has demonstrated antiviral activity *in vivo* by interfering with viral replication[128]. An alternative function of neutrophil-mediated killing is a specific type of programed cell death called NETosis[37]. When NETosis occurs, nuclei of the neutrophil lose their shape, chromatin decondense and a mixture of the nucleus and granules is created[129]. Neutrophils then release these NETs composed of chromatin and granule proteins, that are able to bind, entrap and kill pathogens[37]. These NETs aid in viral immobilization, inactivation and an increase in type I IFNs being released[130]. Influenza A virus has been shown to induce NET formation but the exact mechanism remains unknown[131]. Interestingly influenza A virus-induced NETs do not protect against secondary bacterial infections, leading to the conclusion that influenza induced NETs are functionally different from those generated by other stimuli[132]. Due to their intimate interactions with influenza, neutrophils need to be protected from becoming targets themselves when responding to influenza infection as they exhibit sialic acid receptors that influenza preferentially seeks[38, 133]. Type III IFNs induce the expression of several

ISGs in neutrophils that inhibit viral entry (IFITM)[134], degrade viral RNA (OAS and IFIT)[135] and inhibit virus translation and replication (ISG,OAS,GBP and IFI)[36, 134, 136] leading to a synergistic effect. However, this increase in neutrophils and its byproducts has been shown to lead to neutrophil-mediated lung damage[128, 137, 138].

Due to influenza's ability to subvert the innate immune response, the adaptive immune system is of particular importance. Neutralizing antibodies against the surface proteins HA and NA help prevent attachment or even release of the virions limiting the severity of infection[139]. Due to influenza's proclivity to mutate, neutralizing antibodies against a certain subtype can be ineffective against a newer circulating strain of the virus. The cellular immune response is vital for proper viral clearance due to the production of IFNs[126]. It has been shown in murine models that CD4+ and CD8+ T cell-depleted mice display impaired viral clearance[140]. It is important to note that during the course of influenza infections humans are more susceptible to secondary bacterial infections that can lead to so-called super infections (viral co-infections and/or secondary bacterial infections) and ultimately death due to influenza virus modulating the host immune system[1].

Current prevention and treatment strategies include vaccination and antiviral drugs, both of which have drawbacks[115]. Currently vaccines can increase antibody formation and improve disease outcomes, but the high rate of mutations cause the failure rate of these vaccines to be high[115]. With constant mutations, the virus composition of the vaccines has to be constantly changed to encompass all of the new variants, which is not logistically feasible in a short time frame due to the time required to produce the vaccine in either eggs or cell lines[141]. By the time

current strains are incorporated into new vaccines, those strains may have been replaced in circulation by new variants that the vaccine does not offer protection against. Currently, there are two major classes of antivirals available for prevention and treatment of influenza, M2 inhibitors and neuraminidase inhibitors[142]. Antiviral drugs also face the issue of drug resistance, which is already occurring with M2 inhibitors as they only work against IAV and resistance has emerged in H3N2 and H1N1pdm09[142]. More importantly antiviral drugs are not prescribed until after infection occurs making it ineffective as a preventative measure. Antiviral drugs are most effective if given within 24 hours of initial infection which often is not achievable due to the lag time between the initial infection and when the patient begins to exhibit clinical symptoms[142]. One novel possibility for inactivating influenza could be offered by the use of $OSCN^-$ as an antiviral agent given its activity against H1N1 *in vitro*[101].

***Streptococcus pneumoniae*:**

*Streptococcus pneumoniae* (*Spn*), or pneumococcus, was discovered independently by Pasteur and Sternberg in 1881 and soon after it was recognized as a major pathogen[143]. *Spn* is a Gram-positive, extracellular, opportunistic bacterial pathogen that colonizes the upper respiratory tract[2]. It is the leading cause of bacterial infections such as otitis media, pneumonia, septicemia and meningitis. [2, 3]. Up to 27–65% of children and <10% of adults are carriers of *Spn,* carriage involves a commensal relationship between the bacterium and the host[144], but spread to the bloodstream can result in invasive inflammatory disease[2]. *Spn*

has been designated as a priority pathogen due to the continued high burden of disease and increasing resistance to antibiotics[2]. While the use of the pneumococcal conjugate vaccines has been effective at reducing disease of certain serotypes, *Spn's* ability to remodel its genome via natural competence has led to evasion of vaccine-induced immunity[2, 145]. One of the critical components to *Spn* survival during infection is the polysaccharide capsule[2]. The capsular polysaccharide (CPs) is the immunodominant structure and the main virulence determinant of *Spn* of which 98 distinct CPs have been discovered[2]. It is important to note that there is a group of nonencapsulated *Streptococcus pneumoniae* (NESp) strains that are able to colonize the nasopharyngeal and are not affected by current vaccines[146]. These strains have unique surface proteins that allow for colonization and virulence while void of a capsule even though it was previously thought a capsule was required for successful infection[146].

Upon entering the nasopharynx, the first barrier *Spn* encounters is mucus entrapment. First, the negatively charged CPS inhibits bacterial mucus entrapment[147]. *Spn* also utilizes the enzymes Neuraminidase A (NanA), β-galactosidase (BgaA) to degrade mucus inhibiting mucocillary clearance[148, 149]. Several pneumococcal proteins, including pneumococcal surface protein A (PspA) or choline-binding protein A (CbpA), directly block Complement component 3 (C3) deposition on the *Spn* surface[150, 151]. PspA also binds to lactoferrin to acquire nutrient-rich iron for the bacteria[151]. CbpA is able to induce its own endocytosis by interacting with factor H to facilitate adherence and subsequent internalization of *Spn* via cell glycosaminoglycans[152]. Additionally, *Spn* is able to use its toxin

pneumolysin (Ply) and $H_2O_2$ to damage the epithelium breaking down the epithelial barrier and providing a path for paracellular invasion[153]. CbpA also enables *Spn* to navigate across the endothelium and into the blood stream where the bacteria can then spread systemically. Biologically speaking it is advantageous for *Spn* to survive unnoticed in a commensal state in the nasopharynx where it can then spread to new hosts and continue to propagate. Because of this, *Spn* has developed a wide repertoire to evade and subvert the host innate immune system[2]. *Spn* being an extracellular bacterium is at risk of neutrophil-mediated killing and must evade neutrophils that arrive due to tissue invasion. Neutrophils use several different mechanisms to eliminate extracellular bacteria but *Spn* has evolved several evasion mechanisms as well. Neutrophils are known to release extracellular traps, which contain antimicrobial proteins bound to DNA scaffolds[37]. However, *Spn* utilizes a surface endonuclease (EndA) that allows *Spn* to degrade the DNA scaffold and escape the NETs[154]. Another mechanism *Spn* uses to avoid neutrophils is to evade the neutrophil recruitment that is achieved by CpbE, which cleaves ChoP moieties on platelet-activating factor, a potent activator of neutrophils[155]. If neutrophils are unable to phagocytose *Spn,* they can kill the bacterium by releasing serine proteases from neutrophil granules[156].

*Spn* has a preferred area of colonization and it is the nasopharynx. It also means that *Spn* encounters a diverse microbial community that it must cooperate with or compete against. *Spn* strains interact directly with other bacteria when they co-colonize a single host, these interactions have become exceptionally clinically relevant as *Spn* can dramatically alter the course of infection[157]. Co-colonizing

pneumococci compete with one another through a diverse array of pneumocins and related peptides that have antimicrobial activities[158]. Lysis of susceptible strains not only allows for predation but also provides a source of DNA for genetic exchange through transformation and homologous recombination[157]. *Spn* also interacts with other species of bacteria, and produces $H_2O_2$ at levels exceeding 1mM as an antimicrobial factor to reduce competition by other upper respiratory pathogens, such as *Haemophilus influenzae*, *Neisseria meningitides*, *Moraxella catarrhalis*, and *S. aureus*[159, 160, 161]. It is important to note that *Spn,* a catalase-deficient bacterium, is able to survive endogenous $H_2O_2$ that is sufficient to kill other bacterial species[159]. It was discovered that pyruvate oxidase (SpxB) is the enzyme responsible for $H_2O_2$ production with a dual role that it is also required for *Spn* survival when exposed to high levels of $H_2O_2$[161]. It is hypothesized that SpxB contributes to an $H_2O_2$-resistant energy source that maintains viability during oxidative stress, which is required to resist the toxic $H_2O_2$ of its own activity[161]. *Spn* does not only interact with bacteria, it has a synergistic effect against the immune system when coupled with a viral infection such as influenza. During an influenza infection the virus modulates the expression of proinflammatory chemokines, upregulates receptors used by *Spn* for adherence, compromises the structure of the epithelium and provides more nutrient-rich milieu[2]. All of which are exceedingly beneficial to the bacteria allowing for enhanced susceptibility and increased density of *Spn* colonization[162, 163]. *Spn's* ability to adapt and overcome selective pressures from current therapies, including several classes of antibiotics, and adapting to immunizations demonstrates its clinical relevance. It is important to

gain further insight into *Spn's* interactions with the nasopharynx and airway epithelium as a whole in order to ascertain a solution to combat this ever-evolving pathogen.

# CHAPTER 3

# HYPOTHIOCYANITE PRODUCED BY HUMAN AND RAT EPITHELIAL CELLS INACTIVATES EXTRACELLULAR H1N2 INFLUENZA A VIRUS [9]

[9]Gingerich A, Pang L, Hanson J, Dlugolenski D, Streich R, Lafontaine ER, et al. Inflammation research. 2016;65(1):71-80. Reprinted here with permission of the publisher

**Abstract**

Objective and design. Our aim was to study if an extracellular, oxidative antimicrobial mechanism inherent to tracheal epithelial cells is capable of inactivating influenza virus.

Material or subjects. Epithelial cells were isolated from tracheas of male Sprague-Dawley rats. Both human and rat tracheobronchial epithelial cells were differentiated in air-liquid interface cultures.

Treatment. A/swine/Illinois/02860/09 (swH1N2) influenza A virions were added to the apical side of the airway cells for 1 hour in the presence or absence of lactoperoxidase or thiocyanate.

Methods. Characterization of rat epithelial cells (morphology, DUOX expression) occurred via western blotting, PCR, hydrogen peroxide production measurement and histology. The number of viable virions was determined by plaque assays. Statistical difference of the results was analyzed by ANOVA and Tukey's test.

Results. Our data show that rat tracheobronchial epithelial cells develop a differentiated, polarized monolayer with high trans-epithelial electrical resistance, mucin production and expression of dual oxidases. Influenza A virions are significantly inactivated by human and rat epithelial cells via a dual oxidase-, lactoperoxidase- and thiocyanate-dependent mechanism.

Conclusions. Differentiated air-liquid interface cultures of rat tracheal epithelial cells provide a novel model to study Duox-influenza interactions. The dual oxidase/lactoperoxidase/thiocyanate extracellular oxidative system producing

hypothiocyanite is a fast and potent anti-influenza mechanism inactivating H1N2 viruses prior to infection.

## Introduction

Tracheobronchial epithelial cells (TBEC) in the airways provide the first line of defense against inhaled pathogenic infectious agents[164]. The respiratory epithelium alerts the innate immune system to initiate inflammation[164]. TBECs themselves are capable of fighting pathogens by producing reactive oxygen species, mucins and antimicrobial peptides[164, 165, 166, 167]. TBECs orchestrate an oxidative extracellular antiviral system present in the airway surface liquid consisting of the protein lactoperoxidase (LPO), the thiocyanate anion ($SCN^-$) and hydrogen peroxide ($H_2O_2$) [6, 84, 168]. LPO and $SCN^-$ are both present in large quantities in the airway surface liquid[84, 168]. LPO oxidizes $SCN^-$ using $H_2O_2$ into hypothiocyanite ($OSCN^-$), which has demonstrated antimicrobial effects[43, 169, 170, 171]. This antimicrobial system was originally described in milk and saliva[43, 169, 171], and only recently in the airways[6, 84].

Cellular $H_2O_2$ is derived from two NADPH oxidases highly expressed in TBECs: dual oxidase 1 and 2 (Duox1, Duox2) [6, 47, 172]. Duox1 and Duox2 are the most likely candidates to provide $H_2O_2$ for the $H_2O_2$/LPO/$SCN^-$ antimicrobial system since these enzymes are the dominant NADPH oxidases expressed in TBECs [6, 8, 77], and are ideally localized to the apical membrane to produce extracellular $H_2O_2$ into the airway surface liquid [6, 62]. The $H_2O_2$/LPO/$SCN^-$ antimicrobial system is effective against several microbes including viruses[10, 173]. Its virucidal effects have been described for HIV and RSV but not for other viruses including influenza[10, 173].

Influenza A virus (IAV) causes yearly epidemics with high morbidity and deaths in humans[174, 175]. IAVs have several subtypes that are classified based on their

hemagglutinin (HA) and neuraminidase (NA) surface proteins [176]. The IAV subtypes most commonly infecting humans are H1N1, H1N2, and H3N2 [176]. A recent study showed that enzyme-free $OSCN^-$ has antiviral activity against a pandemic influenza strain (A/H1N1/2009)[11]. Different IAV subtypes (H1N1, H3N2) were shown to elicit discrete responses (Duox up-regulation) in TBECs [72]. These data suggest that the DUOX/$H_2O_2$/LPO/$SCN^-$ system in TBECs is a potent anti-influenza mechanism of the respiratory innate immune system[11, 72]. The potential role of the $H_2O_2$/LPO/$SCN^-$ antimicrobial system in inactivation of the IAV subtype H1N2 has yet to be determined. H1N2 viruses represent a serious public health problem in humans and pigs[177]. The H1N2 IAV subtype resulted from reassortment between H1N1 and H3N2 subtypes [178] and are endemic in the United States [179].

The purpose of this study was to determine if TBECs are capable of inactivating extracellular IAV virions of the H1N2 subtype by the DUOX/$H_2O_2$/LPO/$SCN^-$ system. Air-liquid interface (ALI) cultures of polarized, differentiated TBECs provide the best *in vitro* model of the respiratory epithelium. These cultures develop transepithelial electric resistance (TEER); contain ciliated, goblet and basal cells; produce mucins, and release cytokines upon microbial stimulus [8, 77, 78, 180, 181, 182, 183]. Other labs and we have also shown that TBECs express Duox at their later, differentiated stage in ALI cultures[8, 72, 77, 184]. Human TBECs are difficult to obtain, costly and exhibit large donor-to-donor variation. For this reason, we established a complementary model: ALI cultures of primary rat TBECs. These cells are easier to isolate, cost-effective, less prone to variations and are suitable

for larger scale studies; therefore rat TBECs provide an excellent alternative to human TBECs *in vitro*[185].

In this article, we provide detailed characterization of ALI cultures of primary rat TBECs. Rat cells express both, Duox1 and Duox2, and produce extracellular $H_2O_2$ in a calcium-dependent manner. We also show that rat TBECs inactivate H1N2 IAV to a remarkable extent by the $H_2O_2$/LPO/$SCN^-$ system. These data were further confirmed using ALI cultures of normal human bronchial epithelium (NHBE). In summary, our data establishes rat TBECs as a model to study influenza-Duox interactions and show for the first time that H1N2 subtype of IAV is efficiently inactivated by the Duox/$H_2O_2$/LPO/$SCN^-$ antiviral system.

## Materials and Methods

### Animals.

Male Sprague-Dawley rats were purchased from Harlan Laboratories (South Easton, MA). The animals were between 15-20 weeks old at the time of euthanasia via $CO_2$. All animal-related procedures were approved by the Institutional Animal Care and Use Committee of the University of Georgia (Rada, IACUC protocol numbers: A2012 11-004-Y2-A1, A2015 03-030-Y1-A0.)

### Culture of primary human and rat airway epithelial cells.

Primary normal human bronchial epithelial cells (NHBE) were purchased from Lonza (Walkersville, MD) and cultured as previously described[8]. Briefly, cells were seeded onto 24-well polyester (0.4 micron pore) membrane transwells

(Costar), precoated with 0.3% rat tail collagen I (Sigma), at a density of 2.0 × $10^4$ cells/ well. B-ALI™ growth medium was used in the apical and basal chambers until the cells reached confluence. The upper chamber medium was aspirated and the lower medium was replaced with B-ALI™ Differentiation medium (Lonza, Walkersville, MD). Cells were maintained on the air-liquid interface (ALI) for 4-5 weeks by feeding every other day with ALI differentiation medium (the surface of ALI cultures was washed with sterile HBSS every other day). Antibiotics (penicillin and streptomycin, Life Technologies, Grand Island, NY) were supplemented in the media up to four days before experiments.

Rat tracheas were removed under sterile conditions from male Sprague-Dawley rats. The tracheas were incubated overnight at 4°C in Dulbecco's modified Eagle's medium (DMEM; Sigma) and Ham's nutrient F-12 medium (F-12; Sigma) (1:1) with 5% protease (Sigma). Fetal bovine serum 10% (FBS; Hyclone) was then added to the incubation medium (DMEM/F-12) and TBECs were flushed out. The cells were collected by centrifugation (450g, 4°C, 10 min) and were washed twice with DMEM/F-12 containing 10% FBS. Growth medium for RTE cells consisted of DMEM/F-12 supplemented with 1% L-Glutamine, 1% Pen/Strep, 10 µg/mL insulin, 0.1 µg/mL hydrocortisone, 0.1 µg/mL cholera toxin, 5 µg/mL transferrin, 5ug/mL Transferrin, 25 ng/mL epidermal growth factor (all reagents from Sigma), 1% bovine pituitary extract (Life technologies), 3 mg/mL bovine serum albumin, 50 nM retinoic acid. Polyester permeable membranes on culture inserts (6.5-mm-diameter, 0.4-µm-pore-size; Costar) were precoated with 100 µL 0.3% rat tail collagen I (Sigma). Rat TBECs were plated onto the apical surface of the inserts

with 0.2 mL of growth medium in the upper (apical) compartments of the culture plates ($6.0 \times 10^4$ cells/membrane). Cultures were grown in 95% air and 5% $CO_2$ at 37°C. After 72 hours, media in the apical chamber was changed. The medium was changed every other day using 0.5 and 0.2 mL growth medium in the basal and apical compartments, respectively. After 7 days the cells were confluent (determined visually and by measuring trans epithelial electrical resistance (TEER)). The medium was removed from the apical chamber taking the cells to ALI. The basal chamber was changed every other day. Cells were cultured between 3-4 weeks on ALI when used for experiments. 4 days prior to experiments cells were given antibiotic-free medium. To characterize their morphology, rat TBEC cultures were fixed with 4% paraformaldehyde (GE Healthcare Life Sciences, Pittsburgh, PA) and subjected to H&E and Mayer's mucicarmine staining (detecting mucins) (UGA, Histology Laboratory, Athens, GA) (Figure 1.).

**Influenza A virus.**

Madin-Darby canine kidney (MDCK) cells (ATCC CCL-34) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing high glucose (HyClone) supplemented with 5% heat-inactivated fetal bovine serum (FBS) and maintained at 37°C with 5% $CO_2$. A/swine/Illinois/02860/09 (swH1N2) IAV viral stocks were cultured in MDCK cells using infection medium (DMEM containing high glucose supplemented with 1 mM L-glutamine with 1-μg/ml tosylsulfonyl phenylalanyl chloromethyl ketone [TPCK]-treated trypsin).

**Western blotting**

NHBE cells were lysed by Nonidet P-40 lysis buffer (Boston Bioproducts, Ashland, MA) containing 150 μM PMSF (Fluka Biochemika) and protease inhibitor cocktail (Sigma-Aldrich) [8]. Protein levels were determined using the bicinchoninic acid assay (Pierce, Grand Island, NY). Lysates were electrophoresed on SDS-polyacrylamide gels (8%; Tris-glycine gel, Invitrogen). Gels were blotted on nitrocellulose membrane (Invitrogen) using the iBlot dry blotting system (Life Technologies, Carlsbad, CA). Blots were blocked overnight in TTBS (TBS buffer containing 5% milk powder and 0.05% Tween 20). Blots were incubated with primary anti-Duox antibody (rabbit, polyclonal; 1/2000) [186] followed by incubation with secondary HRP-linked anti-rabbit IgG antibody GE Healthcare; 1/1000). Blots were developed by chemiluminescence using the Lumigen DS detection kit (GE Healthcare).

**RNA isolation and RT-PCR**

RNA was isolated from rat TBECs by RNAzol (Sigma-Aldrich, St. Louis, MO)/chloroform extraction followed by isopropanol precipitation as described [187]. RNA Concentrations and purities were determined using a Nanodrop spectrophotometer. cDNA synthesis was carried out with the Thermoscript cDNA synthesis kit (Life Technologies) using 1 μg total RNA, oligo dT primers and RNaseH treatment. To detect *duox1*, *duox2* and *actin* gene expressions, the following gene-specific primers were used in the PCR reaction (PCR Thermocycler, Eppendorf): rat *duox1* (F: CTGGAGCTCT CCGGGTTT, R:

GGCACTGAGG AGGCTGACTA, product: 767 bp); rat *duox2* (F: GGTGGAGATC AGTGTGGTGA, R: GCTAGGAAGC CCCTCTGC, product: 665 bp); rat *actin* (F: GGAAATGCAC TCCCTTGTGT, R: TGTTAGCTTT GGGGTTCAGG, product: 453 bp). PCR program: 94 C (0.5 min), 62 C (0.5 min), 72 C (1.5 min), 37 cycles.

**Measurement of $H_2O_2$ production.**

$H_2O_2$ production was measured through horseradish peroxidase (HRP)-mediated oxidation of homovanillic acid (HVA) as previously described [77]. Flourescence was measured using Varioskan Flash fluorescence microplate reader (ThermoScientific, excitation wavelength 320nm, emission wavelength 405 nm) for one hour taking readings every minute. $H_2O_2$ production was then calculated and expressed as nmol $H_2O_2$/hr/$10^6$ cells.

**Viral inactivation assay**.

After human or rat TBECs had been on ALI for the respective time, 4 days prior to use cells were switched to antibiotic-free medium and the apical chamber was washed with HBSS (Mediatech, Manassas, VA)once. Each component of the system was tested at the following concentrations 100 μM ATP, 6.5 μg/ml LPO, 400 μM $SCN^-$ (in HBSS as assay medium). The reaction volume was set to 40 μL with the appropriate concentration of each component. Virus was diluted to an MOI of 0.1 (NHBE cells ~5000 viral particles, rat TBECs ~8000 viral particles). Catalase (15,000 U/mL, Sigma-Aldrich, St. Louis, MO) was also used in one of the reactions to inhibit the system. Once the components were assembled, the 40 μL was

pipetted to the apical chamber of the transwell and placed in a 37 °C 5% $CO_2$ incubator for one hour. After one hour, supernatants from each respective well were collected and stored at -80°C. Plaque assays were performed to determine viral concentrations as previously described[177].

**Statistics.**

Data for the viral inactivation assay were log-transformed, significance was calculated using a one-way ANOVA, and a Tukey post-hoc test performed using Minitab17. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

**Results**

**Characterization of ALI cultures of differentiated rat TBECs.**

Recently published results suggest that the Duox/$H_2O_2$/LPO/$SCN^-$ system responds to H1N1 and H3N2 subtypes of IAV[11, 72]. Whether the third IAV subtype commonly infecting humans, H1N2, can be inactivated by TBECs is unknown. To study this, we used ALI cultures of primary, differentiated rat TBECs as an *in vitro* model to study epithelial-influenza interactions. Rat TBECs were cultured on ALI (Fig. 3.1 scheme) in 24-well transwells for 3 weeks and subjected to different assays to characterize them. Hematoxylin and eosin staining revealed that TBECs formed the characteristic monolayer containing polarized epithelial cells with apical cilia (Fig. 3.1A). Basal cells near the transwell membrane support can be visualized (Fig. 3.1A). Mayer's mucicarmine staining detecting mucins as large glycoproteins

identified positively stained (purple), mucin-producing Goblet cells (Fig. 3.1B). Unstained polarized cells are ciliated epithelial cells (Fig. 3.1B). Differentiated TBECs develop high transepithelial electric resistance (TEER) suggesting formation of tight junctions[188]. Figure 3.1C shows the time-dependent development of high TEER values in rat TBECs. These data confirm that rat TBECs form polarized, mucus-producing, ciliated monolayers in our hands providing an excellent *in vitro* model of the respiratory epithelium.

**Rat TBECs express functional Duox1 and Duox2.**

In humans, dual oxidases are expressed in TBECs *in vivo* [6] or *in vitro* in ALI cultures[8, 77, 184, 189]. Duox proteins were also detected in *in vitro* cultures of rat and cow TBECs although detailed description of their culture conditions were not provided[184]. Here we provide detailed characterization of Duox expression and function in rat TBECs. As shown in Figure 3.2A, polarized rat TBECs express Duox protein (~180 kDa molecular weight) to similar extent in all three animals studied. Although the antibody favors detection of Duox1, it is not isoform-specific[190]. Therefore, we detected gene expression levels of each isoform, Duox1 and Duox2, by reverse transcriptase PCR. Figure 3.2B shows that both isoforms are expressed in rat TBECs. Reverse transcriptase was omitted in control samples to show specificity for amplification of DUOX1 and DUOX2 and lack of contaminating host DNA. (Fig. 3.2B). To show that rat TBECs contain functional Duox enzymes, we measured extracellular $H_2O_2$ release by horse radish peroxidase (HRP)-mediated oxidation of homovanillic acid (HVA) – as previously described[62, 77]. Rat TBECs

spontaneously produced extracellular $H_2O_2$ (referred to as basal level) that could be still enhanced by known activators of Duox: ATP and ionomycin (Fig. 3.2C) [8, 78, 191]. In the current study, we used ATP to stimulate Duox activity (see later Figs. 3.4. and 3.5.). Duox requires an increase in cytosolic calcium to be activated[189, 192]. Scavenging extracellular calcium by EGTA largely reduces basal $H_2O_2$ output of rat TBECs suggesting that Duox enzymes are the main $H_2O_2$ producers in rat TBECs, and that extracellular calcium is required for their activation (Fig. 3.2D). In summary, rat TBECs express both Duox isoforms that are the suggested source of $H_2O_2$ production.

**Rat TBECs inactivate H1N2 influenza A virus in a $H_2O_2$/LPO/$SCN^-$-dependent manner.**

Although enzyme-free $OSCN^-$ has virucidal effect against the pandemic A/H1N1/2009 influenza virus [11], and NHBE cells upregulate Duox in response to H1N1 and H3N2 IAV strains [72], there are no data to show direct IAV inactivation by the Duox/$H_2O_2$/LPO/$SCN^-$ system and response of TBECs to the H1N2 subtype. To address this knowledge gap, we exposed TBEC cultures to the H1N2 IAV strain A/Swine/Illinois/02860/09 (swH1N2) at a multiplicity of infection (MOI) of 0.1 for 1.5 hour (see explanatory scheme in Figure 3.3). SwH1N2 is an endemic swine strain originally isolated in the state of Illinois in 2009[177, 193]. Supernatants were collected from infected TBEC cultures to prepare 10-fold serial dilutions (Fig. 3.3). Diluted viral suspensions were added to Madin-Darby Canine Kidney Epithelial Cells (MDCK) (Fig. 3.3). Plaques were counted after three days of incubation, and

changes in viable virion concentrations were calculated (Fig. 3.3). Although Duox enzymes show high spontaneous activity in rat TBECs, ATP was added to enhance their $H_2O_2$ output (Fig. 3.2). LPO and $SCN^-$ were added as indicated at levels found in human airways ($SCN^-$: 400 µM, LPO 6.5 µg/ml) (Fig. 3.4) [184, 189]. Figure 3.4 shows that a significant 2-3 log reduction in the number of viable H1N2 IAV virions occurred when the complete $H_2O_2$/LPO/$SCN^-$ system was reconstituted on top of rat TBEC cultures. Omitting LPO, $SCN^-$ or both resulted in complete loss of virion inactivation (Fig. 3.4), indicating that the full system needs to be present and $OSCN^-$ is responsible for viral inactivation.

**H1N2 IAV inactivation by the $H_2O_2$/LPO/$SCN^-$ system of NHBE.**

To determine if primary cultures of human bronchial epithelial cells behave similarly to rat TBECs, we exposed human NHBE cells to H1N2 IAV under the same conditions as the rat cells (Fig. 3.4) and followed influenza virion inactivation as described. Similarly, to the results shown in Figure 3.4., NHBE caused H1N2 inactivation when the full $H_2O_2$/LPO/$SCN^-$ system was assembled. If LPO, $SCN^-$ or both were left out, influenza inactivation was entirely inhibited (Fig. 3.5). This highlights again the crucial antiviral role of the final product, $OSCN^-$ against influenza, and proves that NHBE cells can produce sufficient $H_2O_2$ by Duox to fuel this antiviral system. In addition, we also show that the $H_2O_2$ scavenger catalase entirely blocks the virion-inactivating effect of the $H_2O_2$/LPO/$SCN^-$ system (Fig. 3.5, see “CAT”). This further suggests that Duox-derived $H_2O_2$ is required and sufficient for influenza inactivation. Overall, the Duox/$H_2O_2$/LPO/$SCN^-$ system of both rat and

human cultures of primary airway epithelial cells have strong antiviral activity against the IAV H1N2 strain. This further confirms that rat TBECs provide an excellent model to study IAV-Duox interactions in the respiratory epithelium.

