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PATHOLOGICAL CONSEQUENCES OF PDI OXIDOREDUCTASE ACTIVITY ON VIRAL PROTEIN MATURATION

A Dissertation Presented

by

Nicolas Chamberlain

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
Specializing in Cellular, Molecular, and Biomedical Sciences

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ABSTRACT

The influenza A virus (IAV) causes severe respiratory illness in humans. Current treatments are rapidly becoming ineffective due to the emergence of viral resistance to available therapies such as oseltamivir and zanamivir. Given the impact of this virus there is an urgent need to explore novel targets for new treatments less susceptible to viral mutation; targeting host proteins utilized by the virus may avoid these limitations. It has been shown *in vitro* that interactions with host ER based protein disulfide isomerases (PDIs) are required for specific IAV proteins to reach their functional conformations. The viral proteins hemagglutinin (HA) and neuraminidase (NA) both contain numerous disulfide bonds necessary for their functionality. Our results demonstrate both HA and NA interact with host PDIA3; a chaperone responsible for the catalysis of disulfide bonds in newly formed glycoproteins. However, it is unknown whether these host-viral protein interactions are required during active infection and whether they represent a putative therapeutic target for the treatment of influenza infection.

In this dissertation I investigated the role of host PDIA3 in the folding of IAV proteins and development of subsequent immunopathology. The impact of PDIA3 during both H1N1 and H3N2 influenza infection was examined using lung epithelial specific PDIA3 knockout mice and inhibitors of PDIs in primary human bronchial epithelial cells and isolated mouse tracheal epithelial cells. Among PDI inhibitors tested, we found LOC14 inhibits PDIA3 at an IC50 of 5µM. Treatment with LOC14 inhibits PDIA3 activity in lung epithelial cells and subsequently decreases disulfide bonds and oligomerization of HA in both H1N1 and H3N2 infected primary lung epithelial cells. Biotin switch assays indicated that LOC14 also decreased disulfide bonds in NA and these differences in oxidative folding correspond to a subsequent decrease in NA activity. Furthermore, following LOC14 treatment we observed a decrease in detectable viral protein in supernatants from infected cells, suggesting potential deficiencies in viral release. Moreover, these decreases in disulfide bonds significantly decrease viral load, and pro-inflammatory responses from primary lung epithelial cells. Lung epithelial specific deletion of PDIA3 in mice results in a significant decrease in viral burden and levels of inflammatory-immune markers in mouse lung, as well as significantly improved airway mechanics. Additionally, *in vivo* administration of LOC14 partially mirrored these results, yielding significant decreases in overall viral burden. Taken together, these data suggest that lung epithelial PDIA3 plays a critical role in the maturation of IAV proteins and propagation of the virus and illustrate the potential of utilizing host PDIs as a target for anti-viral therapies.

CITATIONS

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CHAPTER 1

COMPREHENSIVE LITERATURE REVIEW

Working Title: **Pathological Consequences of the Unfolded Protein Response and Downstream Protein Disulfide Isomerases in Pulmonary Viral Infection and Disease**

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Running title: UPR and PDI activity in pulmonary viral infection and disease

Summary:

Protein folding within the endoplasmic reticulum (ER) exists in a delicate balance; perturbations of this balance can overload the folding capacity of the ER and disruptions of ER homeostasis is implicated in numerous diseases. The unfolded protein response (UPR), a complex adaptive stress response, attempts to restore normal proteostasis, in part, through the up-regulation of various foldases and chaperone proteins including redox-active protein disulfide isomerases (PDIs). There are currently over 20 members of the PDI family each consisting of varying numbers of thioredoxin-like domains which, generally, assist in oxidative folding and disulfide bond rearrangement of peptides. While there is a large amount of redundancy in client proteins of the various PDIs, the size of the family would indicate more nuanced roles for the individual PDIs. However, the role of individual PDIs in disease pathogenesis remains uncertain. The following review briefly discusses recent findings of ER stress, the UPR, and the role of individual PDIs in various respiratory disease states.

Keywords: ER stress, UPR, PDI, Disulfide Bond, pulmonary disease

Introduction

The endoplasmic reticulum (ER) is a highly specialized organelle that plays numerous roles in the cell. It is the primary site of the synthesis of membrane-bound and secreted proteins, and as such maintains an oxidizing redox environment to facilitate the formation of disulfide bonds required for the stabilization of peptide structure[1]. Additionally, numerous posttranslational modifications such as N-inked glycosylation occur solely within the ER[2]. Approximately one-third of all proteins that traffic through the ER contain disulfide bonds and the ER contains a vast array of chaperones and foldases to assist in the folding of newly synthesized peptides[1, 3]. Properly folded proteins are essential for the normal function of the cell, and potentially misfolded proteins are rapidly degraded by the ER-associated degradation (ERAD)[4]. Under basal conditions, approximately thirty percent of newly synthesized peptides are targeted