## Discussion

TBECs are the earliest responders to influenza challenge in the airways[164, 194]. Their reaction to the first invading virions is essential in determining the later fate of the inflammatory response. One of the inflammatory mechanisms by which TBECs fight pathogens is the production of reactive oxygen species[6, 72, 164]. TBECs release hydrogen peroxide into the airway surface liquid that is used by LPO to oxidize its most abundant substrate, $SCN^-$ to produce antimicrobial $OSCN^-$ [84, 85, 168, 195]. LPO is produced primarily in serous acini of submucosal glands in the airways, not in the epithelium[6, 91]. Its main substrate, $SCN^-$ is present extracellularly in submillimolar concentrations in human airways[85]. $SCN^-$ is transported from the blood trough the epithelium via several suggested transport proteins[95, 196, 197]. Both, LPO and $SCN^-$ are abundantly present in airway secretion and $H_2O_2$ production represents the rate-limiting factor in the activity of the system. In intact airways, $H_2O_2$ is primarily provided by two NADPH oxidases, Duox1 and Duox2[6]. Both Duox enzymes and their maturation factors (Duox activators) localize to the apical plasma membrane of bronchial epithelial cells ideally suited to produce extracellular $H_2O_2$[6, 62]. *In vitro* ALI cultures of differentiated and polarized human respiratory epithelium express high amounts of Duox, produce apical $H_2O_2$ and have been shown to kill several microorganisms in an $H_2O_2$-dependent manner [8,

[72, 85, 184]. However, working with cultures of primary human cells has significant limitations: 4-5 weeks of culturing time, cost and most importantly large donor-to-donor variations. We used ALI cultures of rat TBECs to complement our data obtained with human cells. Obtaining primary rat cells is cost effective, the cells are faster to culture, show little variation among donors and allow larger scale studies. Our rat TBECs reconstitute all the features of human cultures and express both dual oxidases (Figs. 3.1-2.). This is in accordance with previous studies that also used rat TBEC ALI cultures[184, 198, 199, 200]. Our data showing that rat TBECs inactivate IAV similarly to human cultures indicates that rat TBECs provide an excellent, alternative model to study influenza-human epithelial cell interactions (Figs. 3.4-5.).

TBECs are capable of producing sufficient $H_2O_2$ to supply the anti-influenza effect of LPO (Figs. 3.4-5.). All three components (Duox/$H_2O_2$, LPO, and $SCN^-$) are necessary to inactivate IAV. Omitting one, two or all three components results in no significant inactivation of IAV. Thus, not only enzyme-free [11] but also enzyme-derived $OSCN^-$ efficiently inactivates IAV. Detailed responses of human epithelial cells to H1N1 and H3N2 subtypes of IAV have been shown but their direct extracellular inactivation by the Duox-based system has not been documented [72]. Our studies are the first to show that extracellular influenza viruses can be inactivated efficiently by epithelial-derived $OSCN^-$. This adds influenza to the growing list of pathogenic infectious agents that the $H_2O_2$/LPO/$SCN^-$ system is capable of inactivating/killing[8, 10, 85, 184]. We also show that Duox and LPO are efficient against H1N2 subtype of IAV.

There are two isoforms of Duox, both of which are highly expressed in TBECs[6, 8, 72, 165, 201]. Which isoform is more important to produce $H_2O_2$ in TBECs is unclear at this point. It is well-accepted that in human TBECs Duox1 is the main Duox isoform expressed[6, 77]. Basal Duox2 expression is lower but Duox2 can be induced largely by microbial stimuli[202]. While the *in vivo* role of Duox1 is still unknown, the main physiological function of Duox2 is clearly to provide $H_2O_2$ for thyroid hormone biosynthesis. This is evident since both Duox2-deficient mice and human patients with Duox2 mutations develop hypothyroidism[65, 203]. Our data indicate that rat TBECs express both Duox1 and Duox2 at similar levels. Although Duox2 has been proposed to be the main isoform in rat TBECs[184], this conclusion was drawn from a siRNA transfection experiment, which showed minimal effect on apical $H_2O_2$ output without showing isoform specificity of the siRNAs. The situation is likely more complicated and both isoforms could be activated by different stimuli or could overtake each other's function to provide redundancy. Therefore, further data are needed to clearly identify the main Duox isoform responsible for the antimicrobial and inflammatory effect of TBECs.

It is important to emphasize the very fast action of the Duox/$H_2O_2$/LPO/$SCN^-$ system to inactivate IAV. Under our experimental conditions, IAV was only added for 1 hour on the top of TBECs. During this time there is a significant decrease in the concentration of viable extracellular virions if the whole system is assembled (Figs. 3.4-5). We assume that this rapid and robust inactivation of IAV by the Duox-based system largely reduces their potential to infect TBECs and to cause inflammation. To our current knowledge very few, if any, airway immune responses

act so quickly[21, 164]. Antimicrobial peptides are already present in the airway surface liquid but we estimate that the $OSCN^-$-generating mechanism has a larger virucidal capacity and is more manipulative (it can be turned on or off fast) [204, 205]. These features make the Duox/$H_2O_2$/LPO/$SCN^-$ antiviral system ideal for pharmaceutical intervention. By enhancing its anti-influenza effect, we could eliminate extracellular virions still on the airway surface before establishing infections in TBECs and triggering inflammation. All the downstream effects of IAV infection could be theoretically prevented at the earliest intervention time point possible.

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**Author contributions**

Conceived and designed experiments: AG. Performed experiments: AG. Analyzed the data: BR . Contributed reagents/materials/analysis tools: RAT. Wrote the paper: AG BR.

## Figures and legends

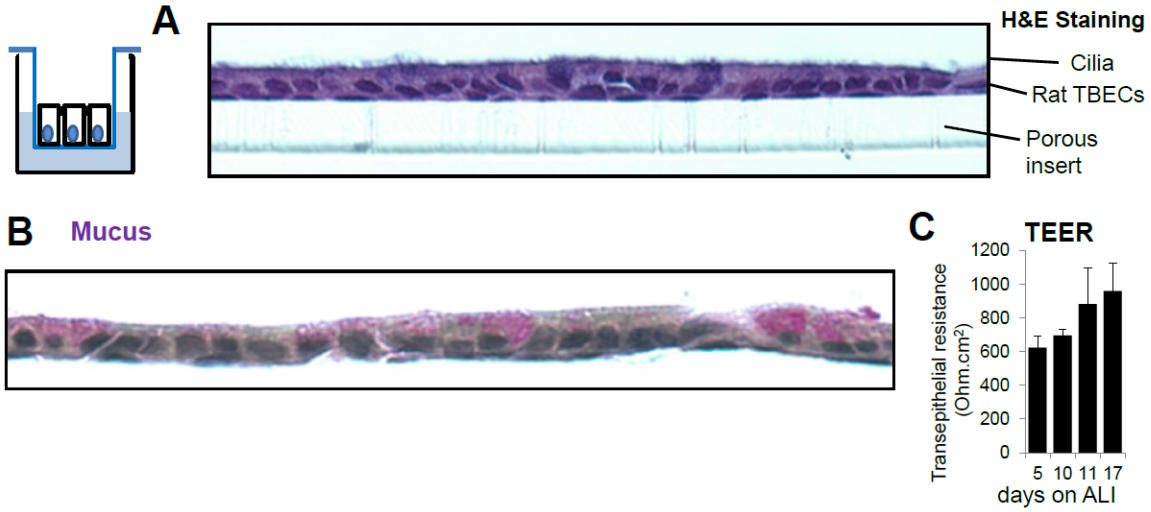



**Figure 3.1 Air-liquid interface cultures of polarized, differentiated rat tracheal epithelial cells provide an excellent *in vitro* model of the bronchial epithelium.** (A) H&E staining shows formation of a ciliated monolayer of polarized cells after 17 days of culture on ALI. One representative results, n=3. (B) Mayer's mucicarmine staining detects mucins. These large glycoproteins were identified by positive staining (purple) originating from mucin-producing Goblet cells. One representative result, n=3. (C) Transepithelial resistance (Ohm·cm$^2$) was measured on days 5, 10, 11, 17 post ALI using a voltohmmeter. Mean+/-S.E.M., n=7. TEER, transepithelial electrical resistance; TBEC, tracheobronchial epithelial cell; ALI, air-liquid interface.

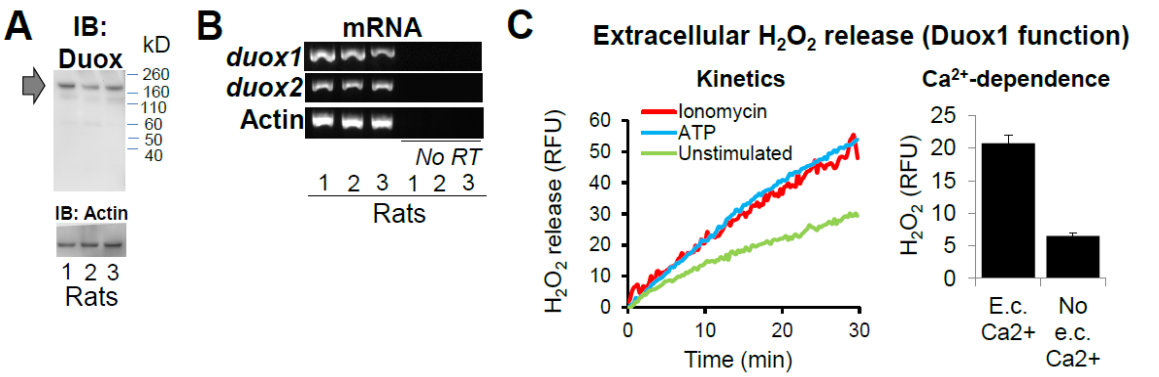



**Figure 3.2 Rat tracheal epithelial cells express Duox and produce extracellular $H_2O_2$ in a calcium-dependent manner.** Air-liquid interface cultures of primary rat tracheal cells were cultured for 17 days and subjected to the following analyses: (A) Duox protein was detected in cell lysates by western blotting. Samples of three animals shown. (B) Gene expression levels of rat duox1, duox2 and actin genes were detected by reverse transcriptase PCR. Samples of three animals shown. (C) Kinetics (left) and endpoint (right) measurements of extracellular hydrogen peroxide production detected by homovanillic acid oxidation assay. Kinetics: one representative result, n=3. ATP (100 μM), ionomycin (1 μM). Endpoint: mean+/-S.E.M., n=3. Extracellular calcium was chelated by addition of 1mM EGTA. IB, immunoblot; RT, reverse transcriptase; RFU, relative fluorescence unit.

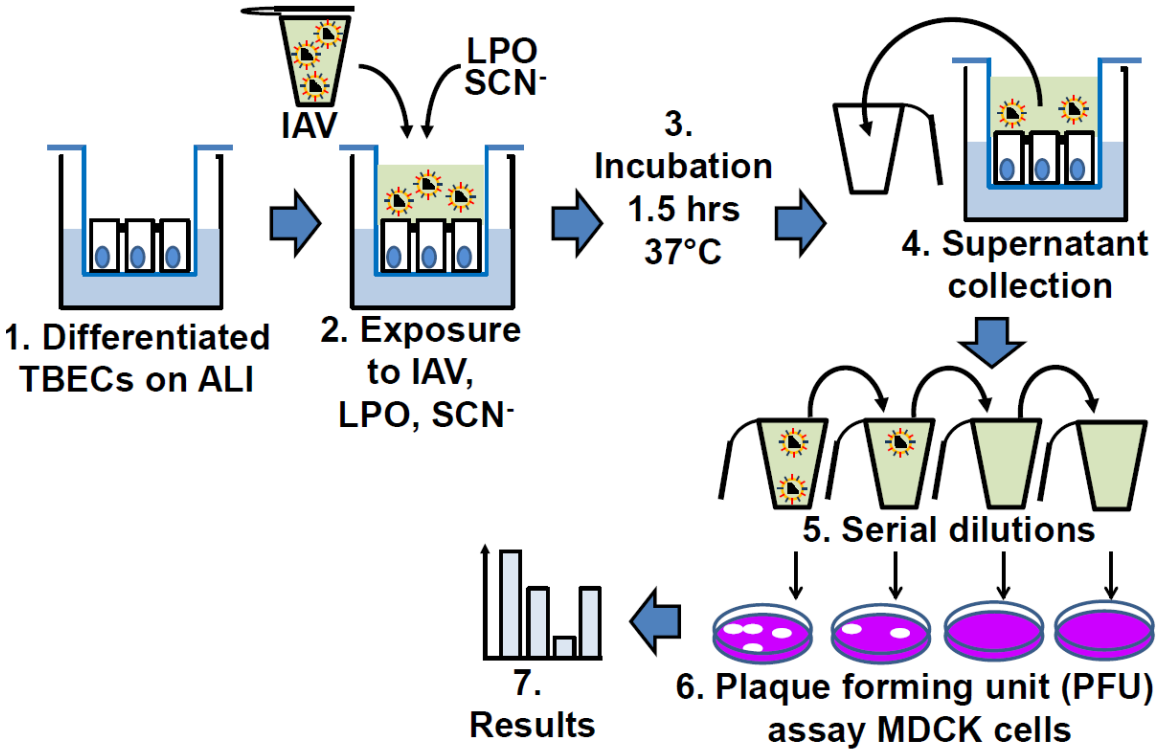
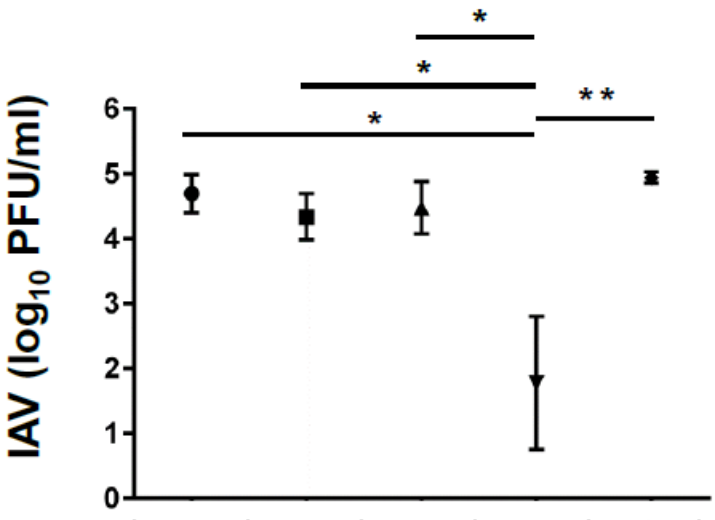
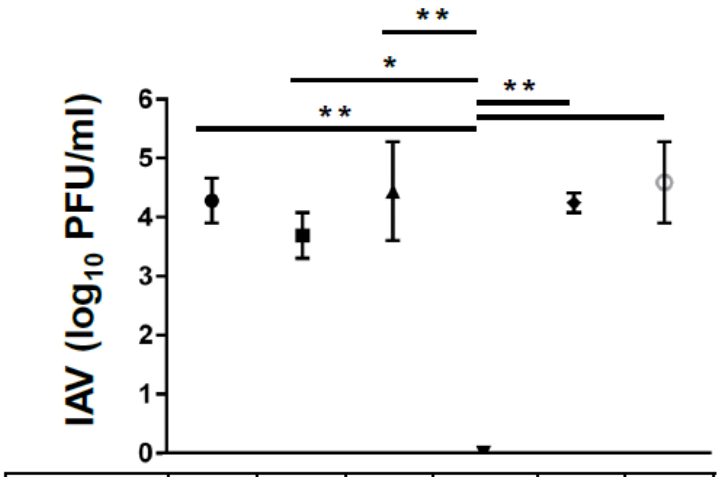



**Figure 3.3 Steps of measuring inactivation of extracellular influenza virions on tracheobronchial epithelial cells.** ALI cultures of differentiated TBECs **(1)** are exposed to IAV in combination with exogenous LPO and $SCN^-$ **(2)** for 1 hour at 37°C **(3)**. Supernatants were then collected **(4)**, 10-fold serial dilutions were performed **(5)** and plaque forming unit assays on MDCK cells **(6)** were used to determine the PFU/mL of virus remaining **(7)**. MDCK, Madin-Darby canine kidney cells; PFU. Plaque forming unit; LPO, lactoperoxidase; $SCN^-$, thiocyanate; IAV, influenza A virus; ALI, air-liquid interface; TBEC, tracheobronchial epithelial cell.




| | | | | | |
|---|---|---|---|---|---|
| ATP | + | + | + | + | - |
| LPO | - | + | - | + | - |
| $SCN^-$ | - | - | + | + | - |

**Figure 3.4 Rat TBECs inactivate influenza virions in a Duox/$H_2O_2$/LPO/$SCN^-$-dependent manner**. ALI cultures of differentiated primary Sprague-Dawley rat TBECs were exposed to 8000 PFU of H1N2 IAV (swH1N2) in presence or absence of ATP (Duox activator, 100 μM), LPO (6.5 ug/ml) or $SCN^-$ (400 μM) in the indicated combinations. After 1 hr incubation supernatants were collected and concentration of viable virus particles was determined by PFU assay using MDCK cells. Mean+/-S.E.M., n=4. ANOVA, Brown-Forsythe test, Tukey's multiple comparisons. *, $p<0.05$; **, $p<0.01$. IAV, influenza A virus; PFU, plaque forming unit.




| | | | | | | |
|---|---|---|---|---|---|---|
| ATP | + | + | + | + | + | - |
| LPO | - | + | - | + | + | - |
| $SCN^-$ | - | - | + | + | + | - |
| CAT | - | - | - | - | + | - |

**Figure 3.5 Human bronchial epithelial cells inactivate influenza virions in a Duox/$H_2O_2$/LPO/$SCN^-$ -dependent manner**. ALI cultures of differentiated human NHBE cells were exposed to 5000 PFU of H1N2 IAV (swH1N2) in presence or absence of ATP (Duox activator, 100 μM), LPO (1 μM), $SCN^-$ (400 μM) or catalase (CAT, 15,000 U/ml) in the indicated combinations. After 1 hr incubation supernatants were collected and concentration of viable virus particles was determined by PFU assay using MDCK cells. Mean+/-S.E.M., N=2. ANOVA, Brown-Forsythe test, Tukey's multiple comparisons. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. IAV, influenza A virus; PFU, plaque forming unit.

# CHAPTER 4

# DUOX1 DEFCIENCY LEADS TO AN INCREASE IN MORTALITY AND MORBIDITY IN A MURINE INFLUENZA INFECTION MODEL

[1]Gingerich, A., Sarr, D. and Rada, B to be submitted to *Plos Pathogens*

**Abstract**

Viral respiratory infections are among the most common illnesses in human and animal populations globally. Influenza virus infections affect millions of people worldwide, causing serious mortality and morbidity that result in a major economic burden. The epithelial cells of the airways represent the first line of defense against respiratory pathogens. One of the proposed innate defense mechanisms respiratory epithelial cells use to prevent infection is an extracellular antimicrobial ion at the airway surface called hypothiocyanite ($OSCN^-$), which is created by lactoperoxidase (LPO), thiocyanate ion ($SCN^-$), and hydrogen peroxide ($H_2O_2$). The source of $H_2O_2$ was unknown until recently, where it was discovered that the enzyme dual oxidase (Duox) was responsible for $H_2O_2$ production. While two isoforms of DUOX exist, DUOX1 is the dominant isoform found in the airways. Our previous study determined that *in vitro* DUOX was capable of driving the production of the antimicrobial ion $OSCN^-$ via the LPO-system. To date the role of DUOX1 in an *in vivo* infection is unknown. By utilizing a *Duox1*-deficient mouse, we demonstrated that *Duox1*-deficiency increased mortality, morbidity and lung viral titers in a murine influenza infection model. These data are the first to show a role for Duox1 that is beneficial to the host in any mammalian organism. The Duox1/LPO system represents a novel innate immune mechanism capable of protecting the host from influenza infection.

## Introduction

Influenza virus is a common cause of seasonal epidemics worldwide, causing it to be a serious public health threat do to our limited ability to control the virus[206]. Developing a better understanding of virus host-interactions at the epithelium is likely to lead to novel therapeutic targets to combat influenza. The first targets of influenza once it enters a host are the surface of epithelial cells that express sialic acids, which the virus binds to with the HA protein. To counter foreign microbes, epithelial cells are able to orchestrate a variety of antimicrobial mechanisms. One such mechanism is the DUOX/$H_2O_2$/LPO/$SCN^-$ system, which has shown the ability to kill a wide variety of pathogens *in vitro*[5]. Apart from *in vitro* studies, very little data has been collected about the effects of this antimicrobial system *in vivo*. Our previous studies demonstrated a role for Duox in inactivating influenza in primary bronchial epithelial cells[9]. The first *in vivo* study involving Duox1 revealed it was responsible for immune mediated damage in an allergic asthma response[73]. As mentioned previously, two isoforms of DUOX exist in higher organisms, DUOX1 and 2. DUOX2 has been extensively described and has a known antiviral role[207, 208, 209, 210, 211], but the function of DUOX1 during a viral infection is yet to be described. Previous studies looking at the role of Duox in an *in vivo* model have been carried out using a complete *Duox1/2* deficient mouse, so a specific *Duox1*-deficient model is needed to determine its role, as it is well described that Duox2 deficiency leads to hypothyroidism[63, 64]. The purpose of this study was to determine the role of Duox1 in an *in vivo* influenza infection model

which to date had not been achieved. Our work is the first to show a positive role of Duox1 in an *in vivo* murine influenza infection model.

## Materials and methods

### Cell lines

Madin-Darby canine kidney cells (MDCK) (ATCC CCL-34) were cultured in Dulbecco's modified Eagle's medium/Nutrient mixture F-12 Ham (DMEM/F-12) (SIGMA, St. Louis, MO) supplemented with 5 % heat-inactivated fetal bovine serum (FBS) and maintained at 37 °C with 5 % $CO_2$.

### Influenza virus propagation

Influenza viruses A/Puerto Rico/8/1934, A/Puerto Rico/8/1934-PA-NanoLuciferase (NLuc) were grown in the allantoic cavity of 9-10 day old specific pathogen free embryonated chicken eggs at 37$^{o}$C. Viral titers were determined by plaque-forming unit (PFU) assay and hemagglutination (HA) assay.

### Mouse infection studies

Both male and female C57BL/6J wild-type mice and *Duox1*-deficient mice, originally generated using a retroviral-based gene-trapping method (Lexicon Pharmaceuticals) and backcrossed onto a C57BL/6J background[212] were used in these experiments. *Duox1*-deficient mice were kindly provided by Dr. Miklós Geiszt (Semmelweis University, Budapest, Hungary). Wild-type and *Duox1*-defcient mice were anesthetized by intraperitoneal (i.p.) injection of Tribromoethanol (Avertin) at the dose of 250 mg/kg. Mice were intranasally inoculated with 40 ul PBS alone or PBS containing A/Puerto Rico/8/1934 H1N1 influenza virus at the indicated dose. After infection, survival and weight changes were monitored daily up to 12 d.p.i or

mice were euthanized at upon losing 30% of starting body weight or at selected days for further analysis. Mice were 6-8 weeks old and equal numbers of female and male mice were used. The infection experiment was repeated three times independently. The Institutional Animal Care and Use Committee (IACUC) of the University of Georgia approved all the protocols and procedures used in the animal experiments: A2017 07-010-Y2-A1.

**Genotyping of *Duox1*-deficient mice**

Oligonucleotide primers were used in separate reactions (LTRRev, 5′-ATAAACCCTCTTGCAGTTGCATC-3′, and For2,5′-CCTTCCTGCTCCTTCTTACC-3′ for the knockout allele; Rev2, 5′-GTGAATCAAGACATAGTTAACC-3′, and For2 for the wild-type allele). Approximately 100 ng of purified tail genomic DNA was used as a template for PCR. For genotyping reactions, DreamTaq (Thermo Fisher Scientific, Waltham, MA) was used according to the manufacturer's instructions in a reaction volume of 50μl. Cycling conditions were as follows: 94 °C for 3 min; then 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s; and finally 72 °C for 2 min. Amplified PCR products were separated on 1.5% agarose gels.

**ELISA assay**

Mouse BAL samples were collected in PBS. A commercial ELISA kit was used to determine LPO (Genway Biotech INC, San Diego, CA.) levels in BAL samples

according to manufacturers instructions. The experimental limits of detection were 0.055 ng/mL

**Statistical analysis**

Significance among multiple samples was calculated using One-way or Two-Way ANOVA followed by Tukey's or Sidak's multiple comparison post-hoc tests. Significance between two samples was calculated using Man-Whitney's test or unpaired t-test. Statistical analysis was performed using Prism 6 for Windows version 6.07 software. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$

## Results

### LPO and $SCN^-$ concentrations in BAL samples of influenza-infected mice

Duox1-derived $H_2O_2$ only represents one component of the Duox1/LPO/$SCN^-$ antimicrobial system. As it has not been previously described in the literature, we examined whether $SCN^-$ and LPO are even present in the murine airways and if they are present at what levels. $SCN^-$ concentration in BAL samples was determined using gas chromatography coupled to high-resolution, accurate-mass mass spectrometry. We found that levels of $SCN^-$ in infected Duox1 and WT Bronchoalveolar Lavage (BAL) samples showed no significant difference during day is 1-5 p.i. (Fig 4.1A). We utilized a commercial murine LPO ELISA to measure BAL samples day 3 and 7 p.i. We found no significant differences between *Duox1*-deficient and WT type mice at either time point when infected (Fig4.1B). These results show that Duox1 deficiency does not appear to alter the other components of the Duox1/LPO/$SCN^-$ antimicrobial system *in vivo*.

### Duox1-deficient mice show increased mortality and morbidity compared to WT in a PR8 influenza challenge

*Duox1*-deficient mice along with wild-type (C57BL/6; WT) m/f were challenged with a 0.5 LD50 dose of influenza A/Puerto Rico/8/1934 (PR8) virus via intranasal administration. At day 12 p.i, WT mice showed a 14% mortality rate while the *Duox1*-deficient mice had a 45% mortality rate (Fig. 4.2). Both groups showed the first signs of mortality at day 7 p.i, with the *Duox1*-deficient mice having the biggest increase in mortality at day 9 p.i. With no obvious phenotype being

seen in unchallenged *Duox1*-deficient mice, this was the first study to show that *Duox1*-deficient mice have increased susceptibility to influenza infection with a significant decrease in survival. Weight loss was recorded over the course of the experiment (Fig 4.3). The *Duox1*-deficient mice demonstrated an earlier and steeper increase in weight loss compared to WT mice over the course of infection (Fig 4.3). At day 3 post infection, the first signs of a difference in weight loss were detected ($p=0.08$) (Fig 4.4), and by day 7, the percent weight loss between the two groups had become significant (*, $P<0.05$). These results support our survival data where the *Duox1*-deficient mice showed increased mortality (Fig 4.2) in addition to the more robust morbidity associated with influenza infection. Based on the results from our *in vitro* data, the role of Duox1 should be the driving force of the LPO antimicrobial system that produces the antiviral anion $OSCN^-$. We assessed viral titers of mice infected with a 0.5 LD50 dose of influenza A/Puerto Rico/8/1934 (PR8) virus via intranasal administration at day 3 and day 5 p.i. Day 3 is the peak of viral titers in lungs in murine infections. At day 3 p, i. lung viral titers of *Duox1*-deficient mice were significantly higher (6.6-logPFU) than the wild type mice (5.2-logPFU). This represents over a 90% increase in viral titers in the lungs of *Duox1*-deficient mice.

## Discussion

The DUOX-$H_2O_2$/LPO/$SCN^-$ system has been studied for its antimicrobial actions against a long list of microbes as mentioned before, nearly all of these studies have been carried out *in vitro.* However the other dual oxidase, DUOX2, has been shown to play a vital role *in vivo* in thyroid hormone biosynthesis, where hydrogen peroxide is utilized to oxidize iodine to allow hormone biosynthesis[45, 46, 60]. Defects in the DUOX2 gene lead to permanent congenital hypothyroidism in both humans and mice[59, 60, 63, 64]. Several studies have carved out the antiviral role of DUOX2[207, 208, 209, 210, 211]. Studies suggest that the production of DUOX2-derived ROS may be responsible for IFN-λ secretion[211], induction of RIG-I and MDA5 [210] and a role for TLR5-dependent inflammatory response of nasal airway epithelium[208]. DUOX1 and 2 have both been shown to be upregulated by influenza infection[210]. However, an important difference exists in that DUOX1 is always producing $H_2O_2$ while DUOX2 must be activated to produce $H_2O_2$[213]. This observation carves out a niche for both enzymes to function in the same area but in vastly different roles. The role of DUOX1, however, has not been thoroughly studied *in vivo,* especially in the context of an infection model. The first study to utilize a murine gene-deficient model found that Duox1 is present in epithelial cells found in the bladder[212]. The study found a lack of Duox1-mediated $H_2O_2$ production in the urothelium leads to altered bladder function and ultimately bladder diseases[212]. This study supports the notion that Duox1 plays a role in a multitude of epithelial cells distributed throughout the body. The earliest study utilizing a murine knockout model of Duox1 in the airways, showed it was responsible for persistent oxidant dependent

epithelial activation of EGFR, which then leads to an increase in type 2 cytokines ultimately leading to immune mediated damage to the host[73]. In *Duox1*-deficient mice the enhanced EGFR phenotype was no longer seen, leading to a decrease in immune mediated damage to the host[73]. We hypothesized that Duox1 plays a positive role in influenza infection based on our *in vitro* data demonstrating its capabilities to reduce viral titers. A previous study laid the groundwork for this using siRNA silencing of Duox1/2 and saw an increase in viral titers[72]. Our study is the first to utilize a global Duox1 deficient mouse in an infectious system, instead of complete Duox1/2 knockout because the role of Duox1 specifically is impossible to elucidate due to hypothyroidism caused by Duox2 deficiency[63, 64]. We utilized the mouse-adapted A/Puerto Rico/8/1934 strain to infect mice with a 0.5 LD50 dose intranasally. After 12 days, we found that the *Duox1*-deficient mice exhibited significantly more mortality (45% vs 14%) and morbidity (Fig 4.10-11) than their WT counterparts (Fig 4.9) did. In the lung, viral titers measured at day 3 and 5 saw a significant increase in lung viral titers in the *Duox1*-deficient (Fig 4.12). These are the first results to date demonstrating a positive role of Duox1 in an *in vivo* infection model. Our *in vitro* data from other studies showing reductions in viral titers fully supported the role Duox-$H_2O_2$/LPO/$SCN^-$ plays in antiviral defense *in vivo,* as a deficiency in Duox1 impaired the system leading to an increase in lung viral titers. This work opens the door for a better understanding of how our innate immune system combats influenza, which ultimately leads to better treatment and prevention options. However, numerous aspects are at play in a complex *in vivo* system. LPO and $SCN^-$ also play a vital role in the function of this system and to date no one had measured LPO or

$SCN^-$ levels in the airways of mice. We confirmed the presence of both LPO and $SCN^-$ with no significant difference seen between the *Duox1*-deficient and the WT. This illustrates the key role Duox1 plays, as without it mice are at a much greater risk of mortality and morbidity. These studies have only scratched the surface of the story, as an increase in viral titers alone is unlikely to cause the increase in mortality that we saw. An effective innate immune response early in viral infections is vital for positive outcomes. Upon influenza infection, neutrophils are among the first innate cells recruited at high levels into the airways and are critical to mounting an effective response to the virus[126]. It has previously been shown in a zebra fish model that Duox1-derived $H_2O_2$ activates the Src family kinase Lyn, which recruits neutrophils to wound sites[214]. A follow up study then found that early Duox1-derived $H_2O_2$ contributed to Cxcl8 expression, a major neutrophil chemoattractant, via the JNK/c-JUN/AP-1 signaling pathway[215]. One of the hallmark antimicrobial products of neutrophils is hypochlorous acid (HOCL) produced by myeloperoxidase[216]. While effective against microbes, it is also highly toxic to the host cells causing tissue injury and excessive inflammation[217, 218]. Interestingly $SCN^-$ is the enzymatically preferred substrate of chordate peroxidases, leading to $OSCN^-$ being produced instead of HOCl[219]. In addition to being the preferred substrate $SCN^-$ is able to reduce HOCl into hypothiocyanous acid (HOSCN)[220, 221]. As such, $SCN^-$ offers a possible solution to mitigating neutrophil mediated damage to the host. Duox1 and neutrophils both have demonstrated key roles during influenza infection; it is likely that an interaction between the two is also occurring that needs to be further studied in murine influenza infection model. One of the vital response systems to viral

infections are IFNs. Future studies should determine whether type I & III IFNs are influencing mortality in influenza infection in our model. It was recently shown that in cervical cancer patients DUOX1 was upregulated in epithelial cells and were further upregulated in patients infected with human papillomavirus (HPV)[222]. Additionally this increased expression of DUOX1 significantly correlated with an increase of type I and III IFNs immune pathways[222]. While not located in the same region as influenza infection occurs normally. This likely points to an interaction between DUOX1 and the IFN pathways that are vital for early control of influenza infection. Type III IFNs act locally at the epithelial surface where they inhibit viral spread, without having to utilize an inflammatory response as type I IFNs do[36]. This is important to note, as lower initial viral burden shifts the IFN response to type III but under higher viral load, it shifts to type I[35, 36]. With Duox1 playing an antiviral role, it is possible that a Duox1 deficiency leads to a shift in the immune response to type I IFNs leading to an exaggerated response ultimately causing increased mortality. Duox1 is potentially playing a role in signaling other branches of the immune system which has been described in other systems[5, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41] and will need to be studied further. While all of our studies focused on the upper respiratory tract (trachea –lungs), how the DUOX1-$H_2O_2$/LPO/$SCN^-$ functions in the nasal epithelium is relevant as other studies have shown a role for Duox2 in that region[72, 210]. One of the drawbacks of our system is that in a global *Duox1*-deficient mouse, we do not know what other effects this deficiency could be having, as it is present in epithelial cells throughout the body and this could potentially be altering the response to infections. Utilizing a tissue specific conditional knockout to target Duox1 specifically in the airways would be the logical next

step. Interestingly DUOX1 and LPO deficiencies have never been reported in human patients. Certain people are more susceptible to respiratory infections, so it is entirely possible that they have SNPs or mutations in DUOX1 or LPO leading to enhanced susceptibility to respiratory infections. Analyzing samples of patients with influenza infection, influenza-like lung disease or of healthy controls could shine light on this question. Our *in vivo* studies demonstrated for the first time a positive role of Duox1 during infection, which is a stark contrast from its negative role in allergy-induced asthma[73]. This system likely has a synergistic effect with other parts of the immune system, and by being one of the first to react; it is more difficult for microbes to evolve a specific resistance to $OSCN^-$ when other stronger selective pressures exist. With our data showing a positive role for Duox1, augmenting this system by controlling some or all of its components offers a potential novel therapeutic with broad-spectrum antimicrobial capabilities.

## Figures

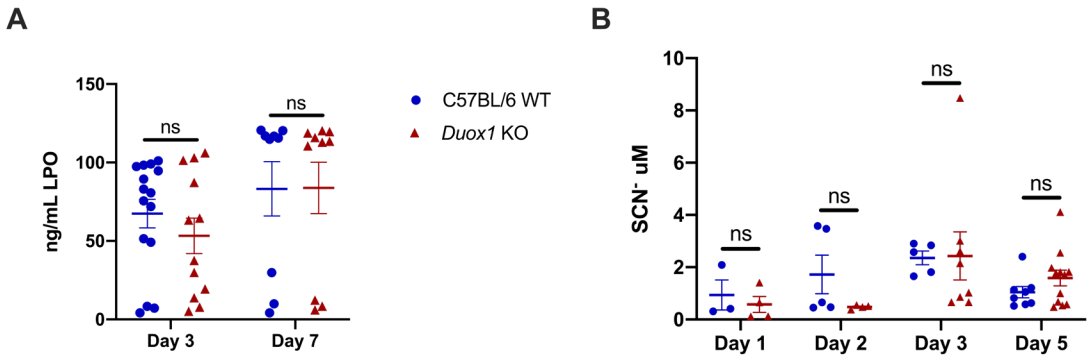
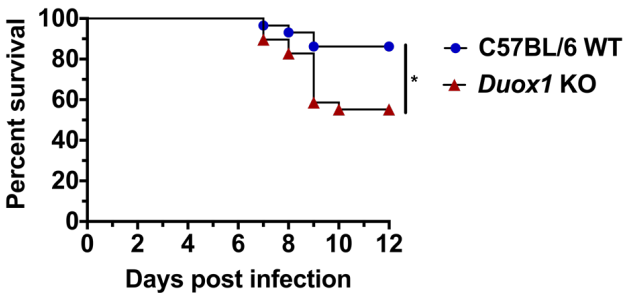
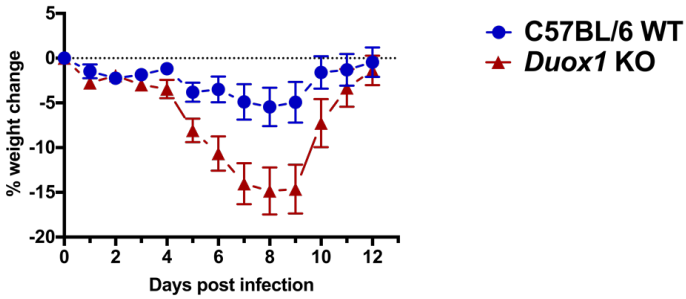



**Figure 4.1: Concentration of LPO and $SCN^-$ in murine BAL samples is not different between *Duox1*-deficient and WT mice.** *Duox1*-deficient mice show no difference in LPO or $SCN^-$ concentration in BAL samples after PR8 challenge. *Duox1*-deficient mice and C57BL/6 WT mice were infected with 0.5 LD50 of influenza virus A/Puerto Rico/8/1934 intranasally (i.n.). (A) 72 and 168 hrs p.i mice were euthanized and BAL was collected and lactoperoxidase in BAL was determined by ELISA in triplicate. N= 26 C57BL/6 WT, N=22 *Duox1* KO M/F (B) 24, 48, 72, 120 hrs later BAL was collected and $SCN^-$ concentration was determined via mass spectrometry N= 20 C57BL/6 WT, N=26 *Duox1* KO M/F. LPO and $SCN^-$ concentration data were analyzed by 2way ANOVA. ns=not significant.




| Mouse background | No. of Subjects | % Survival |
|---|---|---|
| C57BL/6 WT | 29 | 86 |
| *Duox1* KO | 28 | 55 |

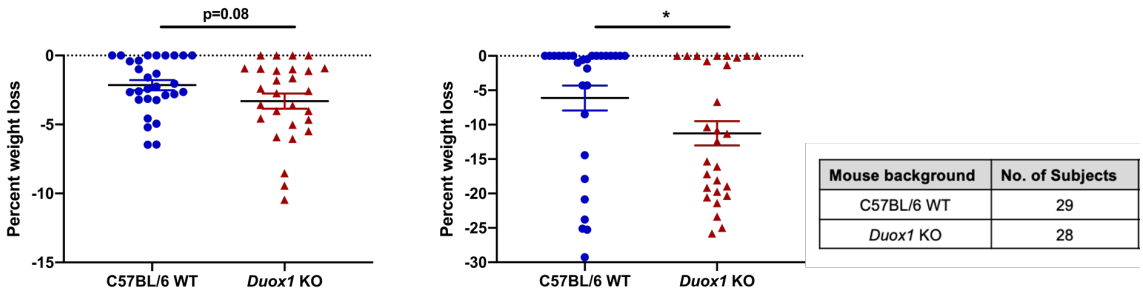
**Figure 4.2: *Duox1*-deficient mice show increased mortality to PR8 challenge.** *Duox1*-deficient mice and C57BL/6 WT mice were infected with 0.5 LD50 of influenza virus A/Puerto Rico/8/1934 intranasally (i.n.). Mortality was monitored for 12 days following infection. Survival was analyzed by log-rank (Mantel-Cox) test. N= 29 C57BL/6 WT, N=28 *Duox*1 KO mice/group M&F. *, $P<0.05$.




| Mouse background | No. of Subjects |
|---|---|
| C57BL/6 WT | 29 |
| *Duox1* KO | 28 |

**Figure 4.3: *Duox1*-deficient mice show increased morbidity to PR8 challenge**

*Duox1*-deficient mice and C57BL/6 WT mice were infected with 0.5 LD50 of influenza virus A/Puerto Rico/8/1934 intranasally (i.n.). Weight change was monitored for 12 days following infection. N= 29 C57BL/6 WT, N=28 *Duox*1 KO mice/group M&F.




| Mouse background | No. of Subjects |
|---|---|
| C57BL/6 WT | 29 |
| *Duox1* KO | 28 |

**Figure 4.4: *Duox1*-deficient mice show increased weight loss at day 3 & 7 p.i.**

*Duox1*-deficient mice and C57BL/6 WT mice were infected with 0.5 LD50 of influenza virus A/Puerto Rico/8/1934 intranasally (i.n.). Weight loss was monitored for 12 days following infection. Weight loss was analyzed by unpaired t test N= 29 C57BL/6 WT, N=28 DUOX1 KO mice/group M&F. *, P<0.05.

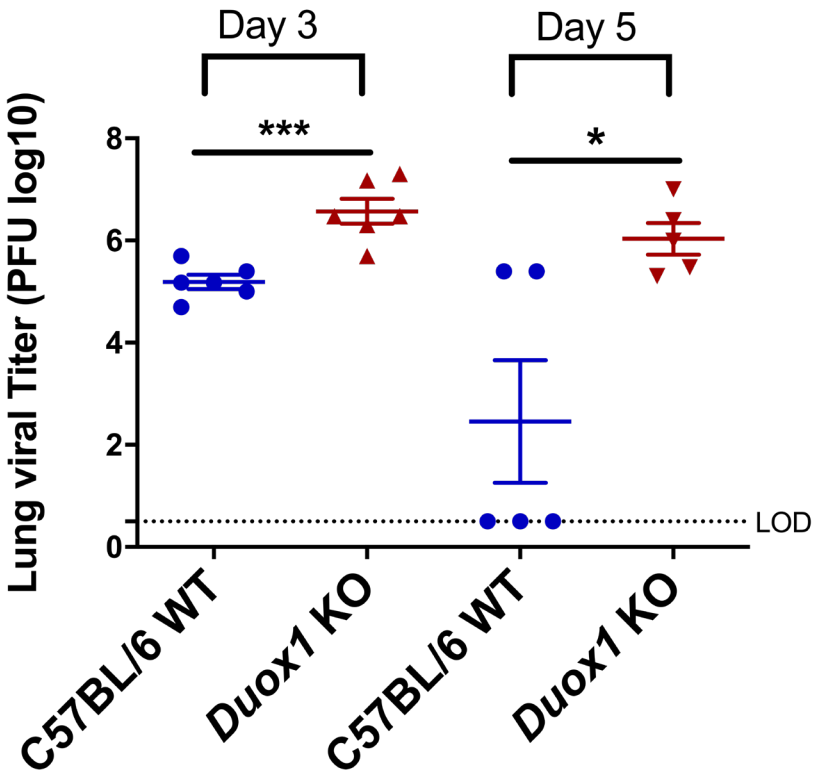



**Figure 4.5: *Duox1*-deficient mice show increased lung viral titers during PR8 challenge** *Duox1*-deficient mice show increased viral titers at day 3 & 5 p.i. *Duox1*-deficient mice and C57BL/6 WT mice were infected with 0.5 LD50 of influenza virus A/Puerto Rico/8/1934 intranasally (i.n.). At 72 hrs and 120hrs after infection, mice were euthanized and virus titers in lung homogenates were determined. Viral titers were analyzed by unpaired t test N= 6 C57BL/6 WT, N=6 *Duox1* KO mice/group day 3 N= 5 C57BL/6 WT, N=5 *Duox1* KO mice/group day 5 M&F. *, P<0.05. ***,P<0.001

# CHAPTER 5

# LPO-DERIVED HYPOTHIOCYANITE INHIBITS INFLUENZA INFECTIVITY BY SURPRESSING VIRAL ATTATCHMENT

[1]Gingerich, A., Rada, B to be submitted to *Plos Pathogens*

**Abstract**

Viral respiratory infections are among the most common illnesses in global human and animal populations. Influenza virus infections affect millions of people worldwide, causing serious mortality and morbidity that result in a major economic burden. The epithelial cells of the airways represent the first line of defense against respiratory pathogens. One of the proposed innate defense mechanisms respiratory epithelial cells use to prevent infection is an extracellular oxidative antimicrobial ion at the airway surface called hypothiocyanite ($OSCN^-$), which is created by lactoperoxidase (LPO), thiocyanate ion ($SCN^-$), and hydrogen peroxide ($H_2O_2$). The innate immune function of this system has been documented against several microbes, but an in depth characterization of its mechanism of action against influenza viruses is still eluding us.

Our results demonstrated that *in vitro* $OSCN^-$ is capable of reducing viral titers of 17 different influenza A (H1N1,H1N2,H3N2), and B (Yamagata and Victoria lineages) strains by 2-6 logs. Additionally, pre/co-incubation of $OSCN^-$ and virus reduces infection of MDCK and primary human tracheobronchial epithelial cells. To elucidate its mechanism of action, we measured neuraminidase (NA) activity of whole virus, and our results showed no change in the presence or absence of hypothiocyanite. $OSCN^-$ treatment of influenza virus led to a 2-4 fold inhibition in binding to HuRBCs.

## Introduction

Airborne microbes that target the respiratory system are responsible for epidemics and periodic pandemics that affect millions of people worldwide; one of the most notorious is influenza virus. Influenza is a single-stranded, negative-sense, segmented RNA virus in the Orthomyxovirus family[106]. Influenza has four genera A, B, C and D that allow it to have unmatched zoonotic potential as it is able to transmit inter- and intra-species. Studies have shown that influenza readily modifies the host immune response and airways in order to evade the host. Current prophylactic options include the quadrivalent influenza vaccine that is adjusted seasonally, and antiviral drugs. There are two major classes of antivirals available for prevention and treatment of influenza infection, M2 inhibitors and neuraminidase inhibitors[142]. M2 inhibitors are facing increasing drug resistance, as they only work against influenza A virus. Resistance has also emerged against neuraminidase inhibitors[223]. The need for broad-spectrum anti-influenza prophylactics is at an all-time high.

The best place to look for novel mechanisms is likely our first line of defense, the innate immune system. While not normally thought of in the same breath as neutrophils and macrophages, airway epithelial cells also employ their own antimicrobial defense systems. Epithelial cells are able to orchestrate an antimicrobial oxidative system present in the airway surface liquid consisting of lactoperoxidase (LPO), its substrate, the anion thiocyanate ($SCN^-$) and hydrogen peroxide ($H_2O_2$), creating the antimicrobial ion hypothiocyanite ($OSCN^-$)[5]. The DUOX1/$H_2O_2$/LPO/$SCN^-$ antimicrobial system has been researched for quite some

time, but not until 2003 was the source of $H_2O_2$ discovered[6]. It was found that the NADPH oxidase Duox1 was responsible for extracellular $H_2O_2$ production in the airways[6]. Our previous study in a *Duox1*-defcient mouse demonstrated that Duox1 deficiency led to increased lung viral titers, morbidity and mortality. This was the first study to demonstrate a positive role in an infection model for Duox1 *in vivo.* Previously studies involving this system's antimicrobial effects have been *in vitro,* where the results have been promising. $OSCN^-$ has shown the ability to kill a wide variety of bacteria, viruses parasites and even fungi[5]. Our study using normal human bronchial epithelial (NHBE) cells, demonstrated the power and efficiency of this system *in vitro* reducing extracellular viral titers by several logs. However, the mechanism $OSCN^-$ utilizes to inactivate influenza virus remains unknown and in this study, we set out to elucidate this mechanism.

## Materials and Methods

### Cell lines and primary cells

Madin-Darby canine kidney cells (MDCK) (ATCC CCL-34) were cultured in Dulbecco's modified Eagle's medium/Nutrient mixture F-12 Ham (DMEM/F-12) (SIGMA, St. Louis, MO) supplemented with 5 % heat-inactivated fetal bovine serum (FBS) and maintained at 37 °C with 5 % $CO_2$. Primary normal human bronchial epithelial (NHBE) cells were purchased from Lonza (Walkersville, MD) and cultured as previously described[71]). Cells were seeded onto 24-well polyester (0.4-micron pore) membrane transwells (Costar), pre-coated with 0.3 % rat-tail collagen II (Sigma), at a density of 3.0 × 10^4 cells/well. B-ALI™ growth medium was used in the apical and basal chambers until the cells reached confluency. The upper chamber medium was aspirated, and the lower medium was replaced with B-ALI™ differentiation medium (Lonza, Walkersville, MD). Cells were maintained on the air–liquid interface (ALI) for 4–5 weeks by feeding every other day with ALI differentiation medium (the surface of ALI cultures was washed with sterile HBSS every other day). Antibiotics (penicillin and streptomycin, Life Technologies, Grand Island, NY) were supplemented in the media up to 4 days before experiments.

### Immunofluorescence

The expression pattern of viral infected NHBE cells was assessed by immunofluorescence. The transwells were washed twice with PBS, then fixed overnight with 10% formalin by adding it to the apical and basal chambers, permeabilized with Triton-X 100 for 15 min, and washed three times with PBS. The

permeabilized cells were then blocked with blocking buffer (PBS with 1% BSA/0.5% Tween 20) for 1 hr. Anti-influenza A nucleoprotein (monoclonal, BEI Resources, NR-43899) was then added to each well (100uL per well) after adding it to PBS with 0.5% Tween 20. After washing, 100uL of anti-murine IgG antibody (Life Technologies) was added to each well. Finally, the cells were stained with DAPI to identify individual cells.

**Influenza virus propagation**

Influenza A and B strains used in this work were purchased from the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH (BEI Resources) and are listed in (Fig 5.3). Influenza viruses were propagated in MDCK cells as described[9]. Briefly, viral strains were cultured in MDCK cells using infection medium (DMEM/F-12 supplemented with 1 mM L-glutamine with 1-µg/ml tosylsulfonyl phenylalanyl chloromethyl ketone [TPCK]-treated trypsin). Viruses were collected 24–48 hrs post-infection. Influenza viruses A/Puerto Rico/8/1934, A/Puerto Rico/8/1934-PA-NanoLuciferase (NLuc) were grown in the allantoic cavity of 9-10 day old specific pathogen free embryonated chicken eggs at 37$^{o}$C. Viral titers were determined by plaque-forming unit (PFU) assay and hemagglutination (HA) assay.

**Hemagglutination inhibition binding assay**

The effect of $OSCN^-$ on virus-induced binding to human red blood cells (HuRBCs) was determined using a hemagglutination assay. Viral solutions (32

HAU) were serially diluted (2-fold) to determine HA titer. Virus was incubated with $OSCN^-$ for 30 min. Each diluted viral solution was mixed with an equal volume of a 1.5% suspension of HuRBCs and incubated at room temperature for 1 hr. After 60 min, hemagglutination titers were determined and expressed as hemagglutination units (HAU) [224].

**Viral purification**

Influenza viruses were purified as previously described[225]. Briefly, supernatants of MDCK cells infected with viruses or allantoic fluid samples from chicken eggs were cleared by low speed centrifugation, and then pelleted through a 25% sucrose cushion in NTE buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA). Purified viruses were pelleted by ultracentrifugation (WX Ultra 80) at 28,000 rpm (104,492g) for 2 hrs, resuspended in 5 mL of PBS buffer, aliquoted, and frozen at −80 °C for future use.

***In vitro* viral inactivation assays**

Inactivation of influenza viruses by $OSCN^-$ was studied in different *in vitro* systems. After NHBE cells had been cultured on ALI for 4-5 weeks, cells were switched to antibiotic-free medium four days prior to experimentation. Each component of the system was tested at the following concentrations: 6.5 μg/mL LPO, 400 μM $SCN^-$ (Sigma-Aldrich, St. Louis, MO). The reaction volume was set to 40 μL with the appropriate concentration of each component. Viral suspensions were diluted to an estimated MOI (multiplicity of infection, IAV/NHBE) of 1 (NHBE

cells ~50000 viral particles/transwell). Catalase (700 U/mL, Sigma-Aldrich, St. Louis, MO) was used to inhibit the system. Once the components were assembled, the 40 µL volume was pipetted to the apical chamber of the transwell and placed in a 37 °C 5 % $CO_2$ incubator. After 1 hr, supernatants from each respective well were collected and stored at −80 °C. Plaque assays were performed to determine viral concentrations as previously described[177].

To measure viral inactivation in the cell-free system, components of the LPO-based antiviral system were used at the following concentrations: 6.5 µg/ml LPO, 400 µM $SCN^-$, glucose (0.005 M) and glucose oxidase 0.01 U/mL. The reaction volume was set to 40 µL with the appropriate concentration of each component mentioned above. Catalase (700 U/mL, Sigma-Aldrich, St. Louis, MO) was also used when indicated to inhibit the system. The components were assembled in a sterile Eppendorf tube with the virus being added last. After incubation for 1hr at 37 $^oC$ , supernatants were stored at -80°C. Plaque assays were performed on MDCK cells to determine viral concentrations as previously described[177].

*In vitro* inactivation of influenza by $OSCN^-$ was also assessed using the 1st line™ immune support product. 1st line™ is an over-the-counter product that is marketed as an immune supplement (distributed by Profound Products). This product uses a proprietary technology to keep $OSCN^-$ stable for a longer period allowing for a better antimicrobial effect and uses bolus of $H_2O_2$ instead of enzymatically generated $H_2O_2$. Briefly, the mix was assembled by sequential additions of reconstituted LPO, $H_2O_2$, $SCN^-$ and poly aluminum chloride, mixed

well and incubated for 30 min at room temperature to allow the generation of $OSCN^-$. Plaque assays were performed on MDCK cells to determine viral concentrations for most of the strains tested as previously described[177].

**Neuraminidase activity assay**

The NA-Fluor ™ Influenza Neuraminidase Assay Kit (Life technologies Carlsbad, CA) was used to determine NA activity of purified influenza viral suspensions. Briefly, 200 μM NA-Fluor™ substrate (10uL) was added to all samples (5x10^5PFU) and incubated at 37$^{o}$C for 60 min in the dark. The reaction was then terminated by adding NA-Fluor™ Stop solution and results were read with a VarioskanFlash™ microplate fluorimeter using excitation wavelength range 365 nm and emission wavelength range 450nm.

**Hemolysis assay**

The RBC hemolysis inhibition assay was modified from a previously described protocol[226]. First, 100 μL of influenza viruses (5x10^5 pfu)/mL) were pre-treated with 100 μL of $OSCN^-$ for 30 min at room temperature. Next, 200 μL of 2% human RBCs were incubated with this mixture for 30 min with gentle shaking. In order to induce HA-mediated RBC hemolysis, 100 μL of 0.5 M sodium citrate (pH 5.2) was incubated with the reaction mixture with intermittent shaking (60 rpm) for 1 hr. The supernatant was collected by centrifugation at 100 × g for 5 min and 300 μL of the reaction mixture was used to determine the released hemoglobin by measuring the absorbance with an Eon Biotek Plate Reader at 540 nm.

**Human RBC isolation**

Human subjects were recruited at the University of Georgia to donate blood. The human blood protocol (UGA# 2012-10769-06) and the consent form were reviewed and approved by the Institutional Review Board (IRB) of the University of Georgia. Enrolled healthy volunteers provided informed consent prior to blood draw. 4mL of human blood was added to 11mL of PBS and centrifuged at 800rpm for 10 min. The supernatant was aspirated and 12mL of PBS was gently added to the pellet and mixed by inverting. The solution was centrifuged at 800rpm for 5 min and the wash step repeated 2 more times. After the final wash, the supernatant was aspirated and PBS was added to make a 10% solution of red blood cells.

**Viral attachment assay**

Binding of fluorescently labeled influenza virus was performed as previously described[227]. Briefly, 200 µL of virus was incubated with 25 µg of Alexa Fluor 488 (Invitrogen A2000) in 1 M NaHCO3 (pH 9.0) for 1 hr at room temperature. Labeled virus were dialyzed against PBS using a 7,000 molecular weight cutoff Slide-A-Lyzer minidialysis unit (Thermo Scientific) overnight at 4 °C. Virus attachment to MDCK cells was measured by fluorescence. MDCK cells cultured in 96-well plates were infected with 128 HAUs of labeled influenza virus A/Aichi/2/68 at 4°C for 4 hrs to allow sufficient binding but no entry. MDCK cells were then washed with ice-cold PBS to remove any unbound virus. Fluorescence was measured via plate

reader (Varioskan flash) at an excitation of 495nm and emission of 519nm. Data are expressed as percentage relative to the untreated virus control.

**Characterization of growth curve of A/Puerto Rico/8/1934-PA-NanoLuciferase (NLuc)**

MDCK cells cultured on 96-well plates ($5x10^3$cells/well) were infected with 128 HAUs of influenza virus A/Puerto Rico/8/1934-PA-NanoLuciferase (NLuc) kindly provided by Dr. Andrew Mehle). This proliferation of this engineered viral strain can be followed by measuring luminescence using the NanoGlo (Promega Madison, WI) assay[228]. The NanoLuc substrate was added to each well and incubated at room temperature for 3 min. Luminescence was then read using the using the VarioskanFlash™ microplate luminometer. Viral titers were reported as relative light units (RLU). Readings were taken at 0, 2,4,6,8 hrs p.i to establish growth curves.

**Time of addition assay**

MDCK cells cultured on 96-well plates (5x10^3cells/well) and incubated at $37^oC$ 5%$CO_2$ overnight. At 0 hr p.i, cells were washed and then infected with 128 HAUs of influenza virus A/Puerto Rico/8/1934-PA-NanoLuciferase. After 1 hr p.i, cells were washed with PBS to remove any unbound virus and media was replaced. At -1-0hrs p.i (pre-infection) and 0-1hrs (attachment) cells were treated with $OSCN^-$. At 8 hrs p.i cells were washed again and the NanoLuc substrate was

added to each well and incubated at room temperature for 3 min. Luminescence was then read using the using the VarioskanFlash™ microplate luminometer.

**Statistical analysis**

Significance among multiple samples was calculated using One-way or Two-Way ANOVA followed by Tukey's or Sidak's multiple comparison post-hoc tests. Significance between two samples was calculated using Man-Whitney's test or unpaired t-test. Statistical analysis was performed using Prism 6 for Windows version 6.07 software. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$

## Results

### NHBE derived $H_2O_2$ is sufficient to inactivate extracellular influenza virus

We had previously shown that H1N2 is inactivated by NHBE cells supplemented with LPO and $SCN^-$[9] . To determine if other strains of influenza could be inactivated, fully differentiated NHBE cells cultured on air-liquid interface were infected in the apical chamber with one of the following influenza strains A/California/07/2009 (H1N1), A/turkey/KS/4880/80 (H) (H1N1), A/Wisconsin/67/2005 (H3N2), B/Florida/4/2006 Yamagata at 1 MOI for 1 hr. The apical chambers were supplemented with physiological levels of LPO (6.2ug/mL), $SCN^-$(400μM) and catalase (700 U). Similar results are shown across all four strains. In figure 5.1, we saw a 1-2 log decrease when LPO/ $SCN^-$ were added to the apical chamber of NHBE cells. The addition of the $H_2O_2$ scavenger catalase inhibited the antiviral effects of the DUOX/$H_2O_2$/LPO/$SCN^-$ antimicrobial system. To confirm $OSCN^-$ was not facilitating viral uptake leading to a decrease in extracellular viral titers, we performed immunofluorescence. After infection, we allowed the virus to infect and replicate for 24 hrs and then stained for nucleoprotein (NP), which would only be seen in replicated virus. We saw a dramatic decrease in the number of infected cells when LPO/$SCN^-$ was added to the apical chamber (Fig 5.2). This once again supports previous data that DUOX derived $H_2O_2$ is necessary and sufficient for antiviral activity[9]. Additionally, this data demonstrates that while differential levels of inactivation are seen, the antiviral effect of $OSCN^-$ is a general mechanism that is capable of inactivating three major influenza A subtypes (H1N, H3N2, H1N2) and influenza B virus.

**Cell-free derived $OSCN^-$ reduces viral titers of both influenza A and B**

While the use of NHBE cells to drive the $H_2O_2$ production for the DUOX/$H_2O_2$/LPO/$SCN^-$ system is physiologically similar to an *in vivo model, i*t is a slow process, NHBE cells take 4-5 weeks to become fully differentiated and capable of producing $H_2O_2$. In order to overcome this hurdle, we utilized a cell-free system capable of producing $OSCN^-$. We validated this system previously by demonstrating that it produced sufficient $OSCN^-$ to inactivate A/swH1N2[229]. It was also found that each individual component of the system (LPO, $SCN^-$, $H_2O_2$) did not significantly alter viral titers[229]. In figure 5.3, we show the 17 different strains of influenza virus we tested in the cell-free system and their susceptibility to $OSCN^-$ after a 1 hr treatment. Susceptibility is defined as reduction in viral titers based on the +Catalase condition – $OSCN^-$ condition viral titer. Catalase is a scavenger of $H_2O_2$; therefore, it inhibits the formation of $OSCN^-$ by preventing LPO from utilizing $H_2O_2$. While we were able to show at least a 1-log reduction in all strains tested, the susceptibility of the strains varied quite a lot. In regards to influenza A virus, we tested 3 different subtypes H1N1, H3N2, and H1N2. We found that in general, all of the H1N1 strains tested were particularly susceptible to $OSCN^-$ exposure except for A/Brisbane/59/2007 H1N1, which only demonstrated a 2-log reduction on average compared to 4-6 reductions of the other H1N1 strains (Fig 5.3). Within the H1N1s we tested two different mutant strains, A/Mississippi/3/01 H275Y and A/Denmark/528/09 275Y, which are both resistant to neuraminidase inhibitors and neither demonstrated a significant difference from the A/Mississippi/3/01 275H WT

counterpart (Fig 5.3). We also tested A/turkey/KS/4880/80 (H) which was isolated from an avian host but of swine origin, suggesting that $OSCN^-$ susceptibility is not exclusive to human isolates[230]. For further validation of this hypothesis, we tested two different H1N2 swine isolates A/Swine/Illinois/02860/09 and A/Swine/OH/115. Both swine strains showed a similar susceptibility of ~5.7-log reduction (Fig 5.3). We investigated the susceptibility of 4 different H3N2 strains A/Aichi/2/68, A/Hong Kong/8/68, A/Wisconsin/67/2005, A/Texas/50/2012. Overall, we found that the H3N2 subtype was more resistant to $OSCN^-$ 1.2-4.2-log reductions than either H1N2 or H1N1 (Fig 5.3). The A/Aichi/2/68, A/Hong Kong/8/68 were the most resistant strains we tested showing ~1.25-log reduction (Fig 5.3). It is important to note that while these H3N2 strains are the most resistant; their 1-log reduction in viral titers still represents over 90% clearance of influenza infectivity. Finally, we tested $OSCN^-$ against influenza B, a species that almost exclusively infects humans. Influenza B -unlike influenza A- is split into two different lineages, Yamagata and Victoria based on the antigenic properties of the HA[108]. Both lineages showed moderate to high susceptibility with 3-7.5-log reductions in viral titers (Fig 5.3). However, the Victoria lineages (B/Brisbane/33/2008 and B/New York/1056/2003) had increased resistance compared to the Yamagata lineage (B/Great Lakes/1739/1954 and B/Florida/4/2006) (Fig 5.3). Overall, based on the species and subtypes we have tested to date, we concluded that $OSCN^-$ has a general antiviral effect against all tested strains but differential levels of susceptibility do exist.

**$OSCN^-$ does not inhibit neuraminidase activity of influenza virus**

The most common antivirals used to treat and prevent influenza infection are neuraminidase inhibitors [113]. Neuraminidase inhibitors attach to the active site on the NA protein inhibiting the enzymatic function[113]. We investigated the potential effects of $OSCN^-$ on influenza NA activity. The NA inhibition assay was performed by measuring the intensity of fluorescence resulting from the cleavage of the substrate MUNANA by the NA. We tested NA activity of purified whole virus suspension of H1N1 (A/California/07/2009, A/Puerto Rico/8/34), H3N2 (A/Aichi/2/68), and Influenza B (B/Florida/4/2006 Yamagata, B/Brisbane/33/2008 Victoria). In fig 5.4, we saw no significant inhibitory effect between virus treated for 1 hr with $OSCN^-$ or LPO+ $SCN^-$+$H_2O_2$+Catalase compared to our virus alone control. To ensure our results and assay were valid, we added the neuraminidase inhibitor Oseltamivir (Tamiflu) to each of the strains tested, ran the neuraminidase activity assay, and saw complete inhibition across all strains tested (Fig 5.4). These results suggest that the neuraminidase surface protein is unaffected by $OSCN^-$ exposure as we saw no difference across the H1N1, H3N2 and influenza B strains that were tested.

**$OSCN^-$ inhibits influenza virus from binding to HuRBCs preventing hemagglutination**

HA is the other surface protein on influenza virus and it is responsible for attachment to host cells. In order to test if $OSCN^-$ is causing a disruption between

the virus-receptor interactions, we utilized a hemagglutination inhibition assay (HAI). When exposed to red blood cells, HA present on the virion binds to RBCs, creating a lattice structure form called hemagglutination. We tested the hemagglutination potential of H1N1 (A/California/07/2009, A/Puerto Rico/8/34), H3N2 (A/Aichi/2/68), and Influenza B (B/Florida/4/2006 Yamagata, B/Brisbane/33/2008 Victoria). In figure 5.5 we saw a significant difference between virus treated for 1 hr with $OSCN^-$ compared to LPO+ $SCN^-$+$H_2O_2$+Catalase or virus alone controls. $OSCN^-$ treatment of virus resulted in a 2-6-fold decrease in HAU compared to the virus alone control for each strain (Fig 5.5). This data suggests that $OSCN^-$ has an inhibitory effect on the HA-sialic acid interaction.

**$OSCN^-$ does not inhibit attached influenza virus from causing hemolysis**

HA's other major function is to facilitate fusion to the endosome and breaking it open once the virus has been endocytosed by the host cell[113]. Our previous results demonstrated that $OSCN^-$ was interacting with HA, so we decided to study if $OSCN^-$ was interfering with the fusion process. To study this process, a hemagglutinin mediated hemolysis assay was utilized. This assay measures the ability of influenza virus to induce membrane fusion and lysis of red blood cells under low-pH conditions, independently of its binding to RBCs. Without an endosome, the virus is unable to drive the fusion process on its own. To trigger lysis of the HuRBCs, acetic acid was added to facilitate the HA conformational change thereby creating pores in the HuRBCs releasing hemoglobin in the solution. We utilized a pre- and post-treatment plan as follows; the pre-treatment

group, virus was incubated with $OSCN^-$ for 30 min and was then exposed to HuRBCs for 30 min to allow for sufficient binding. In the post-treatment group, the virus was incubated with HuRBCs for 30 min then $OSCN^-$ was added for 30 min after the virus was already bound to the RBCs. The pH in both groups was lowered to 5.5 pH to allow for fusion and lysis of the HuRBCs. Figure 5.6 demonstrates that the pre-treatment group reveals a decreased lysis effect while the post-treatment group resulted in no inhibition of lysis. A 40-60% decrease was seen in both strains tested A/Aichi/2/68 H3N2, and B/Brisbane/33/2008 Victoria (Fig 5.6). The addition of catalase to the generation step of $OSCN^-$ once again showed a rescue effect allowing the virus to cause hemolysis similar to the virus alone control (Fig 5.6). These results support our previous data with the HAI assay whereby treating virus with $OSCN^-$ lead to a decrease in the virus's ability to bind to HuRBCs (5.5). Virus that was already attached to the HuRBCs however was unaffected by $OSCN^-$ treatment suggesting that while $OSCN^-$ interacts with HA it does not directly alter the ability of the virus to fuse and cause lysis.

**$OSCN^-$ inhibits alexa-488 labeled influenza virus from binding to MDCK cells**

To further, assess the potential interaction between $OSCN^-$ and the virus' ability to attach to host cells, we utilized a fluorescent binding assay. Due to influenza's small size and replication cycle (6-8 hrs), our immunofluorescence assay is not able to detect the early phases of influenza infection. We therefore tagged the virus with Alexa-488, which binds to the ester bonds found on the viral envelope. We first validated that labeling of the virus does not alter the virus' ability

to bind via HA assay and plaque assays (data not shown) in which we saw no difference in the virus' ability to bind. We first labeled A/Aichi/2/68 H3N2 with Alexa-488, then exposed the virus to $OSCN^-$ and LPO+ $SCN^-$+$H_2O_2$+Catalase for 30 min, and then incubated the virus with MDCK cells for 4 hrs at 4°C. We had to carry out the binding phase of the experiment at 4°C to ensure that binding occurred but endocytosis would not happen. In figure 5.7 we were able show a ~50% reduction in the virus's ability to bind to MDCK cells compared to our virus alone and catalase control. These results support our previous results where we see ~50% reduction in binding when virus is exposed to $OSCN^-$.

**$OSCN^-$ inhibitory effect is due to an interaction with influenza virus and not the host cells**

To investigate the antiviral mechanism of $OSCN^-$, we used a time-of-addition assay with one viral replication cycle to assess replication potential. A time-of-addition assay allows us to introduce $OSCN^-$ at different stages of the viral infection to determine where it is interacting with the virus. We used a viral strain (A/Puerto Rico/8/34/NLuc) that has been modified with the gene for NanoLuc, a small and very bright-engineered variant of luciferase encoded as a polyprotein in the PA gene[228]. The reporter virus enables rapid measurements of viral titers, requiring as little as 8 hrs compared to the 3 days needed for traditional approaches. We validated this early time point in figure 5.8A, where we performed a growth curve assay, our results showed that as early as 6 hrs we were seeing a reliable luminescent signal demonstrating viral replication was occurring. Figure

5.7B demonstrates our treatment schematic, where the -1-0hr is the cells being treated with $OSCN^-$ without virus, to confirm our previous results that $OSCN^-$ is not interacting with surface receptors on cells. At time 0, cells were washed to remove $OSCN^-$ and virus was added for 1 hr. Our 0-1hr condition is co-incubated virus with $OSCN^-$ on the cells for 1 hr. After 1 hr, all cells are washed to remove any unbound virus and residual $OSCN^-$ that was present. Media was added to each well and the cells were incubated for an additional 7 hrs. At 8 hrs p.i, the luminescent substrate was added and luminescence of each condition was measured. When $OSCN^-$ was added at the -1-0hr p.i (Fig 5.8B) no reduction in viral replication was seen, supporting the notion that $OSCN^-$ is interacting with the virus and not the host receptors. However, when $OSCN^-$ was added at the 0-1hr p.i condition, a significant reduction in viral replication was seen. This effect was rescued by the addition of catalase, which prevented the formation of $OSCN^-$ (Fig 5.8B). This suggests that $OSCN^-$ disrupts the early phase of viral infection by interacting with the virion before it attaches to the host cell, leading to a decrease in downstream viral replication.

## Discussion

Our previously published data[9] demonstrated that NHBE cell-derived $OSCN^-$ was capable of reducing viral titers of swH1N2 *in vitro*. In this study, we determined that this result was not strain-specific. Influenza A virus H1N1, H3N2 and influenza B viruses also showed a reduction in extracellular viral titers when exposed to NHBE cell-derived $OSCN^-$ (Fig 5.1). While reduction in viral titers was an encouraging result, it did not exclude the possibility that $OSCN^-$ was leading to an increase in attachment and/or endocytosis. To exclude this possibility, we performed immunofluorescence 24 hrs after infection to determine the number of infected cells. We utilized staining for the NP protein that is only seen in actively replicating virus. Our results (Fig 5.2) showed a decrease in the number of infected cells. Taken together, this data supported our previous data[9] and confirmed our hypothesis that $OSCN^-$ is inactivating influenza virus leading to a decrease in the number of infected cells. We utilized a cell-free method to generate $OSCN^-$ which we previously described[229]. We tested 17 different strains of influenza virus (Fig 5.3) encompassing H1N1, H1N2, H3N2 and influenza B virus of Yamagata and Victoria lineages; we saw a broad effect against all strains leading to reduction in viral titers. Interestingly the susceptibility of these strains varied 1.2-7.5 reduction in viral titers (Fig 5.3). In general, we found that viruses expressing an H1 HA were more susceptible than the H3 HA strains. The potential explanation for this is unclear now, but redox-sensitive disulfide bonds may be different among HA subtypes, could help explain this observation[231]. It is well described in the literature that the HA receptor-binding site is a shallow depression in the globular head at

the membrane-distal end of HA, it is surrounded by structural elements designated 220 loop, 130 loop, 150 loop and 190 helix[232]. Looking at the differences in these regions and comparing them to strains with differential susceptibilities would likely shine light on potential targets of $OSCN^-$. Nonetheless, how $OSCN^-$ exerts its inhibitory effect on the virus remains elusive. Currently, the most effective antivirals against influenza target the surface protein neuraminidase [223]. We found that $OSCN^-$ did not have any inhibitory effect on viral NA activity (Fig 5.4). This was further supported by the viral titer reductions (Fig 5.3) of NA inhibitor resistant strains of the virus A/Mississippi/3/01 H275Y and A/Denmark/528/09 275Y, which showed no difference from the A/Mississippi/3/01 275H WT. The H275Y mutation first appeared in A(H1N1)pdm09 where it became highly resistant to Oseltamivir[233],[234]. These results are quite encouraging as antiviral drug resistance is on the rise; and $OSCN^-$ mechanism of action is independent of NA inhibition and is effective at reducing viral titers of resistant strains. The next likely target of $OSCN^-$ interaction is the HA surface protein, which is responsible for attachment to the host receptor and endosomal fusion. We used a modified HAI assay where we saw between a 2-6 fold reductions in binding to Human red blood cells (HuRBCs) (Fig 5.5). This is the first data to point to a potential mechanism of action of $OSCN^-$. Additionally, this is in direct conflict with a study that showed the same reduction in viral titers but did not show any inhibition of HA-mediated binding to RBCs[235]. Experimentally, the conditions were different as guinea pig RBCs were used in those experiments compared to our use of HuRBCs, and their method for $OSCN^-$ generation was different. We believe our results are valid as we performed

additional assay that supported an inhibition in binding. In our fluorescently labeled binding assay we once again saw ~50% reduction in binding of influenza virus exposed to $OSCN^-$ but this time to MDCK cells (Fig 5.7). This was further supported by a time-of-addition assay. This assay allows for addition of "treatment" at different phases in the viral replication cycle and has been utilized in a wide variety of drug testing experiments against influenza virus[226, 236, 237]. In this assay, we used an engineered NanoLuc virus allowing for detection of viral replication as early as 6 hrs[228]. This assay was crucial as normal IFA techniques take much longer for replicated virus to be observed. Once again, this assay showed influenza virus exposed to $OSCN^-$ before it attached to host cells had a significant inhibitory effect on the downstream replication potential of the virus (Fig 5.8). One potential explanation for the reduction in viral titers was that $OSCN^-$ was interacting with the cells host receptors and not the virus itself. If host receptors were altered in our previous assay, it would give the illusion of the virus being inactivated. The NanoLuc experiments allowed us to rule that out as treating the cells in the absence of the virus and then infecting the cells did not lead to any changes in the downstream replication of the virus, supporting our hypothesis that $OSCN^-$ is acting directly on the virus and not the host cell.

To further investigate $OSCN^-$ mechanism of action, HA-mediated hemolysis assay of HuRBCs was performed. Viral attachment and HA conformational changes are the two events required for hemolysis. It has already been described that small molecules can bind to HA and block the fusion process by either stabilizing or destabilizing HA[238, 239, 240, 241]. Our results indicate that $OSCN^-$

interferes with attachment to the HuRBCs but there is no pH-induced conformational change of HA which is required for virus-induced hemolysis (Fig 5.6). We believe that our *in vitro* results strongly point to an interaction between HA and $OSCN^-$ that ultimately has an inhibitory effect on the virus ability to infect and replicate. However, a question still looms, we see viral titer reductions reaching several logs (~99% inhibition) but the greatest inhibition in binding is only ~70%. Several plausible answers exist to explain the discrepancies we see in viral titer reductions compared to binding inhibition. First, in our assays involving binding to RBCs, the virus and RBCs are not in a 1:1 ratio (1 virus is not only binding to 1 RBC). For hemagglutination to occur it is required for a virus to be bound to multiple RBCs and other viruses. We decided to perform our studies with human RBCs as influenza preferentially binds to specific host erythrocytes[242]. In the case of human isolates, which were used in this study, the preferred binding receptor is the Sa α2,6[243]. According to a previous study, there are predominately Sa α2,6 receptors on human RBCs[207]. A follow up study found that human RBCs perform to a similar level as turkey RBCs, which is the CDC standard RBC of choice[244]. One potential issue with human RBCs is that if improperly washed, anti-HA antibodies could be present from a previous infection and lead to a skewing of results. However, we are confident in the controls we utilized and have ruled that out as a factor in our experiments. The entire surface of the virus is covered in HA and NA protein and it is highly unlikely that $OSCN^-$ would be able to interact and inhibit every single surface protein on a given virion. It is estimated that each virion has 340-400 HA trimers present[245, 246]. Interactions with influenza and the host are multivalent,

meaning there are multiple ligand-receptor bonds acting in parallel to each other[247] not just a 1:1 ratio. In light of this, the discrepancies we see could be partially explained. In a mixed solution such as the HAI assay, even if ~90% of a single virion HAs were inhibited it would still be able to bind to multiple RBCs creating the hemagglutination effect due multiple low affinity bonds. This differs from our plaque assays and NHBE experiments, where the binding targets are static and a higher affinity bond is required for virion uptake/endocytosis and replication to occur [248]. This is further supported by recent studies that have shown influenza virus is able to "crawl" along the epithelium, using the HA-receptor exchange mechanism to find a spot that multiple HA are able to bind and facilitate endocytosis [249]. If $OSCN^-$ is damaging HA it could potentially be more difficult for the virus to find an optimal binding location to enter host cells. An alternative possibility is the viral envelope, which is made of lipid components could be sensitive to oxidative damage[250] leading to $OSCN^-$ breaching the viral envelope and damaging the internal vRNA leading to an inhibition in replication. Other targets of $OSCN^-$ could be cysteine rich regions in viral proteins. HA is rich in cysteine residues and disulfide bonds, and it has been reported that changes in cysteine residues can lead to an alteration in HA function[251]. In bacteria, one of the reported bacterial targets of $OSCN^-$ are cysteine residues found in glycolytic enzymes, such as glyceraldehyde triphosphate dehydrogenase and hexokinase leading to glucolysis-mediated inhibition of bacterial growth [252]. Based on the current literature, cysteine residues on the surface of microbes seem to be the likely target of $OSCN^-$. Our *in vitro* results emphasize that influenza viruses are inactivated by the Duox-

$H_2O_2$/LPO/$SCN^-$ system in both the cell-free and NHBE system in less than 60 minutes. The speed and efficiency of this mechanism is rare in the innate immune system.

Ultimately, this work has revealed a potential mechanism for $OSCN^-$ interaction with influenza virus. Future studies should begin to look for conformation changes to HA, more specifically at the receptor binding sites. This work does not exclude other potential mechanisms such as inhibition of endocytosis or degradation of the viral envelope. It is likely that $OSCN^-$ has a compounding effect on multiple functions, ultimately leading to the decrease in the viral titers we see. The literature fully supports the hypothesis that the DUOX-H2O2/LPO/$SCN^-$ is a powerful system employed by the innate immune system. One paradoxical question does remain, how does a broadly antimicrobial system with seemingly limited resistance raised against it even exist? It is clear commensal microbes have adapted to coexist with this and other antimicrobial systems that target pathogenic microbes. The answer likely lies in its simplicity, antiviral drugs and vaccines both have very specific targets that drive an intense selective pressure. $OSCN^-$ on the other hand is a relatively simple negatively charged ion that clearly reacts with a variety of different targets hence its broad action against viruses, bacteria, fungi and parasites.

**Acknowledgments**

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## Figures and legends

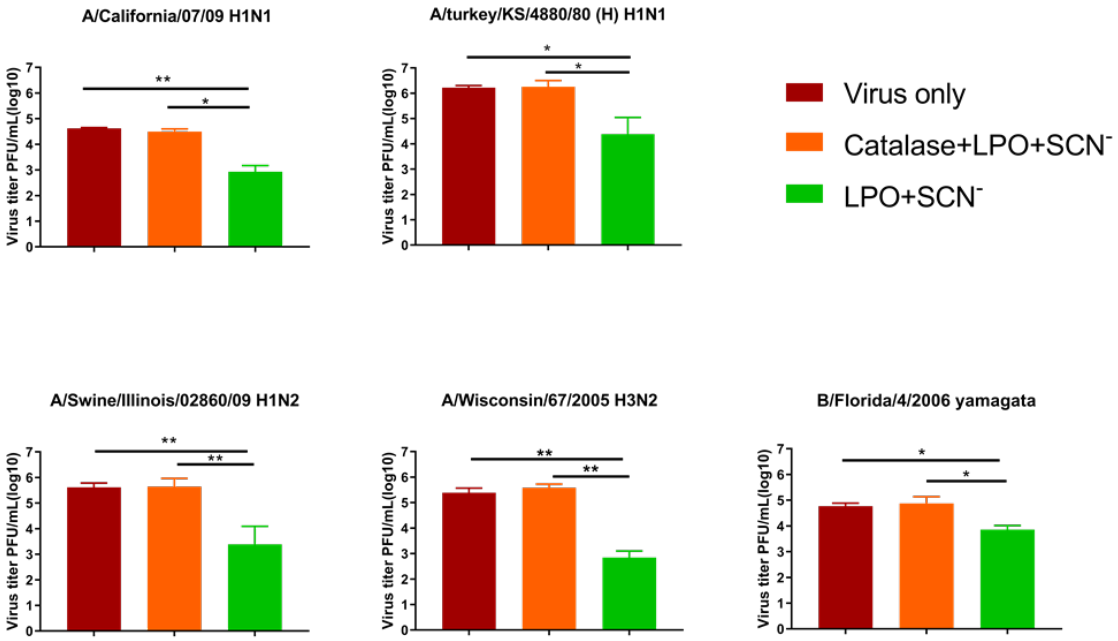


**Figure 5.1 DUOX-derived $H_2O_2$ catalyzes the formation of $OSCN^-$ in NHBE cells capable of inactivating IAV H1N2 and H3N2.** ALI cultures of differentiated human NHBE cells were exposed to 50000 PFU of A/California/07/2009 (H1N1), A/turkey/KS/4880/80 (H) (H1N1), A/Wisconsin/67/2005 (H3N2), B/Florida/4/2006 Yamagata of in presence or absence of, LPO (6.2ug/mL), $SCN^-$ (400 μM) or catalase (CAT, 700 U) in the indicated combinations. After 1 hr incubation supernatants were collected and concentration of viable virus particles was determined by PFU assay using MDCK cells. Mean+/-S.E.M., N=2-4 independent experiments were performed in duplicate. ANOVA, Brown-Forsythe test, Tukey's multiple comparisons. *, $p<0.05$; **, $p<0.01$;. IAV, influenza A virus; PFU, plaque-forming uni

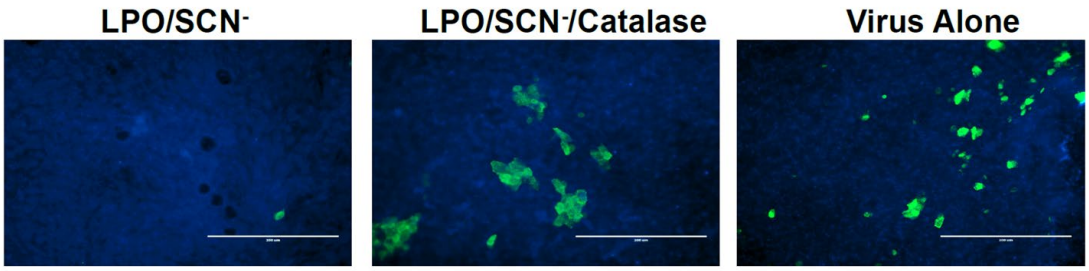


**Figure 5.2: The DUOX1/LPO/SCN$^-$ system is able to inactivate extracellular IAV (H1N2) *in vitro* leading to a decrease in infected cells.** ALI cultures of differentiated human NHBE cells were exposed to 5000 PFU of H1N2 IAV (swH1N2) in presence or absence of LPO (6.2ug/mL), SCN$^-$ (400 μM) or catalase (CAT 700U) in the indicated combinations. After 1 hr, incubation supernatants were collected. 24hrs p.i cells were fixed with 4% paraformaldehyde. Green staining indicates the presence of viral NP. The nuclei were stained with DAPI. Merged images of the viral protein and DAPI-stained nuclei are shown. Images are representative of 3 independent experiments performed in duplicate.

| Viral Strain | Susceptibility to OSCN⁻ (reduction in viral titers pfu/mL log10) | |
|---|---|---|
| A/Denmark/528/09 H1N1 275Y | 3.9 | |
| A/turkey/KS/4880/80 (H) | 5.6 | |
| A/Brisbane/59/2007 H1N1 | 2.0 | |
| A/California/07/2009 H1N1 | 5.6 | |
| A/Mississippi/3/01 275H WT | 6.1 | |
| A/Mississippi/3/01 H275Y | 6.4 | |
| A/Puerto Rico/8/34 H1N1 | 4.4 | H1N1 |
| A/Swine/Illinois/02860/09 H1N2 | 6.1 | |
| A/Swine/OH/115 H1N2 | 5.4 | H1N2 |
| A/Aichi/2/68 H3H2 | 1.2 | |
| A/Hong Kong/8/68 H3N2 | 1.3 | |
| A/Wisconsin/67/2005 H3N2 | 3.3 | H3N2 |
| A/Texas/50/2012 H3N2 | 4.2 | |
| B/Great Lakes/1739/1954 Yamagata | 6.0 | |
| B/Florida/4/2006 Yamagata | 7.5 | |
| B/Brisbane/33/2008 Victoria | 3.7 | Influenza B |
| B/New York/1056/2003 Victoria | 3.2 | |


**Figure 5.3: OSCN⁻ reduces the viral titers of influenza A and B strains in the cell-free system.** Susceptibility of influenza A & B viruses to OSCN⁻. Susceptibility is defined as reduction in viral titers between OSCN⁻+Catalase treated – OSCN⁻ treated virus (log10PFU/mL). N=3-6 independent experiments performed in duplicate for each strain shown.

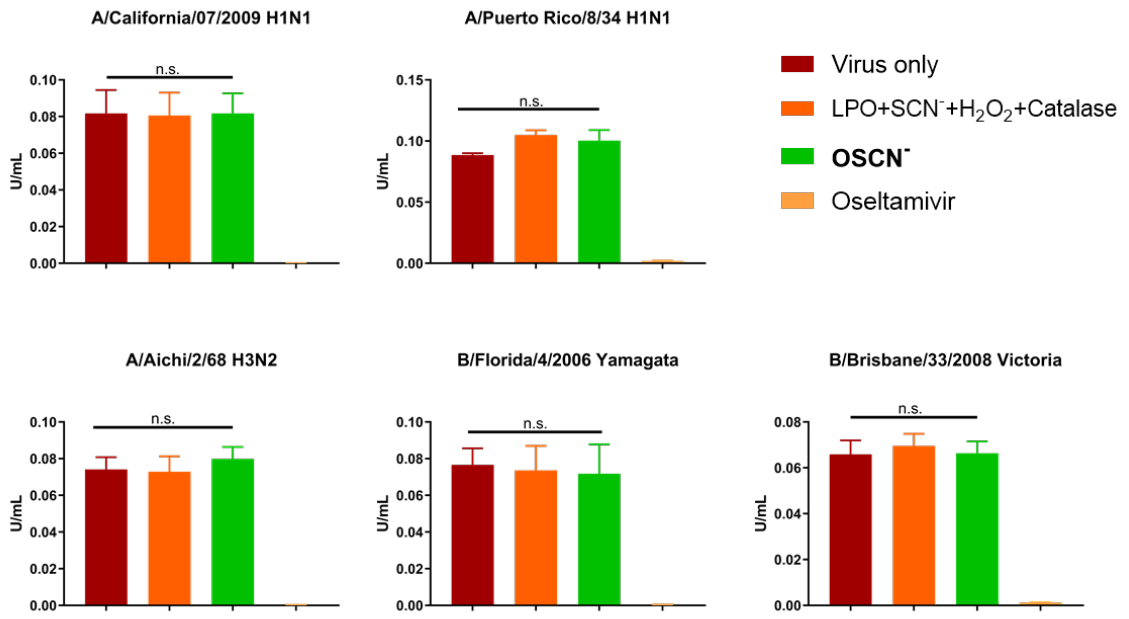


**Figure 5.4: $OSCN^-$ has no inhibitory effect on neuraminidase activity in influenza A and B viruses.** Effect of $OSCN^-$ on the NA activity of influenza virus. Virus (5x10^5 PFU) B/Florida/4/2006 Yamagata, B/Brisbane/33/2008 Victoria, H3N2 (A/Aichi/2/68), H1N1 A/California/07/2009 and A/Puerto Rico/8/34 were incubated with $OSCN^-$, LPO+ $SCN^-$+$H_2O_2$+Catalase or Oseltamivir for 1hr at 37°C. MUNANA (200µM) was added and incubated for 1hr at 37°C. Stop solution was used to terminate the reaction and the fluorescence was measured with a microplate reader at an excitation of 365nm and an emission wavelength of 450nm. Samples were then tested using a neuraminidase assay kit. Mean+/- S.E.M., N=4 independent experiments in triplicate. ANOVA, Brown-Forsythe test, Tukey's multiple comparisons.

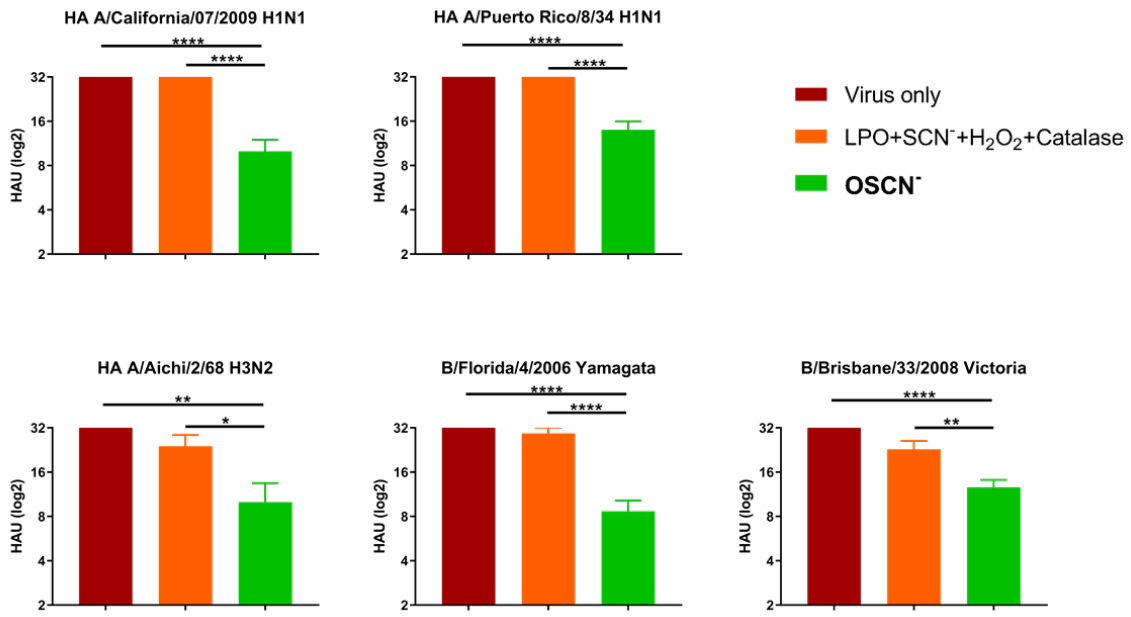


**Figure 5.5: $OSCN^-$ decreases influenzas ability to bind to HuRBCs.** Influenza virus (32HAU) B/Florida/4/2006 Yamagata, B/Brisbane/33/2008 Victoria, H3N2 (A/Aichi/2/68), H1N1 A/California/07/2009 and A/Puerto Rico/8/34 were incubated with $OSCN^-$or LPO+$SCN^-$+$H_2O_2$+Catalase for 1hr at 37°C. Human red blood cells (HuRBCs) were incubated with influenza virus for 1hr at RT. Blood from three different donors (A+, O+, B+). Mean+/-S.E.M., N=4-7 independent experiments performed in duplicate. ANOVA, Brown-Forsythe test, Tukeys multiple comparisons. *, $p<0.05$; **, $p<0.01$; ****,$p<0.0001$

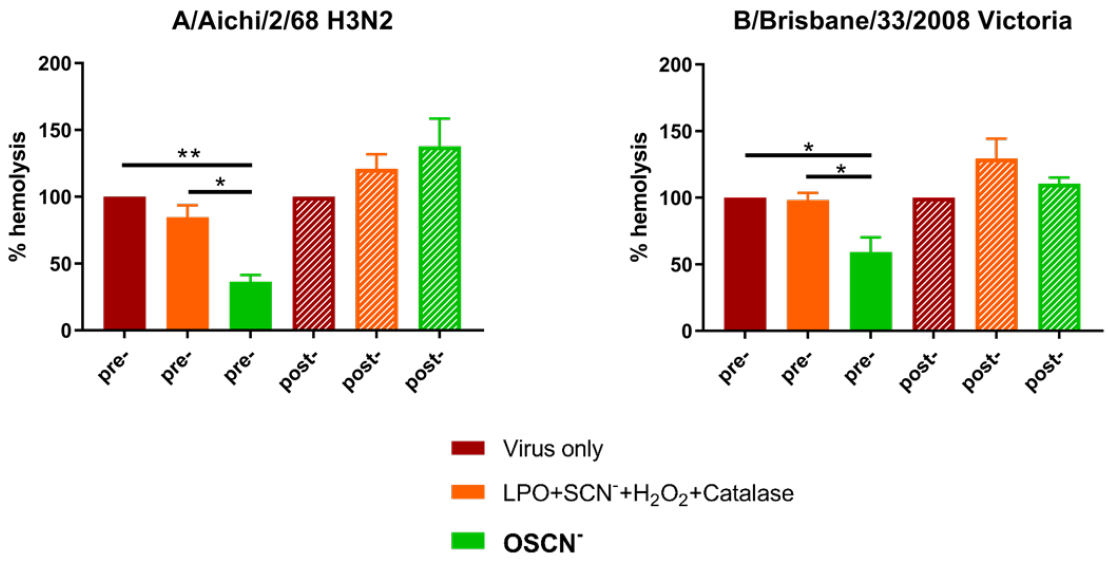


**Figure 5.6: $OSCN^-$ does not alter attached influenza virus ability to cause pH-induced lysis.** O $SCN^-$does not alter attached influenza virus ability to cause pH-induced lysis. Influenza virus, (A) H3N2 (A/Aichi/2/68) and (B) B/Brisbane/33/2008 Victoria were pre-treated with $OSCN^-$ or LPO+ $SCN^-$+$H_2O_2$+Catalase for 30 min at 37°C. HuRBCs were incubated with treated virus for 30 min at 37°C. Post-treated influenza virus was incubated with HuRBCs for 30 min at 37°C. Influenza virus bound to the RBCs was treated with $OSCN^-$ or LPO+ $SCN^-$+$H_2O_2$+Catalase for 30 min at 37°C. The mixtures were then acidified (pH 5.2). The suspension was then incubated for 1hr at 37°C. The supernatant was collected by centrifugation and released hemoglobin was measured at 540 nm. Data are expressed as percentage relative to the untreated virus control. Mean+/-S.E.M., N=5 independent experiments performed in triplicate. ANOVA, Brown-Forsythe test, Tukey's multiple comparisons. *, $p<0.05$; **, $p<0.01$.

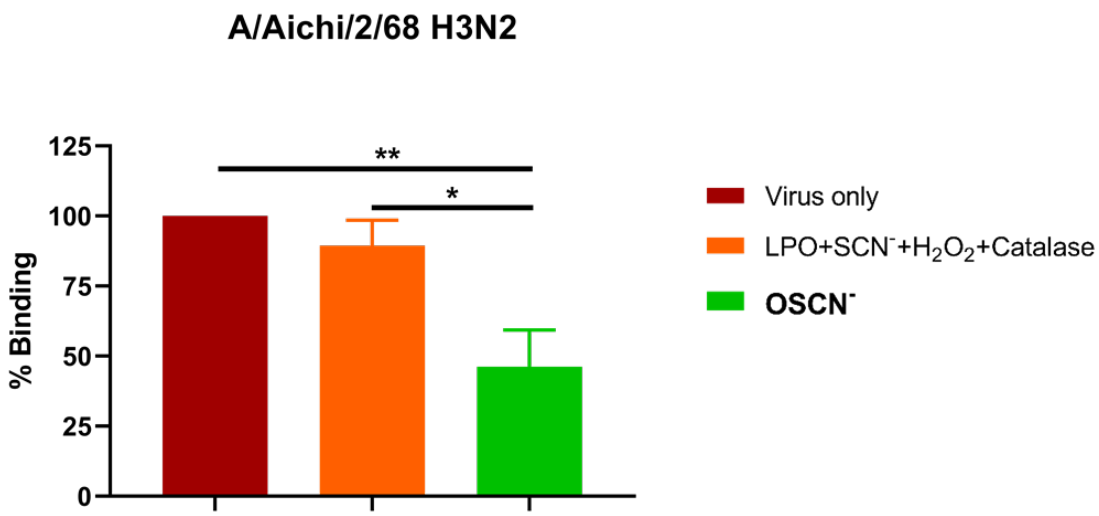


**Figure 5.7: $OSCN^-$ reduces binding of Alexa 488 labeled virus (H3N2) to MDCK cells.** $OSCN^-$ reduces binding of Alexa 488 labeled virus (H3N2) to MDCK cells. Virus attachment to MDCK cells was measured by fluorescence. MDCK cells were infected with 128 HAUs of labeled influenza virus A/Aichi/2/68 at 4°C for 4hrs to allow sufficient binding but no entry. MDCK cells were then washed with ice-cold PBS to remove any unbound virus. Fluorescence was measured via plate reader VarioskanFlash™ at an excitation of 495nm and emission of 519nm. Data are expressed as percentage relative to the untreated virus control. Mean+/-S.E.M., N=5 independent experiments performed in triplicate. ANOVA, Brown-Forsythe test, Tukey's multiple comparisons. *, $p<0.05$; **, $p<0.01$.

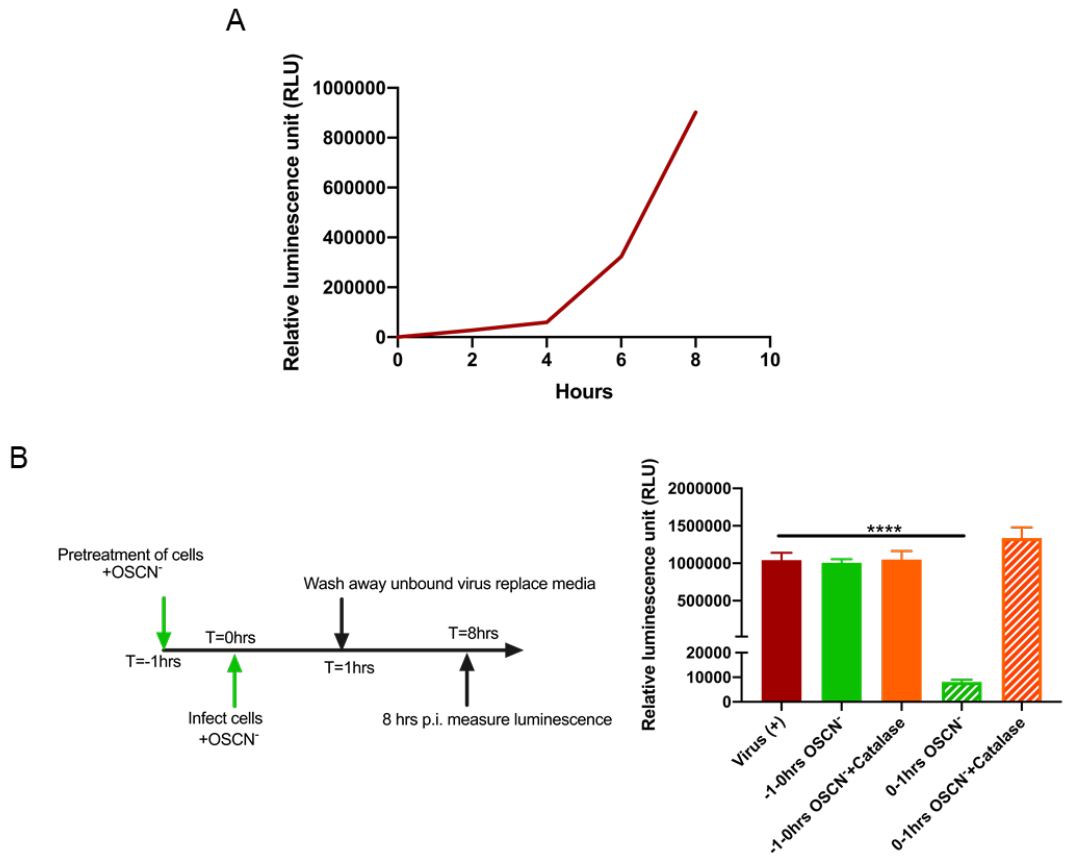


**Figure 5.8: OSCN- treatment does not alter host receptors but interacts directly with the virus to reduce attachment and downstream replication.** OSCN- treatment does not alter host receptors but interacts directly with the virus to reduce attachment and downstream replication. (A) MDCK cells cultured on 96-well plates were infected with 128 HAUs of influenza virus A/Puerto Rico/8/1934-PA-NanoLuciferase (NLuc). The NanoLuc substrate was added to each well and Luminescence was then measured using the VarioskanFlash™ microplate luminometer. Readings were taken at 0, 2, 4, 6, 8 hrs p.i to establish growth curves. (B) MDCK cells were infected with 128 HAUs of influenza virus A/Puerto Rico/8/1934-PA-NanoLuciferase (NLuc) at time 0, OSCN- was added to the cells for 1hr at the indicated times. At 8hrs pi cells were washed again Luminescence

was then read. Mean+/-S.E.M., N=4 independent experiments performed in triplicate. ANOVA, Brown-Forsythe test, Tukey's multiple comparisons. ****,p<0.0001

# CHAPTER 6

# OXIDATIVE KILLING OF ENCAPSULATED AND NONENCAPSULATED *STREPTOCOCCUS PNEUMONIAE* BY LACTOPEROXIDASE-GENERATED HYPOTHIOCYANITE

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**Abstract**

*Streptococcus pneumoniae* (Pneumococcus) infections affect millions of people worldwide, cause serious mortality and represent a major economic burden. Despite recent successes due to pneumococcal vaccination and antibiotic use, Pneumococcus remains a significant medical problem. Airway epithelial cells, the primary responders to pneumococcal infection, orchestrate an extracellular antimicrobial system consisting of lactoperoxidase, thiocyanate anion and hydrogen peroxide. Lactoperoxidase oxidizes thiocyanate-using $H_2O_2$ into the final product hypothiocyanite that has antimicrobial effects against a wide range of microorganisms, but its effect on Pneumococcus has never been studied. Our aim was to determine whether hypothiocyanite can kill *Streptococcus pneumoniae.* We measured bacterial killing in a cell-free *in vitro* system where pneumococcal survival was determined by colony forming units on agar plates and bacterial growth inhibition by measuring changes in optical density of bacteria in liquid cultures. Our results indicate that hypothiocyanite generated by lactoperoxidase exerted robust killing of both encapsulated and nonencapsulated pneumococcal strains. Killing of *Streptococcus pneumoniae* by a commercially available hypothiocyanite-generating product was even more pronounced than that achieved with laboratory reagents. Catalase, an $H_2O_2$ scavenger, inhibited pneumococcal growth by hypothiocyanite under all circumstances. $H_2O_2$ generated by Pneumococcus had only minimal effect on the antimicrobial action of hypothiocyanite as a bacterial mutant deficient in pyruvate oxidase, the main bacterial $H_2O_2$ source, had somewhat enhanced susceptibility to hypothiocyanite

than its wild-type strain. Overall, we found that numerous pneumococcal strains are susceptible to lactoperoxidase-generated hypothiocyanite *in vitro*, which presents an opportunity to explore hypothiocyanite as a potential, anti-pneumococcal therapy.

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## Introduction

*Streptococcus pneumoniae (Spn)* is a leading cause of bacterial infections such as otitis media, pneumonia, septicemia and meningitis. [2, 3]. Colonization can occur at any point in a person's life but occurs most commonly in young children where *Spn* prevalence reaches over 50% in hosts 2-3 years old [157]. Worldwide, *Spn* is a major cause of infant mortality with 1.2 million deaths reported every year[3, 253]. Current pneumococcal vaccines target the capsular polysaccharide of *Spn*, but these vaccines only provide serotype-specific protection [254]. *Spn* infections can also be controlled with antibiotics, but widespread antibiotic use has led to accelerated antibiotic resistance in *Spn*[255]. These challenges have led to the need for novel therapeutics and a better understanding of *Spn* interactions with the host.

The airway epithelium represents one of the largest physical and immune barriers against airborne microbes such as *Spn* [256]. Lactoperoxidase (LPO) is a heme peroxidase found in the airway surface liquid (ASL) where it performs its antimicrobial activity[5]. The LPO-based antimicrobial system requires two other components to function properly. LPO needs a source of $H_2O_2$ to function. In the human airways, $H_2O_2$ is mainly supplied by the NADPH oxidase Dual oxidase 1, DUOX1[6, 7, 8]. LPO then uses $H_2O_2$ to oxidize the pseudohalide thiocyanate ($SCN^-$) abundantly present in ASL into the antimicrobial ion hypothiocyanite ($OSCN^-$)[6, 165]. The LPO-based system has previously been shown to be an effective *in vitro* neutralizer of a wide variety of viruses[9, 10, 11] and bacteria[5, 12]. Interestingly, even though *Spn* represents an enormous health burden, the effectiveness of the LPO-based system against *Spn* has not been determined to date.

Due to the relevance of *Spn* in public health combined with the emergence of antibiotic resistance and a vaccine with limited efficacy, the LPO-based system may provide valuable insight and a possible new therapeutic option for management of *Spn* infections. We hypothesized that the LPO-based system is effective at killing *Spn in vitro.* We found that both encapsulated and nonencapsulated pneumococci were susceptible to $OSCN^-$-mediated killing in a cell-free experimental system.

## Materials and Methods

### Bacteria

*Spn* strains EF3030 (encapsulated serotype 19F) [257], MNZ41 (nonencapsulated) [258], TIGR4 [259] and TIGR4Δ*spxB* [260] (encapsulated serotype 4) were first inoculated overnight on sheep blood agar plates (BAP) at 37°C in 5% $CO_2$. Then colonies were picked and grown @37°C in 5% $CO_2$ in TH+Y media until bacteria reached log phase. Bacteria was then collected and harvested by centrifugation at 10,000g for 5 minutes, washed twice with Hank's balanced salt solution (HBSS), and resuspended in HBSS. Bacterial density was then determined by measuring optical density (OD) at 600 nm. The bacterial density was set to 0.6, which is representative of $10^9$ CFU/mL *Spn* that was confirmed by performing serial dilutions, plating bacteria on BAP and counting colonies. The identities of the *Spn* strains were confirmed by 16S rRNA Gene Sequencing (Genewiz, South Plainfield, NJ, USA). Optochin-sensitivity of the *Spn* strains used was also confirmed for each experiment using BD BBL™ Taxo™ P Discs (Fisher Scientific, Pittsburgh, PA, USA).

### Bacterial killing measured by colony counting

Components of the LPO-based antibacterial system were used as described previously[5]. Briefly, the following concentrations were used: 6.5 μg/ml LPO, 400 μM $SCN^-$, 5 mM glucose and 0.1 U/mL glucose oxidase. The reaction volume was set to 120 μL. Catalase (700 U/mL) was also used when indicated to inhibit the system by scavenging $H_2O_2$. The components were assembled in a

sterile 96-well microplate with the bacteria being added last at a maximal concentration of $5x10^5$ CFU/ml. The plates were then placed in a 37°C incubator with 5% $CO_2$. After 6 hours of incubation, 40 µL was spread onto BAP in triplicate and incubated at 37°C with 5% $CO_2$. After 24 hours, the colonies were counted and CFU/mL was determined. Agar plates exposed to only the assay medium without *Spn* were always used to ensure that no potential contaminants were detected. A time 0 condition was also counted to make sure that bacterial death was due to $OSCN^-$ and not related to an unknown variable. All the reagents were ordered from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

**Bacteriostatic effects measured by a microplate-based growth assay**

Bacterial growth was measured by a microplate-based assay described previously [261]. Briefly, the components (mentioned in the cell-free assay) were assembled in a sterile 96-well plate with the bacteria being added last. Bacterial growth was measured in a microplate spectrophotometer [Eon (BioTek Instruments Inc., Winooski, VT, USA) or Varioskan Flash (Thermo Scientific, Rochester, NY)] based on following increases in OD as a measure of bacterial density. *Spn* strains were grown at 37°C for 14 hours, and OD at 600 nm was measured every 3 minutes. Each sample was run in triplicate. The time required for the positive control (*Spn* alone) to reach an OD of 0.4 (exponential growth phase) was used as the reference point for all other conditions, and OD values of other samples were compared to this.

**The commercial 1st line™ immune support product**

*1st line*™ is an over-the-counter product that is marketed as an immune supplement (distributed by Profound Products). This product uses a proprietary technology to keep $OSCN^-$ stable for a longer period of time allowing for a better antimicrobial effect. We tested this product in conjunction with our previously described cell-free system. Briefly, 0.1 g of LPO was reconstituted in 25 mL of HBSS. 750 µL of $H_2O_2$ solution was added to a 15 mL conical tube followed by the addition of 700 U/mL of catalase. The solution was incubated for 10 minutes to allow catalase to scavenge all $H_2O_2$ present. 12.5 mL of LPO solution was then added to each tube and mixed. Following this step, 750 µL of $SCN^-$ was also added to each sample and mixed thoroughly. Finally, 750 µL poly aluminum chloride was administered to each solution and mixed well. Samples were then incubated for 30 minutes at room temperature allowing the generation of $OSCN^-$. By the end of this incubation time, the solution separates into two distinct phases. The top, clear phase containing $OSCN^-$ was used for experiments while the pelleted precipitate was discarded.

**Bacterial $H_2O_2$ production**

The ROS-GloTM luminescence kit following the manufacturer's instructions (Promega Corporation, Madison, WI, USA) measured generation of $H_2O_2$ in bacterial suspension. This sensitive assay enables specific and direct detection of

low amounts of $H_2O_2$. TIGR4 wild-type or *ΔspxB* bacteria in suspension ($5x10^6$/ml) of HBSS buffer were incubated for 30 minutes, followed by centrifugation to collect supernatants for analysis of $H_2O_2$ production. Ros-GLo™ reagent was added to bacterium-free supernatants and luminescence was read using a Varioskan Flash microplate luminometer (Thermo Scientific, Rochester, NY). Results of biological triplicates are expressed as relative luminescence units (RLU).

**Quantitation of $OSCN^-$ generation**

Production of $OSCN^-$ was assessed using the photometric 5-thio-2-nitrobenzoic acid (TNB) oxidation assay [262]. $OSCN^-$ converts TNB that absorbs light at 412 nm, into a colorless disulfide (5,5'-dithio-bis-[2-nitrobenzoic acid]) (DNTB, Ellman's reagent). $OSCN^-$ production is measured as decrease in OD at 412 nm and is calculated based on the Lambert-Beer Law and the absorption coefficient $\varepsilon_{412}$ = 14,100 $M^{-1}$ $cm^{-1}$ [263]. $OSCN^-$ production is expressed as concentration of $OSCN^-$ produced in the volume of the cell-free system under different conditions in 30 minutes. Short-lived TNB cannot be purchased and was generated by reducing DTNB with the help of β-mercaptoethanol (both purchased at Sigma-Aldrich, St. Louis, MO, USA).

**Statistical analysis**

Significance among multiple samples was calculated using One-way or Two-Way ANOVA followed by Tukey's or Sidak's multiple comparison post-hoc tests. Significance between two samples was calculated using Man-Whitney's test.

Statistical analysis was performed using Prism 6 for Windows version 6.07 software. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

## Results

### LPO-derived hypothiocyanite kills both encapsulated and nonencapsulated Spn strains

To explore the effects of $OSCN^-$ on *Spn*, we used our previously established cell-free *in vitro* system that generates $OSCN^-$ at levels comparable to those measured in human airways [229]. This system utilizes the enzyme glucose oxidase, which oxidizes its substrate glucose to produce D-gluconolactate[264]. $H_2O_2$ is a byproduct of the reaction and allows us to mimic the nature of $H_2O_2$ production *scn* by Dual oxidases[5, 229]. Previously, we have successfully used this experimental system to show that LPO-generated $OSCN^-$ inactivates a wide range of influenza strains[9, 264]. Using this $H_2O_2$/LPO/$SCN^-$ cell-free system, we wanted to determine the effectiveness of $OSCN^-$ against *Spn*. We utilized physiologically relevant levels of each component of the system: 400 µM $SCN^-$ [85] and 6.5 µg/ml LPO[8]. $H_2O_2$ production by glucose oxidase was set to a rate of 0.01 U/ml, which is similar to what is seen in primary normal human bronchial epithelial (NHBE) cells by Duox[9, 77]. We tested both encapsulated (TIGR4, EF3030) and nonencapsulated (MNZ41) *Spn* strains, and we observed that the cell-free $H_2O_2$/LPO/$SCN^-$ system effectively killed all three strains of *Spn* (Fig 6.1A). $OSCN^-$ is generated reproducibly to achieve a final $OSCN^-$ concentration of 41.2 ± 4.2 µM (mean ± S.E.M., n=4) (Fig 6.1B). Our data also demonstrate that the $OSCN^-$ detection method (TNB oxidation assay) does not detect $H_2O_2$ (Fig 6.1B). Since $H_2O_2$ alone is capable of killing *Spn* [265], we exposed EF3030 *Spn* to the same levels of glucose oxidase without $SCN^-$ or LPO to ensure our results were $OSCN^-$-mediated and not

due to $H_2O_2$. The results show that *Spn* survival is not impaired by the concentration of $H_2O_2$ that was generated in this assay (Fig 6.1C).

**Catalase prevents hypothiocyanite-mediated bacteriostatic effects**

To confirm our findings that *Spn* is being inhibited by $OSCN^-$, we utilized a kinetic growth assay to measure bacterial growth and an inhibitor of the $H_2O_2$/LPO/$SCN^-$ cell-free system. Catalase is an enzyme found in almost all living organisms that catalyzes the decomposition of $H_2O_2$ to water and oxygen [266]. The use of catalase in our cell-free system eliminates $H_2O_2$ and ultimately prevents the formation of $OSCN^-$ (Fig 6.2A), rendering the cell-free system nonfunctional while also ensuring that neither $SCN^-$ nor LPO have an antimicrobial effect independent of $OSCN^-$ formation. Results shown in (Fig 6.2B) indicate that *Spn* bacteria exposed to $OSCN^-$ have inhibited bacterial growth compared to those treated with catalase or unexposed to $OSCN^-$. Both *Spn* strains tested show the same trend, where $OSCN^-$ treatment significantly reduces bacterial growth ($p<0.0001$) and addition of catalase entirely rescues this effect ($p<0.0001$) (Fig 5.2B). The nonencapsulated *Spn* strain, MNZ41, could not be tested in this assay because it did not grow in liquid cultures used under these experimental conditions. Taken together, these data show for the first time in an *in vitro* model that $OSCN^-$ has antimicrobial/bacteriostatic action against different strains of *Spn* that is catalase-sensitive.

**LPO-derived $OSCN^-$ inhibits *Spn* growth in a $SCN^-$ concentration-dependent manner**

Physiological levels of $SCN^-$ in the airways have been measured to be around 400 μM[85]. We decided to test $SCN^-$ in a supra- and superphysiological range between 40 μM-4 mM to determine if bacteriostatic effects on one of the *Spn* strains, EF3030, can be enhanced. Our data show that supraphysiological levels (40 μM) of $SCN^-$ inhibit *Spn* EF3030 in a robust manner (Fig 6.3A-B). Increasing the $SCN^-$ concentration showed a dose-dependent response, where bacterial growth inhibition continued to increase, all the way up to 4 mM of $SCN^-$ (Fig 6.3). From this data, we conclude that the reported physiological $SCN^-$ concentration is sufficient to support the antibacterial activity of the LPO system against *Spn*.

**Commercially available 1st line™ effectively kills Spn via $OSCN^-$**

The $H_2O_2$/LPO/$SCN^-$ cell-free system has proven to be effective at killing *Spn in vitro.* A drawback of this system is, however, that $OSCN^-$ has a very short life span (less than 60 minutes after it has been generated) requiring it to constantly be produced *in vitro* in order to test its effect on microbes. This is why we utilized glucose oxidase, not bolus-like addition of $H_2O_2$, to allow a steady production of $H_2O_2$ and $OSCN^-$[264]. As the next step, we took advantage of and tested a commercially available product that generates $OSCN^-$ and claims to keep it stable for much longer. This product, *1st line*™, utilizes a stabilizing molecule to allow $OSCN^-$ to persist for up to 12 hours. We compared inhibited growth of *Spn* EF3030 and TIGR4 by the *1st line*™ product. Our results demonstrate that $OSCN^-$

generated by 1st line™ resulted in robust *Spn* growth inhibition (Fig 6.4A-B). The addition of catalase during the generation of $OSCN^-$ with *1st line*™ inhibited the bacteriostatic effect (Fig 6.4); similar to what was previously shown in our cell-free system (Fig 6.2). These results provide further evidence that $OSCN^-$ is solely responsible for antimicrobial effects against *Spn*.

### *SpxB-deficient Spn* is more sensitive to $OSCN^-$-mediated antimicrobial effects

Interestingly, not only human cells but *Spn* itself is capable of producing $H_2O_2$ [267]. *Sp*n-generated $H_2O_2$ could interfere with the killing effect of $OSCN^-$. *Sp*n-generated $H_2O_2$ could provide additional $H_2O_2$ for $OSCN^-$ generation by LPO leading to improved bacterial killing or it could prime bacteria against oxidative stress resulting in impaired *Spn* killing by $OSCN^-$. Additionally, an excess of $H_2O_2$ could have an inhibitory effect on LPO. To explore these possibilities, we tested a mutant TIGR4 *Spn* strain (Δ*spxB*) deficient in pyruvate oxidase, the main $H_2O_2$ producer in *Spn*[260], for susceptibility to $OSCN^-$. As expected, the *spxB*-deficient TIGR4 strain had an $H_2O_2$ generation that was reduced by 72.5±0.9% (mean ± SEM, N=2) compared to the wild-type TIGR4 counterpart (Fig 6.5A). The TIGR4Δ*spxB* mutant and its parental strain were exposed to $OCSN^-$ produced by 1st line™, and bacterial growth and killing was evaluated with both, CFU-based counting and microplate-based bacterial growth assays. The TIGR4Δ*spxB* mutant was also susceptible to the antimicrobial effect of $OSCN^-$ that was partially inhibited by catalase (Fig 6.5B). We next calculated "susceptibility to $OSCN^-$" in two ways:

1) decrease in $\log_{10}$ of the colony counts, and 2) as percent decrease in bacterial density measured as OD at 600 nm. CFU count results obtained at two different time points of incubation show that the difference at 6 hours of incubation reached the level of significance (p=0.029) while that after 2 hours did not (p=0.229) (Fig 6.5C). When the potential difference in $OSCN^-$-mediated growth inhibition was addressed using the microplate-based growth assay, no significant difference (p=0.059) was observed although the *p* value was close to the threshold and the data clearly showed a trend toward enhanced $OSCN^-$ susceptibility of TIGR4Δ*spxB* (Fig 6.5D). Overall, we conclude that pyruvate oxidase provides some protection in *Spn* survival following $OSCN^-$ exposure.

## Discussion

While the LPO-based system has been shown to be effective at killing numerous species of bacteria and viruses *in vitro*, no report to date studied the interaction between this system and *Spn*. *Spn* first encounters epithelial cells at the apical surface of the nasal cavity, a region that is a hotbed of defense mechanisms. The LPO-based system kills microbes in the extracellular space before they enter the epithelium and establish infection[5]. We were able to demonstrate that $OSCN^-$ kills and inhibits growth of *Spn* effectively. The ability of $OSCN^-$ to kill a variety of microbes in the extracellular spaces makes it a very interesting innate immune mechanism to study.

It is possible that *Spn* stimulates one of the main cellular sources of $H_2O_2$, DUOX1, since we had previously shown that bacterial ligands of *P. aeruginosa* participate in DUOX1 activation[165]. *Spn* has been shown to trigger $H_2O_2$ production in airway epithelial cells in a pneumolysin-independent but lytA-dependent manner[268]. We utilized the enzyme catalase that converts $H_2O_2$ into $H_2O$ and $O_2$, to inhibit the production of $OSCN^-$. This was necessary because *Spn* is a catalase-negative bacterium and is capable of producing its own $H_2O_2$[269]. *Spn*-derived $H_2O_2$ is sufficient to mediate bactericidal activity of other bacteria and to stimulate DNA damage and apoptosis in epithelial cells leading to tissue damage during infection[270]. It was found that in the absence of common antioxidant proteins, *Spn* utilizes pyruvate oxidase (spxB) that has a dual role of creating $H_2O_2$, and protecting itself from oxidative damage[161]. Pyruvate oxidase, the main $H_2O_2$ source in *Spn*[260], has been found to be important to initiate oxidative attacks on host

cells[270]. Our data indicate that spxB provides a moderate protection against the oxidative attack of $OSCN^-$. Our results are in line with previous findings of other groups where spxB was determined to be useful against oxidative stress of different origins[161, 271, 272]. $H_2O_2$ generation by spxB in *Spn* likely enhances its antioxidant capacity and thereby improves its defense against oxidative stress. Our results indicate a new, protective consequence of SpxB expression in *Spn.* The *in vivo* relevance of this finding remains to be confirmed in animal models.

*Spn* utilizes a wide spectrum of virulence factors, one of the most important ones being the polysaccharide capsule that forms the outermost layer of the bacteria [273]. This capsule provides protection against phagocytosis, complement components, mucus and spontaneous or antibiotic-induced autolysis [274]. Some *Spn* strains, however, do not possess a capsule[146, 275]. Our results show that both encapsulated and nonencapsulated *Spn* strains are susceptible to $OSCN^-$. Thus, it is likely that $OSCN^-$ is able to penetrate the capsule and interact with the cell wall or other internal bacterial components.

$OSCN^-$ has a wide microbial target spectrum[5]. Its mechanism of action likely involves oxidative attack on one or more microbe-specific molecules or cellular mechanisms that essentially will lead to a bactericidal action. Previous studies have shown that $OSCN^-$ is oxidizing bacterial sulfhydryls [276, 277, 278], thereby inhibiting bacterial respiration [75], but no further research has been published to support this possible mechanism of action.

The LPO-based system presents a novel therapeutic target, since it is an effective antimicrobial innate mechanism that does not have many drawbacks due

to its final product being nontoxic to host cells and its broad activity against a wide range of pathogens [93]. By testing the *1st line*™ product (alongside our cell-free method of $OSCN^-$ generation), we observed the same efficient *Spn* killing and growth inhibition results. This is a promising foundation for $OSCN^-$ as a potential therapeutic. While we experienced similar levels of killing of *Spn* when comparing our glucose oxidase system and the *1st line*™ product, there are some subtle differences that would likely affect the efficacy of *in vivo* studies. The greatest benefit to the *1st line*™ system is the stabilizing aspect of the compound. Allowing $OSCN^-$ to persist for over 12 hours would likely increase the efficacy and potency of the system, which could be a major advantage over the non-stabilized, natural $OSCN^-$ anion. This also allows for higher concentrations of $OSCN^-$ to be achieved without having toxicity issues.

We determined the effectiveness of $OSCN^-$ against *Spn* in this proof-of-concept study as it had not been reported previously. We were able to demonstrate that $OSCN^-$ effectively kills both encapsulated and nonencapsulated strains of *Spn* in a cell-free system. We also successfully utilized a commercially available product, *1st line*™, to demonstrate the therapeutic potential of the LPO system in an *in vitro* model. These studies warrant further research to elucidate the molecular mechanism of $OSCN^-$ and its potential as an *in vivo* therapeutic.

## Figures and Legends

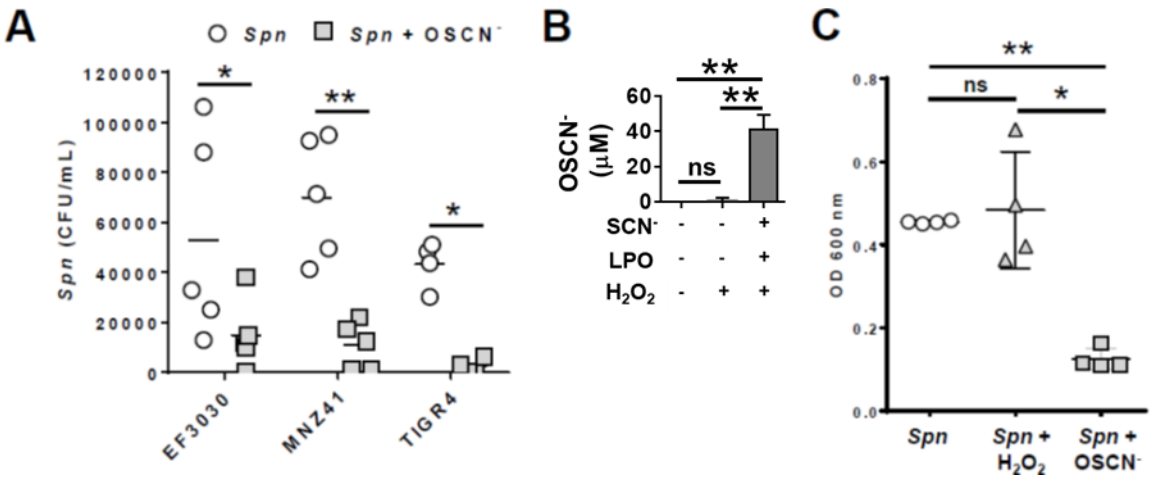


**Fig 6.1 LPO-derived hypothiocyanite kills both encapsulated and nonencapsulated *Spn* strains.**

**A)** $OSCN^-$-mediated *Spn* killing was tested in the cell-free system against EF3030 encapsulated serotype 19F (Mean+/-SEM, N=5 independent experiments in duplicate), MNZ41 nonencapsulated (Mean+/-SEM, N=5 independent experiments in duplicate) and TIGR4 encapsulated serotype 4 (Mean+/-SEM, N=4 independent experiments in duplicate). Bacteria were incubated for 6hrs with or without $OSCN^-$ generated by the LPO/$SCN^-$/$H_2O_2$ system and bacterial killing was quantified by colony counts on BAP. Mann-Whitney's test. **B)** Reproducible $OSCN^-$ production in the cell-free system measured by the DTNB oxidation method. (Mean+/-SEM, N=4 independent experiments in triplicate). One-way ANOVA and Holm-Sidak's multiple comparisons test. **C)** *Spn* EF3030 is inhibited only by $OSCN^-$ and not by $H_2O_2$. $OSCN^-$ was generated in presence of the complete cell-free system (LPO+$SCN^-$+glucose/GO) while $H_2O_2$ was produced by the glucose/GO system in the absence of LPO and $SCN^-$. Bacterial growth was assessed using the microplate-based growth assay (Mean+/-SEM, N=4 independent experiments in

triplicate). Two-way ANOVA and Sidak's multiple comparisons test. **(**Ns, not significant; *, $p<0.05$; **, $p<0.01$. CFU, colony-forming unit; LPO, lactoperoxidase; $OSCN^-$, hypothiocyanite; $SCN^-$, thiocyanate; *Spn*, *Streptococcus pneumoniae*.

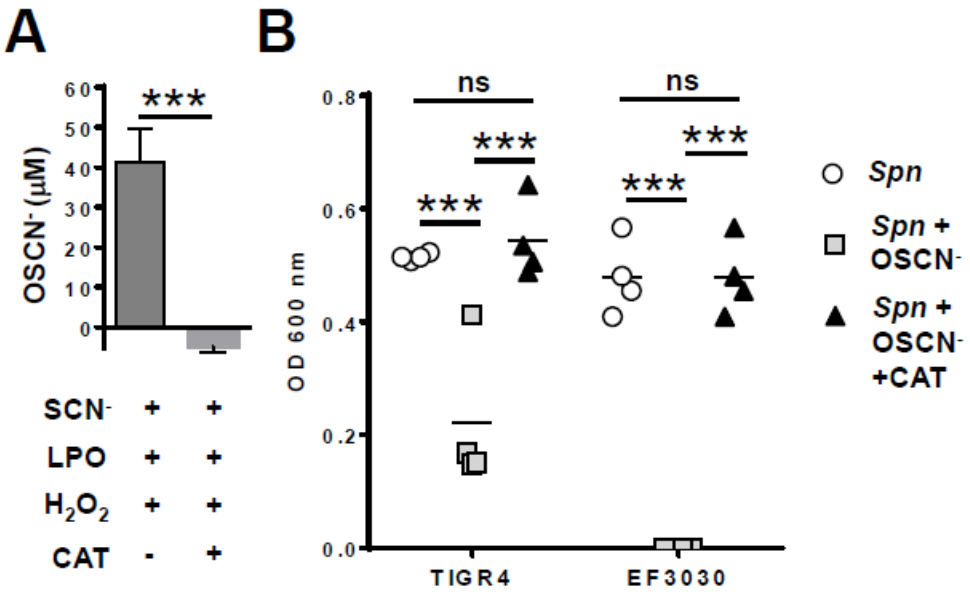


**Fig 6.2 Catalase rescues *Spn* from $OSCN^-$-mediated growth inhibition.**

**(A)** Addition of catalase to the cell-free system blocks $OSCN^-$ generation measured by the DTNB assay. (Mean+/-SEM, N=4 independent experiments in triplicate). Mann-Whitney test. **(B)** Catalase inhibits killing of TIGR4 and EF3030 *Spn* strains (Mean+/-SEM, N=4 independent experiments in triplicate). Two-way ANOVA, Tukey's multiple comparison test. Ns, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. CAT, catalase.

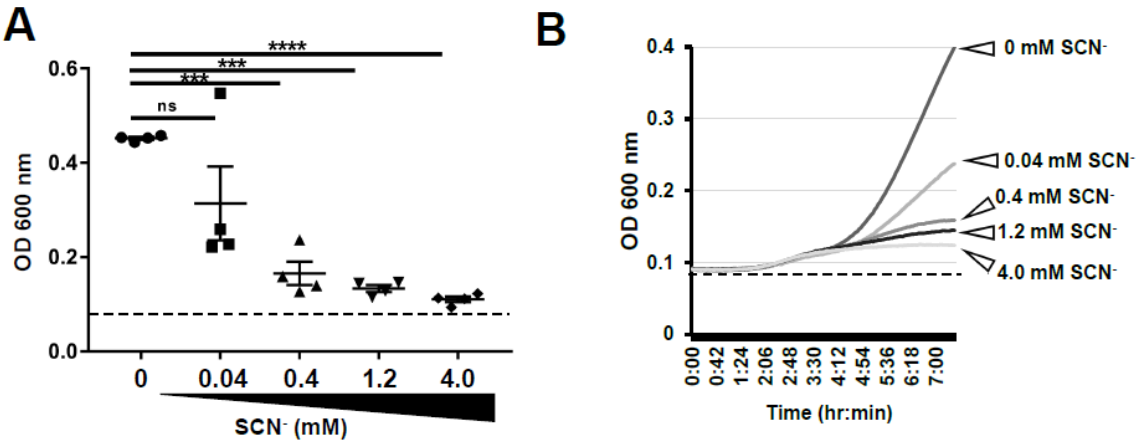


**Fig 6.3 OSCN$^-$ exhibits a thiocyanate dose-dependent manner bacteriostatic effect on *Spn* EF3030.**

**(A)** Increasing the concentration of SCN$^-$ in the cell-free system leads to improved growth inhibition of *Spn* EF3030. Bacteria were incubated for 6 hrs with OSCN$^-$, and bacterial growth was quantified by the microplate-based growth assay (Mean+/-SEM, N=4 independent experiments in triplicate). One-way ANOVA and Tukey's multiple comparison test. **(B)** Representative kinetics of EF3030 growth curves. ***, $p<0.001$; ns, not significant. Dotted lines indicate the OD background of the growth medium without bacteria that was not subtracted in these experiments.

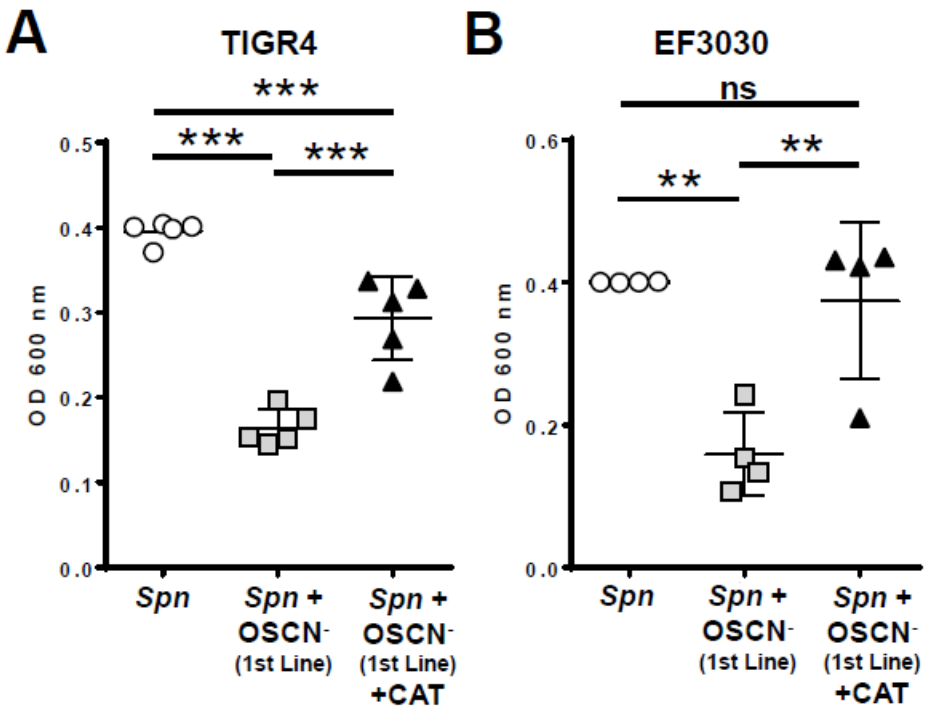


**Fig 6.4 OSCN$^-$ generated using commercially available 1st line™ effectively inhibits *Spn* growth.**

The antimicrobial action of the hypothiocyanite-producing 1st line™ product efficiently inhibits growth **(A)** TIGR4 (Mean+/-SEM, N=5 independent experiments in triplicate) and **(B)** EF3030 (Mean+/-SEM, N=4 independent experiments in triplicate) *Spn* strains. Catalase reversed this inhibitory effect partially (TIGR4) or fully (EF3030). Bacterial survival was measured by the microplate-based growth assay. One-way ANOVA, Tukey's multiple comparison test. **, $p<0.01$; ***, $p<0.001$; ns, not significant. CAT, catalase.

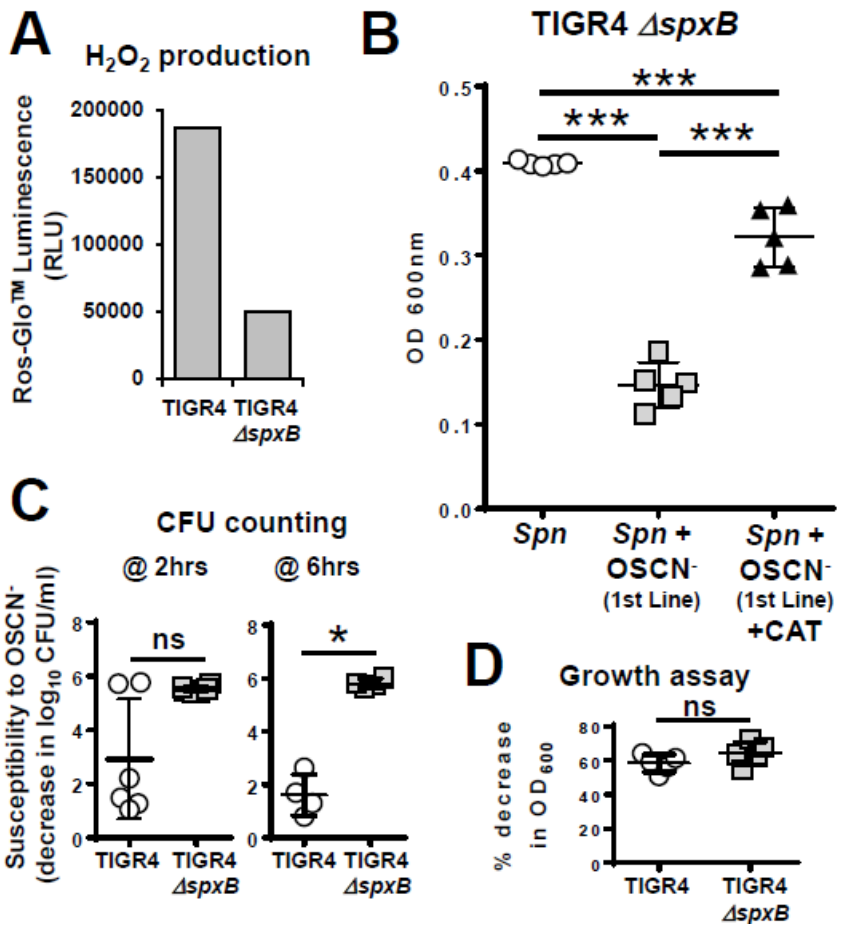


**Fig 6.5 Pyruvate oxidase-deficiency increases susceptibility of *Spn* to OSCN$^-$**

**.**

**(A)** $H_2O_2$ generation was quantitated in wild-type and *spxB*-deficient (*ΔspxB*) TIGR4 *Spn* strains using the Ros-GLo™ luminescence $H_2O_2$ quantitation kit. Mean, N=2 independent experiments performed in triplicate. **(B)** OSCN$^-$-mediated (1st line™) growth inhibition of the TIGR4Δ*spxB Spn* strain was measured by the microplate-based growth assay in presence or absence of catalase (Mean+/-SEM, N=5 independent experiments performed in triplicate). One-way ANOVA, Tukey's multiple comparison test. **(C)** Killing by OSCN$^-$ generated via 1st line™ of wild-type and TIGR4Δ*spxB Spn* strains was compared at two different incubation times (2

and 6hrs) via CFU counting (Mean+/-SEM, N=4 independent experiments performed in duplicate). Mann-Whitney's test. **(D)** Growth inhibition by $OSCN^-$ generated via 1st line™ of wild-type and TIGR4Δ*spxB Spn* strains was evaluated using the microplate-based growth assay (Mean+/-SEM, N=5 independent experiments performed in triplicate). Mann-Whitney's test. ***, $p<0.001$; *, $p<0.05$; ns, not significant. RLU, relative luminescence unit; CAT, catalase.

# CHAPTER 7

# CONCLUSIONS

The airways present the front lines of defense against airborne pathogens. Understanding the innate immune system at this front line, is of the utmost importance as modifying and understanding currently existing mechanisms presents our best chance at combating pathogens. In this work, we describe and evaluate the potential of a broad-spectrum immune mechanism the DUOX1-$H_2O_2$/LPO/$SCN^-$ system that produces the antimicrobial anion $OSCN^-$. This system was first studied almost a century ago. Nevertheless, its effectiveness had not yet been tested against two of the most common, dangerous airborne pathogens: influenza virus and *Streptococcus pneumoniae*. We hypothesized that DUOX1 supplies the $H_2O_2$ for the LPO/$SCN^-$ antimicrobial defense system producing hypothiocyanite in the host bronchial epithelium which is capable of inactivating extracellular influenza virus and killing *Spn*, altering the course of respiratory infections. To address this hypothesis, we examined the following specific aims:

**Specific Aim 1:** To identify the mechanism of influenza inactivation by the DUOX1/LPO/$SCN^-$ system. The working hypothesis is that $OSCN^-$ produced by the DUOX1/LPO/$SCN^-$ system interacts with surface proteins leading to a decrease in infectivity. The data in chapter 3 shows that differentiated NHBE cells express DUOX1 and are capable of producing $H_2O_2$ validating the model system.

The addition of LPO and $SCN^-$ to the apical surface of differentiated NHBE elicited the formation of $OSCN^-$ in a cell-based system, which resulted in extracellular inactivation of A/swine/Illinois/02860/09 (swH1N2). The data in chapter 5 illustrated a more detailed look at the interaction between $OSCN^-$ and influenza virus. We tested A/California/07/2009 (H1N1), A/turkey/KS/4880/80 (H) (H1N1), A/Wisconsin/67/2005 (H3N2), and B/Florida/4/2006 Yamagata in the same cell based system to confirm the previous results were not strain specific. In addition to reduction in extracellular viral titers, we also saw a decrease in the number of infected cells 24 hrs p.i via IFA, when LPO and $SCN^-$ were added to the apical surface of the NHBE cells. A cell-free system was established to produce $OSCN^-$ *in vitro* and tested against 17 different strains of influenza including three major influenza A subtypes H1N1, H1N2 and H3N2 and the two lineages of influenza B, Yamagata and Victoria. The data demonstrates that $OSCN^-$ has a general inhibitory effect against all the strains tested, however, we did see a large degree of variability in the susceptibility, with the H1 being more susceptible than the H3 strains. Pointing to a possible interaction with the HA protein. We tested the activity of the NA protein after $OSCN^-$ exposure and saw no inhibitory effect leading us to conclude that NA was not the target of $OSCN^-$. Upon using a HAI assay, we did find a significant inhibitory effect on the viruses' ability to bind to receptors on the surface of red blood cells, pointing to HA as a likely target of $OSCN^-$. This data was further supported by the use of a time-of-addition assay and a binding assay whose results showed that after $OSCN^-$ exposure influenzas ability to bind to host receptors was inhibited. These results suggest that the decrease in viral titers

seen is due to $OSCN^-$ interacting with the HA protein on the surface of the cell likely inhibiting the binding ability of the virus to host receptors.

**Specific Aim 2:** To identify and evaluate the role of Duox1 in survival and lung viral titers in a murine influenza infection model. The working hypothesis is that *Duox1*-deficient mice will demonstrate and increased susceptibility to influenza infection. In chapter 5, our *in vitro* results demonstrated the DUOX1/LPO/$SCN^-$ system is capable of inactivating extracellular influenza virus. In chapter 4, we then tested the effectiveness of this system *in vivo* in a *Duox1*-deficient mouse model. We infected WT and *Duox1*-deficient mice with influenza virus intranasally and examined mortality and morbidity. During the infection, *Duox1*-defcient mice showed significantly increased morbidity and mortality compared to the WT mice. Additionally, we evaluated lung viral titers at day 3 and 7 post infection; *Duox1*-deficient mice had significantly more virus in their lungs than WT mice. These results suggest that *Duox1*-deficient mice are more susceptible to influenza due to an impaired ability to fight off early virus exposure leading to elevated lung viral titers and enhanced morbidity and mortality as a downstream consequence.

**Specific Aim 3:** Determine if the $H_2O_2$/LPO/$SCN^-$ system is effective at killing *Streptococcus pneumoniae*. The working hypothesis is that $OSCN^-$ produced by the $H_2O_2$/LPO/$SCN^-$ will kill *Streptococcus pneumoniae in vitro*. In chapter 6, we tested the bactericidal and bacteriostatic effects of $OSCN^-$ against

encapsulated and unencapsulated strains of *Spn*. $OSCN^-$ had both a bactericidal and bacteriostatic effect on *Spn in vitro.* Additionally, we used the spxB mutant strain deficient in pyruvate-oxidase, a gene responsible for $H_2O_2$ production and protection against ROS in our killing and growth inhibition assays. The data suggested that the spxB has some role in protection against $OSCN^-$, as the spxB mutant was more sensitive than the WT strain. These results show for the first time that $OSCN^-$ is capable of killing *Spn*.

Taken together, the results of these studies suggest the DUOX1/LPO/$SCN^-$ systems' antimicrobial product $OSCN^-$ has a greater range of effectiveness than previously believed. This work also furthers our understanding of the antimicrobial systems utilized by epithelial cells. Our results showed for the first time the role of Duox1 in an infection model as it had not previously been reported. Targeting this system may have therapeutic potential, which is especially important with the rise of antiviral and antibiotic resistance, and waning efficacy in current vaccines.

Future studies need to investigate the interaction with $OSCN^-$ and the HA protein of influenza virus. While our results demonstrated a decrease in binding efficiency, a 50% reduction does not necessarily explain several log reductions in viral titers, meaning there is likely other mechanisms at play. There is even less known about the mechanism of action of $OSCN^-$ against bacteria, specifically *Spn*. It is likely $OSCN^-$ is interacting with sulfhydryl groups present on the bacteria thereby affecting bacterial respiration. These studies set the proof of concept of $OSCN^-$ as a potential therapeutic option for airborne pathogens. The *in vivo* results with the *Duox1*-deficient mice demonstrated the importance of having a functional

Duox1. Future studies should strive to understand the downstream effects of this gene and if it is interacting with any other immune mechanisms, or if it solely exists as a direct antimicrobial system. Additionally, human DUOX1 deficiencies or mutations need investigation, as currently that information is unknown. Patients who are susceptible to respiratory infections could have a *DUOX1* mutation leading to a decrease in their ability to fend off airborne pathogens. Finally, if studied further the DUOX1/LPO/$SCN^-$ system presents a unique opportunity to harness a conserved antimicrobial system as a potential powerful therapeutic target in our eternal battle against pathogenic microbes.

**References**


1. Morris, D.E., Cleary, D.W. & Clarke, S.C. Secondary Bacterial Infections Associated with Influenza Pandemics. *Frontiers in microbiology* **8**, 1041-1041 (2017).

2. Weiser, J.N., Ferreira, D.M. & Paton, J.C. Streptococcus pneumoniae: transmission, colonization and invasion. *Nature reviews. Microbiology* **16**, 355-367 (2018).

3. Denny, F.W. & Loda, F.A. Acute respiratory infections are the leading cause of death in children in developing countries. *The American journal of tropical medicine and hygiene* **35**, 1-2 (1986).

4. Hiemstra, P.S., McCray, P.B., Jr. & Bals, R. The innate immune function of airway epithelial cells in inflammatory lung disease. *The European respiratory journal* **45**, 1150-1162 (2015).

5. Sarr, D., Toth, E., Gingerich, A. & Rada, B. Antimicrobial actions of dual oxidases and lactoperoxidase. *J Microbiol* **56**, 373-386 (2018).

6. Geiszt, M., Witta, J., Baffi, J., Lekstrom, K. & Leto, T.L. Dual oxidases represent novel hydrogen peroxide sources supporting mucosal surface host defense. *FASEB J* **17**, 1502-1504 (2003).

7. Moskwa, P. *et al.* A novel host defense system of airways is defective in cystic fibrosis. *American journal of respiratory and critical care medicine* **175**, 174-183 (2007).

8. Rada, B., Lekstrom, K., Damian, S., Dupuy, C. & Leto, T.L. The Pseudomonas toxin pyocyanin inhibits the dual oxidase-based antimicrobial system as it imposes oxidative stress on airway epithelial cells. *Journal of immunology (Baltimore, Md. : 1950)* **181**, 4883-4893 (2008).

9. Gingerich, A. *et al.* Hypothiocyanite produced by human and rat respiratory epithelial cells inactivates extracellular H1N2 influenza A virus. *Inflammation research : official journal of the European Histamine Research Society ... [et al.]* **65**, 71-80 (2016).

10. Pourtois, M. *et al.* Inhibition of HIV infectivity by lactoperoxidase-produced hypothiocyanite. *Journal de biologie buccale* **18**, 251-253 (1990).

11. Cegolon, L. *et al.* In vitro antiviral activity of hypothiocyanite against A/H1N1/2009 pandemic influenza virus. *International journal of hygiene and environmental health* **217**, 17-22 (2014).

12. Moreau-Marquis, S., Coutermarsh, B. & Stanton, B.A. Combination of hypothiocyanite and lactoferrin (ALX-109) enhances the ability of tobramycin and aztreonam to eliminate Pseudomonas aeruginosa biofilms growing on cystic fibrosis airway epithelial cells. *The Journal of antimicrobial chemotherapy* **70**, 160-166 (2015).

13. Vareille, M., Kieninger, E., Edwards, M.R. & Regamey, N. The Airway Epithelium: Soldier in the Fight against Respiratory Viruses. *Clinical Microbiology Reviews* **24**, 210-229 (2011).

14. Tam, A., Wadsworth, S., Dorscheid, D., Man, S.F. & Sin, D.D. The airway epithelium: more than just a structural barrier. *Therapeutic advances in respiratory disease* **5**, 255-273 (2011).

15. Roche, W.R., Montefort, S., Baker, J. & Holgate, S.T. Cell adhesion molecules and the bronchial epithelium. *The American review of respiratory disease* **148**, S79-82 (1993).

16. Hong, K.U., Reynolds, S.D., Watkins, S., Fuchs, E. & Stripp, B.R. In vivo differentiation potential of tracheal basal cells: evidence for multipotent and unipotent subpopulations. *American journal of physiology. Lung cellular and molecular physiology* **286**, L643-649 (2004).

17. Knight, D.A. & Holgate, S.T. The airway epithelium: structural and functional properties in health and disease. *Respirology (Carlton, Vic.)* **8**, 432-446 (2003).

18. De Water, R. *et al.* Ultrastructural localization of bronchial antileukoprotease in central and peripheral human airways by a gold-labeling technique using monoclonal antibodies. *The American review of respiratory disease* **133**, 882-890 (1986).

19. Lumsden, A.B., McLean, A. & Lamb, D. Goblet and Clara cells of human distal airways: evidence for smoking induced changes in their numbers. *Thorax* **39**, 844-849 (1984).

20. Evans, M.J. & Plopper, C.G. The role of basal cells in adhesion of columnar epithelium to airway basement membrane. *The American review of respiratory disease* **138**, 481-483 (1988).

21. Tamura, S. & Kurata, T. Defense mechanisms against influenza virus infection in the respiratory tract mucosa. *Japanese journal of infectious diseases* **57**, 236-247 (2004).

22. Hamann, K.J. *et al.* Expression of Fas (CD95) and FasL (CD95L) in human airway epithelium. *American journal of respiratory cell and molecular biology* **19**, 537-542 (1998).

23. Griffith, T.S., Brunner, T., Fletcher, S.M., Green, D.R. & Ferguson, T.A. Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science (New York, N.Y.)* **270**, 1189-1192 (1995).

24. Ank, N. *et al.* An important role for type III interferon (IFN-lambda/IL-28) in TLR-induced antiviral activity. *Journal of immunology (Baltimore, Md. : 1950)* **180**, 2474-2485 (2008).

25. Diebold, S.S. Recognition of viral single-stranded RNA by Toll-like receptors. *Adv Drug Deliv Rev* **60**, 813-823 (2008).

26. Schroder, N.W. *et al.* Lipoteichoic acid (LTA) of Streptococcus pneumoniae and Staphylococcus aureus activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and

CD14, whereas TLR-4 and MD-2 are not involved. *The Journal of biological chemistry* **278**, 15587-15594 (2003).

27. Malley, R. *et al.* Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 1966-1971 (2003).

28. Albiger, B. *et al.* Toll-like receptor 9 acts at an early stage in host defence against pneumococcal infection. *Cellular microbiology* **9**, 633-644 (2007).

29. Ank, N. *et al.* Lambda interferon (IFN-lambda), a type III IFN, is induced by viruses and IFNs and displays potent antiviral activity against select virus infections in vivo. *Journal of virology* **80**, 4501-4509 (2006).

30. McNab, F., Mayer-Barber, K., Sher, A., Wack, A. & O'Garra, A. Type I interferons in infectious disease. *Nature Reviews Immunology* **15**, 87-103 (2015).

31. Joyce, E.A., Popper, S.J. & Falkow, S. Streptococcus pneumoniae nasopharyngeal colonization induces type I interferons and interferon-induced gene expression. *BMC Genomics* **10**, 404 (2009).

32. LeMessurier, K.S., Hacker, H., Chi, L., Tuomanen, E. & Redecke, V. Type I interferon protects against pneumococcal invasive disease by inhibiting bacterial transmigration across the lung. *PLoS pathogens* **9**, e1003727 (2013).

33. Biron, C.A. Interferons alpha and beta as immune regulators--a new look. *Immunity* **14**, 661-664 (2001).

34. Davidson, S., Crotta, S., McCabe, T.M. & Wack, A. Pathogenic potential of interferon αβ in acute influenza infection. *Nature Communications* **5**, 3864 (2014).

35. Okabayashi, T. *et al.* Type-III interferon, not type-I, is the predominant interferon induced by respiratory viruses in nasal epithelial cells. *Virus research* **160**, 360-366 (2011).

36. Galani, I.E. *et al.* Interferon-lambda Mediates Non-redundant Front-Line Antiviral Protection against Influenza Virus Infection without Compromising Host Fitness. *Immunity* **46**, 875-890.e876 (2017).

37. Brinkmann, V. *et al.* Neutrophil extracellular traps kill bacteria. *Science (New York, N.Y.)* **303**, 1532-1535 (2004).

38. Ivan, F.X. *et al.* Neutrophils infected with highly virulent influenza H3N2 virus exhibit augmented early cell death and rapid induction of type I interferon signaling pathways. *Genomics* **101**, 101-112 (2013).

39. Standish, A.J. & Weiser, J.N. Human neutrophils kill Streptococcus pneumoniae via serine proteases. *Journal of immunology (Baltimore, Md. : 1950)* **183**, 2602-2609 (2009).

40. Urban, C.F. *et al.* Neutrophil Extracellular Traps Contain Calprotectin, a Cytosolic Protein Complex Involved in Host Defense against Candida albicans. *PLoS pathogens* **5**, e1000639 (2009).

41. Sorensen, G.L., Husby, S. & Holmskov, U. Surfactant protein A and surfactant protein D variation in pulmonary disease. *Immunobiology* **212**, 381-416 (2007).

42. Wilson, S.S., Wiens, M.E. & Smith, J.G. Antiviral Mechanisms of Human Defensins. *Journal of molecular biology* **425**, 10.1016/j.jmb.2013.1009.1038 (2013).

43. Klebanoff, S.J., Clem, W.H. & Luebke, R.G. The peroxidase-thiocyanate-hydrogen peroxide antimicrobial system. *Biochimica et biophysica acta* **117**, 63-72 (1966).

44. Magnani, F. & Mattevi, A. Structure and mechanisms of ROS generation by NADPH oxidases. *Current opinion in structural biology* **59**, 91-97 (2019).

45. Rigutto, S. *et al.* Activation of dual oxidases Duox1 and Duox2: differential regulation mediated by camp-dependent protein kinase and protein kinase C-dependent phosphorylation. *The Journal of biological chemistry* **284**, 6725-6734 (2009).

46. Donko, A., Peterfi, Z., Sum, A., Leto, T. & Geiszt, M. Dual oxidases. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **360**, 2301-2308 (2005).

47. Dupuy, C. *et al.* Purification of a novel flavoprotein involved in the thyroid NADPH oxidase. Cloning of the porcine and human cdnas. *The Journal of biological chemistry* **274**, 37265-37269 (1999).

48. Lambeth, J.D. NOX enzymes and the biology of reactive oxygen. *Nature reviews. Immunology* **4**, 181-189 (2004).

49. Bedard, K., Lardy, B. & Krause, K.H. NOX family NADPH oxidases: not just in mammals. *Biochimie* **89**, 1107-1112 (2007).

50. Bedard, K. & Krause, K.H. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev* **87**, 245-313 (2007).

51. Geiszt, M. & Leto, T.L. The Nox family of NAD(P)H oxidases: host defense and beyond. *J Biol Chem* **279**, 51715-51718 (2004).

52. Panday, A., Sahoo, M.K., Osorio, D. & Batra, S. NADPH oxidases: an overview from structure to innate immunity-associated pathologies. *Cellular & molecular immunology* **12**, 5-23 (2015).

53. Kim, J.H. *et al.* NADPH oxidase 4 is required for the generation of macrophage migration inhibitory factor and host defense against Toxoplasma gondii infection. *Sci Rep* **7**, 6361 (2017).

54. Ryu, J.C. *et al.* Neutrophil pyroptosis mediates pathology of P. aeruginosa lung infection in the absence of the NADPH oxidase NOX2. *Mucosal Immunol* **10**, 757-774 (2017).

55. Chen, C., Li, L., Zhou, H.J. & Min, W. The Role of NOX4 and TRX2 in Angiogenesis and Their Potential Cross-Talk. *Antioxidants (Basel)* **6** (2017).

56. Prieto-Bermejo, R. & Hernandez-Hernandez, A. The Importance of NADPH Oxidases and Redox Signaling in Angiogenesis. *Antioxidants (Basel)* **6** (2017).

57. Mistry, R.K. & Brewer, A.C. Redox regulation of gasotransmission in the vascular system: A focus on angiogenesis. *Free radical biology & medicine* **108**, 500-516 (2017).

58. Lipinski, S. *et al.* DUOX2-derived reactive oxygen species are effectors of NOD2-mediated antibacterial responses. *J Cell Sci* **122**, 3522-3530 (2009).

59. De Deken, X., Corvilain, B., Dumont, J.E. & Miot, F. Roles of DUOX-mediated hydrogen peroxide in metabolism, host defense, and signaling. *Antioxidants & redox signaling* **20**, 2776-2793 (2014).

60. De Deken, X., Wang, D., Dumont, J.E. & Miot, F. Characterization of ThOX proteins as components of the thyroid H(2)O(2)-generating system. *Experimental cell research* **273**, 187-196 (2002).

61. Grasberger, H. & Refetoff, S. Identification of the maturation factor for dual oxidase. Evolution of an eukaryotic operon equivalent. *The Journal of biological chemistry* **281**, 18269-18272 (2006).

62. Morand, S. *et al.* Duox maturation factors form cell surface complexes with Duox affecting the specificity of reactive oxygen species generation. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **23**, 1205-1218 (2009).

63. Grasberger, H., De Deken, X., Miot, F., Pohlenz, J. & Refetoff, S. Missense mutations of dual oxidase 2 (DUOX2) implicated in congenital hypothyroidism have impaired trafficking in cells reconstituted with DUOX2 maturation factor. *Molecular endocrinology (Baltimore, Md.)* **21**, 1408-1421 (2007).

64. Grasberger, H. *et al.* Mice deficient in dual oxidase maturation factors are severely hypothyroid. *Molecular endocrinology (Baltimore, Md.)* **26**, 481-492 (2012).

65. Moreno, J.C. *et al.* Inactivating mutations in the gene for thyroid oxidase 2 (THOX2) and congenital hypothyroidism. *The New England journal of medicine* **347**, 95-102 (2002).

66. Allaoui, A., Botteaux, A., Dumont, J.E., Hoste, C. & De Deken, X. Dual oxidases and hydrogen peroxide in a complex dialogue between host mucosae and bacteria. *Trends in molecular medicine* **15**, 571-579 (2009).

67. Wright, J.M. *et al.* Respiratory epithelial gene expression in patients with mild and severe cystic fibrosis lung disease. *American journal of respiratory cell and molecular biology* **35**, 327-336 (2006).

68. Ganesan, S. & Sajjan, U.S. Repair and Remodeling of airway epithelium after injury in Chronic Obstructive Pulmonary Disease. *Current respiratory care reports* **2**, 10.1007/s13665-13013-10052-13662 (2013).

69. Tian, Z. *et al.* Cigarette Smoke Impairs A(2A) Adenosine Receptor Mediated Wound Repair through Up-regulation of Duox-1 Expression. *Scientific reports* **7**, 44405 (2017).

70. Gorissen, S.H. *et al.* Dual oxidase-1 is required for airway epithelial cell migration and bronchiolar reepithelialization after injury. *American journal of respiratory cell and molecular biology* **48**, 337-345 (2013).

71. Rada, B., Lekstrom, K., Damian, S., Dupuy, C. & Leto, T.L. The Pseudomonas toxin pyocyanin inhibits the Dual oxidase-based antimicrobial system as it imposes oxidative stress on airway epithelial

cells. *Journal of immunology (Baltimore, Md. : 1950)* **181**, 4883-4893 (2008).

72. Strengert, M. *et al.* Mucosal reactive oxygen species are required for antiviral response: role of Duox in influenza a virus infection. *Antioxid Redox Signal* **20**, 2695-2709 (2014).

73. Habibovic, A. *et al.* DUOX1 mediates persistent epithelial EGFR activation, mucous cell metaplasia, and airway remodeling during allergic asthma. *JCI insight* **1**, e88811 (2016).

74. Little, A.C. *et al.* PARADOXICAL ROLES OF DUAL OXIDASES IN CANCER BIOLOGY. *Free radical biology & medicine* (2017).

75. Thomas, E.L. & Aune, T.M. Lactoperoxidase, peroxide, thiocyanate antimicrobial system: correlation of sulfhydryl oxidation with antimicrobial action. *Infection and immunity* **20**, 456-463 (1978).

76. Derscheid, R.J. & Ackermann, M.R. Perinatal lamb model of respiratory syncytial virus (RSV) infection. *Viruses* **4**, 2359-2378 (2012).

77. Rada, B., Boudreau, H.E., Park, J.J. & Leto, T.L. Histamine stimulates hydrogen peroxide production by bronchial epithelial cells via histamine

H1 receptor and dual oxidase. *American journal of respiratory cell and molecular biology* **50**, 125-134 (2014).

78. Rada, B. & Leto, T.L. Characterization of hydrogen peroxide production by Duox in bronchial epithelial cells exposed to Pseudomonas aeruginosa. *FEBS Lett* **584**, 917-922 (2010).

79. Kizys, M.M.L. *et al.* DUOX2 Mutations Are Associated With Congenital Hypothyroidism With Ectopic Thyroid Gland. *The Journal of clinical endocrinology and metabolism* **102**, 4060-4071 (2017).

80. Peters, C. *et al.* DUOX2/DUOXA2 Mutations Frequently Cause Congenital Hypothyroidism that Evades Detection on Newborn Screening in the United Kingdom. *Thyroid : official journal of the American Thyroid Association* **29**, 790-801 (2019).

81. Bafort, F., Parisi, O., Perraudin, J.P. & Jijakli, M.H. Mode of action of lactoperoxidase as related to its antimicrobial activity: a review. *Enzyme research* **2014**, 517164 (2014).

82. Allen, P.Z. & Morrison, M. Lactoperoxidase. VI. Immunochemical studies on lactoperoxidase from the milk of several species. *Arch Biochem Biophys* **113**, 540-547 (1966).

83. Goldman, A.S. & Smith, C.W. Host resistance factors in human milk. *J Pediatr* **82**, 1082-1090 (1973).

84. Conner, G.E., Salathe, M. & Forteza, R. Lactoperoxidase and hydrogen peroxide metabolism in the airway. *American journal of respiratory and critical care medicine* **166**, S57-61 (2002).

85. Wijkstrom-Frei, C. *et al.* Lactoperoxidase and human airway host defense. *American journal of respiratory cell and molecular biology* **29**, 206-212 (2003).

86. Xiang, Y. *et al.* The critical role of myeloperoxidase in Streptococcus pneumoniae clearance and tissue damage during mouse acute otitis media. *Innate Immunity* **23**, 296-306 (2017).

87. Vlasova, II. Peroxidase Activity of Human Hemoproteins: Keeping the Fire under Control. *Molecules (Basel, Switzerland)* **23** (2018).

88. Courtois, P., van Beers, D., de Foor, M., Mandelbaum, I.M. & Pourtois, M. Abolition of herpes simplex cytopathic effect after treatment with peroxidase generated hypothiocyanite. *Journal de biologie buccale* **18**, 71-74 (1990).

89. Welk, A. *et al.* Effect of lactoperoxidase on the antimicrobial effectiveness of the thiocyanate hydrogen peroxide combination in a quantitative suspension test. *BMC microbiology* **9**, 134 (2009).

90. Popper, L. & Knorr, D. Inactivation of yeast and filamentous fungi by the lactoperoxidase-hydrogen peroxide-thiocyanate-system. *Die Nahrung* **41**, 29-33 (1997).

91. Salathe, M. *et al.* Isolation and characterization of a peroxidase from the airway. *American journal of respiratory cell and molecular biology* **17**, 97-105 (1997).

92. Zamocky, M., Furtmuller, P.G. & Obinger, C. Evolution of catalases from bacteria to humans. *Antioxidants & redox signaling* **10**, 1527-1548 (2008).

93. Chandler, J.D. & Day, B.J. Thiocyanate: a potentially useful therapeutic agent with host defense and antioxidant properties. *Biochemical pharmacology* **84**, 1381-1387 (2012).

94. Suzuki, S. *et al.* Induction of Airway Allergic Inflammation by Hypothiocyanite via Epithelial Cells. *The Journal of biological chemistry* **291**, 27219-27227 (2016).

95. Lorentzen, D. *et al.* Concentration of the antibacterial precursor thiocyanate in cystic fibrosis airway secretions. *Free radical biology & medicine* **50**, 1144-1150 (2011).

96. Alstad, K.S. The effects of thiocyanate on basal and supplemental blood pressures. *British heart journal* **11**, 249-256 (1949).

97. Estes, J.E. & Keith, N.M. Hypothyroidism and mild myxedema from thiocyanate intoxication. *The American journal of medicine* **1**, 45-52 (1946).

98. Barnett, H.J., Jackson, M.V. & Spaulding, W.B. Thiocyanate psychosis. *Journal of the American Medical Association* **147**, 1554-1558 (1951).

99. Aas, K. & Thingstad, R. Thiocyanate therapy of hypertension; further experiences. *Acta medica Scandinavica* **139**, 229-241 (1951).

100. Vesey, C.J. & Cole, P.V. Blood cyanide and thiocyanate concentrations produced by long-term therapy with sodium nitroprusside. *British journal of anaesthesia* **57**, 148-155 (1985).

101. Cegolon, L. *et al.* In vitro antiviral activity of hypothiocyanite against A/H1N1/2009 pandemic influenza virus. *International journal of hygiene and environmental health* **217**, 17-22 (2014).

102. Chandler, J.D., Min, E., Huang, J., Nichols, D.P. & Day, B.J. Nebulized thiocyanate improves lung infection outcomes in mice. *British journal of pharmacology* **169**, 1166-1177 (2013).

103. Johnson, N.P. & Mueller, J. Updating the accounts: global mortality of the 1918-1920 "Spanish" influenza pandemic. *Bulletin of the history of medicine* **76**, 105-115 (2002).

104. Dolin, R. Influenza--interpandemic as well as pandemic disease. *The New England journal of medicine* **353**, 2535-2537 (2005).

105. Martin, P.M. & Martin-Granel, E. 2,500-year evolution of the term epidemic. *Emerging infectious diseases* **12**, 976-980 (2006).

106. Hause, B.M. *et al.* Characterization of a novel influenza virus in cattle and Swine: proposal for a new genus in the Orthomyxoviridae family. *mBio* **5**, e00031-00014 (2014).

107. Knipe, D.M. & Howley, P.M. *Fields virology*, 6th edn. Wolters Kluwer/Lippincott Williams & Wilkins Health: Philadelphia, PA, 2013.

108. Paul Glezen, W., Schmier, J.K., Kuehn, C.M., Ryan, K.J. & Oxford, J. The burden of influenza B: a structured literature review. *American journal of public health* **103**, e43-51 (2013).

109. Influenza-associated pediatric deaths--United States, September 2010-August 2011. *MMWR. Morbidity and mortality weekly report* **60**, 1233-1238 (2011).

110. Hatta, M. & Kawaoka, Y. The NB Protein of Influenza B Virus Is Not Necessary for Virus Replication In Vitro. *Journal of Virology* **77**, 6050-6054 (2003).

111. Dugan, V.G. *et al.* The evolutionary genetics and emergence of avian influenza viruses in wild birds. *PLoS pathogens* **4**, e1000076 (2008).

112. Rota, P.A. *et al.* Cocirculation of two distinct evolutionary lineages of influenza type B virus since 1983. *Virology* **175**, 59-68 (1990).

113. von Itzstein, M. The war against influenza: discovery and development of sialidase inhibitors. *Nature reviews. Drug discovery* **6**, 967-974 (2007).

114. Ghedin, E. *et al.* Large-scale sequencing of human influenza reveals the dynamic nature of viral genome evolution. *Nature* **437**, 1162-1166 (2005).

115. Kim, H., Webster, R.G. & Webby, R.J. Influenza Virus: Dealing with a Drifting and Shifting Pathogen. *Viral immunology* **31**, 174-183 (2018).

116. de Graaf, M. & Fouchier, R.A. Role of receptor binding specificity in influenza A virus transmission and pathogenesis. *The EMBO journal* **33**, 823-841 (2014).

117. Air, G.M. Influenza virus-glycan interactions. *Current opinion in virology* **7**, 128-133 (2014).

118. Luo, M. Influenza virus entry. *Advances in experimental medicine and biology* **726**, 201-221 (2012).

119. Worch, R. Structural biology of the influenza virus fusion peptide. *Acta biochimica Polonica* **61**, 421-426 (2014).

120. Boulo, S., Akarsu, H., Ruigrok, R.W. & Baudin, F. Nuclear traffic of influenza virus proteins and ribonucleoprotein complexes. *Virus research* **124**, 12-21 (2007).

121. Rossman, J.S. & Lamb, R.A. Influenza virus assembly and budding. *Virology* **411**, 229-236 (2011).

122. Hartshorn, K.L. *et al.* Evidence for a protective role of pulmonary surfactant protein D (SP-D) against influenza A viruses. *The Journal of clinical investigation* **94**, 311-319 (1994).

123. Daher, K.A., Selsted, M.E. & Lehrer, R.I. Direct inactivation of viruses by human granulocyte defensins. *J Virol* **60**, 1068-1074 (1986).

124. Fernandez-Sesma, A. *et al.* Influenza virus evades innate and adaptive immunity via the NS1 protein. *J Virol* **80**, 6295-6304 (2006).

125. Mibayashi, M. *et al.* Inhibition of retinoic acid-inducible gene I-mediated induction of beta interferon by the NS1 protein of influenza A virus. *J Virol* **81**, 514-524 (2007).

126. Lamichhane, P.P. & Samarasinghe, A.E. The Role of Innate Leukocytes during Influenza Virus Infection. *Journal of immunology research* **2019**, 8028725 (2019).

127. Hayden, F.G. *et al.* Local and systemic cytokine responses during experimental human influenza A virus infection. Relation to symptom formation and host defense. *The Journal of clinical investigation* **101**, 643-649 (1998).

128. Barlow, P.G. *et al.* Antiviral activity and increased host defense against influenza infection elicited by the human cathelicidin LL-37. *PloS one* **6**, e25333 (2011).

129. Fuchs, T.A. *et al.* Novel cell death program leads to neutrophil extracellular traps. *The Journal of cell biology* **176**, 231-241 (2007).

130. Schönrich, G. & Raftery, M.J. Neutrophil Extracellular Traps Go Viral. *Frontiers in immunology* **7**, 366-366 (2016).

131. Tripathi, S., Verma, A., Kim, E.J., White, M.R. & Hartshorn, K.L. LL-37 modulates human neutrophil responses to influenza A virus. *Journal of leukocyte biology* **96**, 931-938 (2014).

132. Narayana Moorthy, A. *et al.* In vivo and in vitro studies on the roles of neutrophil extracellular traps during secondary pneumococcal pneumonia after primary pulmonary influenza infection. *Frontiers in immunology* **4** (2013).

133. Hartshorn, K.L. *et al.* Neutrophil deactivation by influenza A virus. Role of hemagglutinin binding to specific sialic acid-bearing cellular proteins. *Journal of immunology (Baltimore, Md. : 1950)* **154**, 3952-3960 (1995).

134. Brass, A.L. *et al.* The IFITM proteins mediate cellular resistance to influenza A H1N1 virus, West Nile virus, and dengue virus. *Cell* **139**, 1243-1254 (2009).

135. Sadler, A.J. & Williams, B.R.G. Interferon-inducible antiviral effectors. *Nature Reviews Immunology* **8**, 559-568 (2008).

136. Durfee, L.A., Lyon, N., Seo, K. & Huibregtse, J.M. The ISG15 conjugation system broadly targets newly synthesized proteins: implications for the antiviral function of ISG15. *Molecular cell* **38**, 722-732 (2010).

137. Jorens, P.G. *et al.* Interleukin 8 (IL-8) in the bronchoalveolar lavage fluid from patients with the adult respiratory distress syndrome (ARDS) and patients at risk for ARDS. *Cytokine* **4**, 592-597 (1992).

138. Narasaraju, T. *et al.* Excessive Neutrophils and Neutrophil Extracellular Traps Contribute to Acute Lung Injury of Influenza Pneumonitis. *The American Journal of Pathology* **179**, 199-210 (2011).

139. Chen, X. *et al.* Host Immune Response to Influenza A Virus Infection. *Frontiers in immunology* **9**, 320 (2018).

140. de Bree, G.J. *et al.* Selective accumulation of differentiated CD8+ T cells specific for respiratory viruses in the human lung. *The Journal of experimental medicine* **202**, 1433-1442 (2005).

141. Manini, I. *et al.* Egg-Independent Influenza Vaccines and Vaccine Candidates. *Vaccines* **5** (2017).

142. McKimm-Breschkin, J.L. Influenza neuraminidase inhibitors: antiviral action and mechanisms of resistance. *Influenza and other respiratory viruses* **7 Suppl 1**, 25-36 (2013).

143. White, B., Robinson, E.S. & Barnes, L.A. *The Biology of Pneumococcus: The Bacteriological, Biochemicl, and Immunological Characters and Activites of Diplococcus Pneumoniae*. The Commonwealth Fund, 1938.

144. Yahiaoui, R.Y. *et al.* Prevalence and antibiotic resistance of commensal Streptococcus pneumoniae in nine European countries. *Future microbiology* **11**, 737-744 (2016).

145. von Gottberg, A. *et al.* Effects of vaccination on invasive pneumococcal disease in South Africa. *The New England journal of medicine* **371**, 1889-1899 (2014).

146. Keller, L.E., Robinson, D.A. & McDaniel, L.S. Nonencapsulated Streptococcus pneumoniae: Emergence and Pathogenesis. *mBio* **7**, e01792 (2016).

147. Nelson, A.L. *et al.* Capsule enhances pneumococcal colonization by limiting mucus-mediated clearance. *Infection and immunity* **75**, 83-90 (2007).

148. Uchiyama, S. *et al.* The surface-anchored NanA protein promotes pneumococcal brain endothelial cell invasion. *The Journal of experimental medicine* **206**, 1845-1852 (2009).

149. Limoli, D.H., Sladek, J.A., Fuller, L.A., Singh, A.K. & King, S.J. BgaA acts as an adhesin to mediate attachment of some pneumococcal strains to human epithelial cells. *Microbiology (Reading, England)* **157**, 2369-2381 (2011).

150. Andre, G.O. *et al.* Role of Streptococcus pneumoniae Proteins in Evasion of Complement-Mediated Immunity. *Front Microbiol* **8**, 224 (2017).

151. Tu, A.H., Fulgham, R.L., McCrory, M.A., Briles, D.E. & Szalai, A.J. Pneumococcal surface protein A inhibits complement activation by Streptococcus pneumoniae. *Infection and immunity* **67**, 4720-4724 (1999).

152. Zhang, J.R. *et al.* The polymeric immunoglobulin receptor translocates pneumococci across human nasopharyngeal epithelial cells. *Cell* **102**, 827-837 (2000).

153. Rayner, C.F. *et al.* Interaction of pneumolysin-sufficient and -deficient isogenic variants of Streptococcus pneumoniae with human respiratory mucosa. *Infection and immunity* **63**, 442-447 (1995).

154. Beiter, K. *et al.* An endonuclease allows Streptococcus pneumoniae to escape from neutrophil extracellular traps. *Current biology : CB* **16**, 401-407 (2006).

155. Hergott, C.B. *et al.* Bacterial exploitation of phosphorylcholine mimicry suppresses inflammation to promote airway infection. *The Journal of clinical investigation* **125**, 3878-3890 (2015).

156. Standish, A.J. & Weiser, J.N. Human Neutrophils Kill Streptococcus pneumoniae via Serine Proteases. *The Journal of Immunology* **183**, 2602-2609 (2009).

157. Shak, J.R., Vidal, J.E. & Klugman, K.P. Influence of bacterial interactions on pneumococcal colonization of the nasopharynx. *Trends in microbiology* **21**, 129-135 (2013).

158. Miller, E.L., Abrudan, M.I., Roberts, I.S. & Rozen, D.E. Diverse Ecological Strategies Are Encoded by Streptococcus pneumoniae Bacteriocin-Like Peptides. *Genome biology and evolution* **8**, 1072-1090 (2016).

159. Park, B., Nizet, V. & Liu, G.Y. Role of Staphylococcus aureus catalase in niche competition against Streptococcus pneumoniae. *Journal of bacteriology* **190**, 2275-2278 (2008).

160. Regev-Yochay, G., Trzcinski, K., Thompson, C.M., Malley, R. & Lipsitch, M. Interference between Streptococcus pneumoniae and Staphylococcus aureus: In vitro hydrogen peroxide-mediated killing by Streptococcus pneumoniae. *Journal of bacteriology* **188**, 4996-5001 (2006).

161. Pericone, C.D., Park, S., Imlay, J.A. & Weiser, J.N. Factors contributing to hydrogen peroxide resistance in Streptococcus pneumoniae include

pyruvate oxidase (SpxB) and avoidance of the toxic effects of the fenton reaction. *Journal of bacteriology* **185**, 6815-6825 (2003).

162. Nakamura, S., Davis, K.M. & Weiser, J.N. Synergistic stimulation of type I interferons during influenza virus coinfection promotes Streptococcus pneumoniae colonization in mice. *The Journal of clinical investigation* **121**, 3657-3665 (2011).

163. McCullers, J.A. & Rehg, J.E. Lethal synergism between influenza virus and Streptococcus pneumoniae: characterization of a mouse model and the role of platelet-activating factor receptor. *The Journal of infectious diseases* **186**, 341-350 (2002).

164. Vareille, M., Kieninger, E., Edwards, M.R. & Regamey, N. The airway epithelium: soldier in the fight against respiratory viruses. *Clin Microbiol Rev* **24**, 210-229 (2011).

165. Rada, B. & Leto, T.L. Oxidative innate immune defenses by Nox/Duox family NADPH oxidases. *Contributions to microbiology* **15**, 164-187 (2008).

166. Rose, M.C. & Voynow, J.A. Respiratory tract mucin genes and mucin glycoproteins in health and disease. *Physiological reviews* **86**, 245-278 (2006).

167. Voynow, J.A., Gendler, S.J. & Rose, M.C. Regulation of mucin genes in chronic inflammatory airway diseases. *American journal of respiratory cell and molecular biology* **34**, 661-665 (2006).

168. Conner, G.E., Wijkstrom-Frei, C., Randell, S.H., Fernandez, V.E. & Salathe, M. The lactoperoxidase system links anion transport to host defense in cystic fibrosis. *FEBS letters* **581**, 271-278 (2007).

169. Klebanoff, S.J. & Luebke, R.G. The Antilactobacillus System of Saliva. Role of Salivary Peroxidase. *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine* **118**, 483-486 (1965).

170. Zeldow, B.J. Studies on the antibacterial action of human saliva. III. Cofactor requirements of Lactobacillus bactericidin. *Journal of immunology* **90**, 12-16 (1963).

171. Bjorck, L., Rosen, C., Marshall, V. & Reiter, B. Antibacterial activity of the lactoperoxidase system in milk against pseudomonads and other gram-negative bacteria. *Applied microbiology* **30**, 199-204 (1975).

172. De Deken, X. *et al.* Cloning of two human thyroid cDNAs encoding new members of the NADPH oxidase family. *The Journal of biological chemistry* **275**, 23227-23233 (2000).

173. Mikola, H., Waris, M. & Tenovuo, J. Inhibition of herpes simplex virus type 1, respiratory syncytial virus and echovirus type 11 by peroxidase-generated hypothiocyanite. *Antiviral research* **26**, 161-171 (1995).

174. Poland, G.A., Jacobson, R.M. & Targonski, P.V. Avian and pandemic influenza: an overview. *Vaccine* **25**, 3057-3061 (2007).

175. Thompson, W.W. *et al.* Mortality associated with influenza and respiratory syncytial virus in the United States. *Jama* **289**, 179-186 (2003).

176. Steel, J. & Lowen, A.C. Influenza A virus reassortment. *Current topics in microbiology and immunology* **385**, 377-401 (2014).

177. Dlugolenski, D. *et al.* Swine Influenza Virus PA and Neuraminidase Gene Reassortment into Human H1N1 Influenza Virus Is Associated with an

Altered Pathogenic Phenotype Linked to Increased MIP-2 Expression. *J Virol* **89**, 5651-5667 (2015).

178. Karasin, A.I., Olsen, C.W. & Anderson, G.A. Genetic characterization of an H1N2 influenza virus isolated from a pig in Indiana. *J Clin Microbiol* **38**, 2453-2456 (2000).

179. Karasin, A.I. *et al.* Genetic characterization of H1N2 influenza A viruses isolated from pigs throughout the United States. *J Clin Microbiol* **40**, 1073-1079 (2002).

180. Rada, B. & Leto, T.L. Redox warfare between airway epithelial cells and Pseudomonas: dual oxidase versus pyocyanin. *Immunol Res* **43**, 198-209 (2009).

181. Hauser, M.J. *et al.* Antiviral responses by Swine primary bronchoepithelial cells are limited compared to human bronchoepithelial cells following influenza virus infection. *PloS one* **8**, e70251 (2013).

182. Oshansky, C.M., Barber, J.P., Crabtree, J. & Tripp, R.A. Respiratory syncytial virus F and G proteins induce interleukin 1alpha, CC, and CXC chemokine responses by normal human bronchoepithelial cells. *The Journal of infectious diseases* **201**, 1201-1207 (2010).

183. Oshansky, C.M. *et al.* Avian influenza viruses infect primary human bronchial epithelial cells unconstrained by sialic acid alpha2,3 residues. *PloS one* **6**, e21183 (2011).

184. Moskwa, P. *et al.* A novel host defense system of airways is defective in cystic fibrosis. *American journal of respiratory and critical care medicine* **175**, 174-183 (2007).

185. Matsuura, S., Shirakami, G., Iida, H., Tanimoto, K. & Fukuda, K. The effect of sevoflurane on ciliary motility in rat cultured tracheal epithelial cells: a comparison with isoflurane and halothane. *Anesthesia and analgesia* **102**, 1703-1708 (2006).

186. El Hassani, R.A. *et al.* Dual oxidase2 is expressed all along the digestive tract. *American journal of physiology. Gastrointestinal and liver physiology* **288**, G933-942 (2005).

187. Rada, B., Gardina, P., Myers, T.G. & Leto, T.L. Reactive oxygen species mediate inflammatory cytokine release and EGFR-dependent mucin secretion in airway epithelial cells exposed to Pseudomonas pyocyanin. *Mucosal Immunol* **4**, 158-171 (2011).

188. Pascual, J.M., McKenzie, A., Yankaskas, J.R., Falck, J.R. & Zeldin, D.C. Epoxygenase metabolites of arachidonic acid affect electrophysiologic properties of rat tracheal epithelial cells1. *The Journal of pharmacology and experimental therapeutics* **286**, 772-779 (1998).

189. Forteza, R., Salathe, M., Miot, F., Forteza, R. & Conner, G.E. Regulated hydrogen peroxide production by Duox in human airway epithelial cells. *American journal of respiratory cell and molecular biology* **32**, 462-469 (2005).

190. Pachucki, J., Wang, D., Christophe, D. & Miot, F. Structural and functional characterization of the two human ThOX/Duox genes and their 5'-flanking regions. *Molecular and cellular endocrinology* **214**, 53-62 (2004).

191. Boots, A.W. *et al.* ATP-mediated activation of the NADPH oxidase DUOX1 mediates airway epithelial responses to bacterial stimuli. *The Journal of biological chemistry* **284**, 17858-17867 (2009).

192. Ameziane-El-Hassani, R. *et al.* Dual oxidase-2 has an intrinsic Ca2+-dependent H2O2-generating activity. *The Journal of biological chemistry* **280**, 30046-30054 (2005).

193. Dlugolenski, D. *et al.* Bat cells from Pteropus alecto are susceptible to influenza A virus infection and reassortment. *Influenza Other Respir Viruses* **7**, 900-903 (2013).

194. Nicholls, J.M. The battle between influenza and the innate immune response in the human respiratory tract. *Infection & chemotherapy* **45**, 11-21 (2013).

195. Gerson, C. *et al.* The lactoperoxidase system functions in bacterial clearance of airways. *American journal of respiratory cell and molecular biology* **22**, 665-671 (2000).

196. Fragoso, M.A. *et al.* Transcellular thiocyanate transport by human airway epithelia. *J Physiol* **561**, 183-194 (2004).

197. Pedemonte, N. *et al.* Thiocyanate transport in resting and IL-4-stimulated human bronchial epithelial cells: role of pendrin and anion channels. *Journal of immunology* **178**, 5144-5153 (2007).

198. Pang, M. *et al.* Recombinant rat CC16 protein inhibits LPS-induced MMP-9 expression via NF-kappaB pathway in rat tracheal epithelial cells. *Exp Biol Med (Maywood)* (2015).

199. Poussin, C. *et al.* The species translation challenge-a systems biology perspective on human and rat bronchial epithelial cells. *Sci Data* **1**, 140009 (2014).

200. St-Laurent, J., Boulet, L.P. & Bissonnette, E. Cigarette smoke differently alters normal and ovalbumin-sensitized bronchial epithelial cells from rat. *J Asthma* **46**, 577-581 (2009).

201. Harper, R.W. *et al.* Differential regulation of dual NADPH oxidases/peroxidases, Duox1 and Duox2, by Th1 and Th2 cytokines in respiratory tract epithelium. *FEBS letters* **579**, 4911-4917 (2005).

202. Gattas, M.V. *et al.* Oxidative epithelial host defense is regulated by infectious and inflammatory stimuli. *Free radical biology & medicine* **47**, 1450-1458 (2009).

203. Grasberger, H. Defects of thyroidal hydrogen peroxide generation in congenital hypothyroidism. *Molecular and cellular endocrinology* **322**, 99-106 (2010).

204. Ganz, T. Antimicrobial polypeptides in host defense of the respiratory tract. *J Clin Invest* **109**, 693-697 (2002).

205. Hertz, C.J. *et al.* Activation of Toll-like receptor 2 on human tracheobronchial epithelial cells induces the antimicrobial peptide human beta defensin-2. *Journal of immunology* **171**, 6820-6826 (2003).

206. Shim, J.M., Kim, J., Tenson, T., Min, J.-Y. & Kainov, D.E. Influenza Virus Infection, Interferon Response, Viral Counter-Response, and Apoptosis. *Viruses* **9**, 223 (2017).

207. Ito, T. *et al.* Receptor specificity of influenza A viruses correlates with the agglutination of erythrocytes from different animal species. *Virology* **227**, 493-499 (1997).

208. Joo, J.H. *et al.* Dual oxidase 2 is essential for the toll-like receptor 5-mediated inflammatory response in airway mucosa. *Antioxidants & redox signaling* **16**, 57-70 (2012).

209. Kim, B.J. *et al.* Intranasal delivery of Duox2 DNA using cationic polymer can prevent acute influenza A viral infection in vivo lung. *Applied microbiology and biotechnology* **102**, 105-115 (2018).

210. Kim, H.J. *et al.* The Induction of Pattern-Recognition Receptor Expression against Influenza A Virus through Duox2-Derived Reactive Oxygen

Species in Nasal Mucosa. *American journal of respiratory cell and molecular biology* **53**, 525-535 (2015).

211. Kim, H.J. *et al.* Reactive oxygen species induce antiviral innate immune response through IFN-lambda regulation in human nasal epithelial cells. *American journal of respiratory cell and molecular biology* **49**, 855-865 (2013).

212. Donko, A. *et al.* Urothelial cells produce hydrogen peroxide through the activation of Duox1. *Free radical biology & medicine* **49**, 2040-2048 (2010).

213. Jeon, Y.J. & Kim, H.J. Duox2-induced innate immune responses in the respiratory epithelium and intranasal delivery of Duox2 DNA using polymer that mediates immunization. *Applied microbiology and biotechnology* **102**, 4339-4343 (2018).

214. Yoo, S.K., Starnes, T.W., Deng, Q. & Huttenlocher, A. Lyn is a redox sensor that mediates leukocyte wound attraction in vivo. *Nature* **480**, 109-112 (2011).

215. de Oliveira, S., Boudinot, P., Calado, Â. & Mulero, V. Duox1-Derived H<sub>2</sub>O<sub>2</sub> Modulates Cxcl8 Expression and

Neutrophil Recruitment via JNK/c-JUN/AP-1 Signaling and Chromatin Modifications. *The Journal of Immunology* **194**, 1523-1533 (2015).

216. Klebanoff, S.J., Kettle, A.J., Rosen, H., Winterbourn, C.C. & Nauseef, W.M. Myeloperoxidase: a front-line defender against phagocytosed microorganisms. *Journal of leukocyte biology* **93**, 185-198 (2013).

217. Souza, C.E. *et al.* Hypochlorous acid-induced heme degradation from lactoperoxidase as a novel mechanism of free iron release and tissue injury in inflammatory diseases. *PloS one* **6**, e27641 (2011).

218. Klebanoff, S.J. Myeloperoxidase: friend and foe. *Journal of leukocyte biology* **77**, 598-625 (2005).

219. van Dalen, C.J., Whitehouse, M.W., Winterbourn, C.C. & Kettle, A.J. Thiocyanate and chloride as competing substrates for myeloperoxidase. *The Biochemical journal* **327 ( Pt 2)**, 487-492 (1997).

220. Xulu, B.A. & Ashby, M.T. Small molecular, macromolecular, and cellular chloramines react with thiocyanate to give the human defense factor hypothiocyanite. *Biochemistry* **49**, 2068-2074 (2010).

221. Ashby, M.T., Carlson, A.C. & Scott, M.J. Redox buffering of hypochlorous acid by thiocyanate in physiologic fluids. *Journal of the American Chemical Society* **126**, 15976-15977 (2004).

222. Cho, S.Y. *et al.* Dual oxidase 1 and NADPH oxidase 2 exert favorable effects in cervical cancer patients by activating immune response. *BMC Cancer* **19**, 1078-1078 (2019).

223. Samson, M., Pizzorno, A., Abed, Y. & Boivin, G. Influenza virus resistance to neuraminidase inhibitors. *Antiviral Res* **98**, 174-185 (2013).

224. Hirst, G.K. THE QUANTITATIVE DETERMINATION OF INFLUENZA VIRUS AND ANTIBODIES BY MEANS OF RED CELL AGGLUTINATION. *The Journal of experimental medicine* **75**, 49-64 (1942).

225. Byrd-Leotis, L. *et al.* Shotgun glycomics of pig lung identifies natural endogenous receptors for influenza viruses. *Proceedings of the National Academy of Sciences* **111**, E2241-E2250 (2014).

226. Lin, T.J., Lin, C.F., Chiu, C.H., Lee, M.C. & Horng, J.T. Inhibition of endosomal fusion activity of influenza virus by Rheum tanguticum (da-huang). *Scientific reports* **6**, 27768 (2016).

227. Byrd-Leotis, L. *et al.* Shotgun glycomics of pig lung identifies natural endogenous receptors for influenza viruses. *Proceedings of the National Academy of Sciences of the United States of America* **111**, E2241-2250 (2014).

228. Karlsson, E.A. *et al.* Measuring Influenza Virus Infection Using Bioluminescent Reporter Viruses for In Vivo Imaging and In Vitro Replication Assays. *Methods in molecular biology (Clifton, N.J.)* **1836**, 431-459 (2018).

229. Patel, U. *et al.* Susceptibility of influenza viruses to hypothiocyanite and hypoiodite produced by lactoperoxidase in a cell-free system. *PloS one* **13**, e0199167-e0199167 (2018).

230. Hinshaw, V.S., Webster, R.G., Bean, W.J., Downie, J. & Senne, D.A. Swine influenza-like viruses in turkeys: potential source of virus for humans? *Science (New York, N.Y.)* **220**, 206-208 (1983).

231. Kemble, G.W., Bodian, D.L., Rose, J., Wilson, I.A. & White, J.M. Intermonomer disulfide bonds impair the fusion activity of influenza virus hemagglutinin. *J Virol* **66**, 4940-4950 (1992).

232. Skehel, J.J. & Wiley, D.C. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annual review of biochemistry* **69**, 531-569 (2000).

233. Influenza activity--United States, 2012-13 season and composition of the 2013-14 influenza vaccine. *MMWR. Morbidity and mortality weekly report* **62**, 473-479 (2013).

234. Hurt, A.C. *et al.* Antiviral resistance during the 2009 influenza A H1N1 pandemic: public health, laboratory, and clinical perspectives. *The Lancet. Infectious diseases* **12**, 240-248 (2012).

235. Sugita, C. *et al.* Antiviral activity of hypothiocyanite produced by lactoperoxidase against influenza A and B viruses and mode of its antiviral action. *Acta virologica* **62**, 401-408 (2018).

236. Zhu, L. *et al.* Inhibition of influenza A virus (H1N1) fusion by benzenesulfonamide derivatives targeting viral hemagglutinin. *PloS one* **6**, e29120 (2011).

237. Hsieh, C.F. *et al.* Mechanism by which ma-xing-shi-gan-tang inhibits the entry of influenza virus. *Journal of ethnopharmacology* **143**, 57-67 (2012).

238. Luo, G. *et al.* Molecular mechanism underlying the action of a novel fusion inhibitor of influenza A virus. *J Virol* **71**, 4062-4070 (1997).

239. Cianci, C. *et al.* pH-dependent changes in photoaffinity labeling patterns of the H1 influenza virus hemagglutinin by using an inhibitor of viral fusion. *Journal of virology* **73**, 1785-1794 (1999).

240. Plotch, S.J. *et al.* Inhibition of influenza A virus replication by compounds interfering with the fusogenic function of the viral hemagglutinin. *J Virol* **73**, 140-151 (1999).

241. Russell, R.J. *et al.* Structure of influenza hemagglutinin in complex with an inhibitor of membrane fusion. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 17736-17741 (2008).

242. Rogers, G.N. & Paulson, J.C. Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. *Virology* **127**, 361-373 (1983).

243. Stephenson, I., Wood, J.M., Nicholson, K.G. & Zambon, M.C. Sialic acid receptor specificity on erythrocytes affects detection of antibody to avian influenza haemagglutinin. *Journal of medical virology* **70**, 391-398 (2003).

244. Makkoch, J. *et al.* Erythrocyte binding preference of human pandemic influenza virus a and its effect on antibody response detection. *Ann Lab Med* **32**, 276-282 (2012).

245. Harris, A. *et al.* Influenza virus pleiomorphy characterized by cryoelectron tomography. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 19123-19127 (2006).

246. Inglis, S.C., Carroll, A.R., Lamb, R.A. & Mahy, B.W. Polypeptides specified by the influenza virus genome I. Evidence for eight distinct gene products specified by fowl plague virus. *Virology* **74**, 489-503 (1976).

247. Muller, M., Lauster, D., Wildenauer, H.H.K., Herrmann, A. & Block, S. Mobility-Based Quantification of Multivalent Virus-Receptor Interactions: New Insights Into Influenza A Virus Binding Mode. *Nano letters* **19**, 1875-1882 (2019).

248. Edinger, T.O., Pohl, M.O. & Stertz, S. Entry of influenza A virus: host factors and antiviral targets. *Journal of General Virology* **95**, 263-277 (2014).

249. Sakai, T., Nishimura, S.I., Naito, T. & Saito, M. Influenza A virus hemagglutinin and neuraminidase act as novel motile machinery. *Scientific reports* **7**, 45043-45043 (2017).

250. Sakudo, A., Misawa, T., Shimizu, N. & Imanishi, Y. N(2) gas plasma inactivates influenza virus mediated by oxidative stress. *Frontiers in bioscience (Elite edition)* **6**, 69-79 (2014).

251. Xu, S. *et al.* Mutations of two transmembrane cysteines of hemagglutinin (HA) from influenza A H3N2 virus affect HA thermal stability and fusion activity. *Virus Genes* **47**, 20-26 (2013).

252. Nagy, P., Jameson, G.N.L. & Winterbourn, C.C. Kinetics and Mechanisms of the Reaction of Hypothiocyanous Acid with 5-Thio-2-nitrobenzoic Acid and Reduced Glutathione. *Chemical Research in Toxicology* **22**, 1833-1840 (2009).

253. Berkley, J.A. *et al.* Bacteremia among children admitted to a rural hospital in Kenya. *The New England journal of medicine* **352**, 39-47 (2005).

254. Douglas, R.M., Paton, J.C., Duncan, S.J. & Hansman, D.J. Antibody response to pneumococcal vaccination in children younger than five years of age. *The Journal of infectious diseases* **148**, 131-137 (1983).

255. Nagai, K. *et al.* Antimicrobial susceptibility of Streptococcus pneumoniae, Haemophilus influenzae, and Moraxella catarrhalis clinical isolates from children with acute otitis media in Japan from 2014 to 2017. *Journal of infection and chemotherapy : official journal of the Japan Society of Chemotherapy* (2018).

256. van Alphen, L., Jansen, H.M. & Dankert, J. Virulence factors in the colonization and persistence of bacteria in the airways. *Am J Respir Crit Care Med* **151**, 2094-2099; discussion 2099-2100 (1995).

257. Andersson, B. *et al.* Identification of an active disaccharide unit of a glycoconjugate receptor for pneumococci attaching to human pharyngeal epithelial cells. *J Exp Med* **158**, 559-570 (1983).

258. Keller, L.E. *et al.* Draft Genome Sequences of Five Multilocus Sequence Types of Nonencapsulated Streptococcus pneumoniae. *Genome Announc* **1** (2013).

259. Tettelin, H. *et al.* Complete genome sequence of a virulent isolate of Streptococcus pneumoniae. *Science* **293**, 498-506 (2001).

260. Bryant, J.C. *et al.* Pyruvate oxidase of Streptococcus pneumoniae contributes to pneumolysin release. *BMC Microbiol* **16**, 271 (2016).

261. Rada, B.K., Geiszt, M., Kaldi, K., Timar, C. & Ligeti, E. Dual role of phagocytic NADPH oxidase in bacterial killing. *Blood* **104**, 2947-2953 (2004).

262. Riddles, P.W., Blakeley, R.L. & Zerner, B. Ellman's reagent: 5,5'-dithiobis(2-nitrobenzoic acid)--a reexamination. *Anal Biochem* **94**, 75-81 (1979).

263. Gau, J., Arnhold, J. & Flemmig, J. Reactivation of peroxidase activity in human saliva samples by polyphenols. *Arch Oral Biol* **85**, 70-78 (2018).

264. Patel, U. *et al.* Susceptibility of influenza viruses to hypothiocyanite and hypoiodite produced by lactoperoxidase in a cell-free system. *PloS one* **13**, e0199167 (2018).

265. Echlin, H. *et al.* Pyruvate Oxidase as a Critical Link between Metabolism and Capsule Biosynthesis in Streptococcus pneumoniae. *PLoS pathogens* **12**, e1005951 (2016).

266. Chelikani, P., Fita, I. & Loewen, P.C. Diversity of structures and properties among catalases. *Cellular and molecular life sciences : CMLS* **61**, 192-208 (2004).

267. Yesilkaya, H., Andisi, V.F., Andrew, P.W. & Bijlsma, J.J. Streptococcus pneumoniae and reactive oxygen species: an unusual approach to living with radicals. *Trends Microbiol* **21**, 187-195 (2013).

268. Zahlten, J. *et al.* Streptococcus pneumoniae-Induced Oxidative Stress in Lung Epithelial Cells Depends on Pneumococcal Autolysis and Is Reversible by Resveratrol. *J Infect Dis* **211**, 1822-1830 (2015).

269. Andisi, V.F. *et al.* Pneumococcal gene complex involved in resistance to extracellular oxidative stress. *Infection and immunity* **80**, 1037-1049 (2012).

270. Rai, P. *et al.* Streptococcus pneumoniae secretes hydrogen peroxide leading to DNA damage and apoptosis in lung cells. *Proceedings of the National Academy of Sciences of the United States of America* **112**, E3421-3430 (2015).

271. Carvalho, S.M. *et al.* Pyruvate oxidase influences the sugar utilization pattern and capsule production in Streptococcus pneumoniae. *PLoS One* **8**, e68277 (2013).

272. Lisher, J.P. *et al.* Biological and Chemical Adaptation to Endogenous Hydrogen Peroxide Production in Streptococcus pneumoniae D39. *mSphere* **2** (2017).

273. Yother, J. Capsules of Streptococcus pneumoniae and other bacteria: paradigms for polysaccharide biosynthesis and regulation. *Annu Rev Microbiol* **65**, 563-581 (2011).

274. Kadioglu, A., Weiser, J.N., Paton, J.C. & Andrew, P.W. The role of Streptococcus pneumoniae virulence factors in host respiratory colonization and disease. *Nature reviews. Microbiology* **6**, 288-301 (2008).

275. Dixit, C. *et al.* Nonencapsulated Streptococcus pneumoniae as a cause of chronic adenoiditis. *IDCases* **4**, 56-58 (2016).

276. Bafort, F., Parisi, O., Perraudin, J.P. & Jijakli, M.H. Mode of action of lactoperoxidase as related to its antimicrobial activity: a review. *Enzyme research* **2014**, 517164-517164 (2014).

277. Ashby, M.T. Inorganic Chemistry of Defensive Peroxidases in the Human Oral Cavity. *Journal of Dental Research* **87**, 900-914 (2008).

278. Aune, T.M. & Thomas, E.L. Oxidation of protein sulfhydryls by products of peroxidase-catalyzed oxidation of thiocyanate ion. *Biochemistry* **17**, 1005-1010 (1978).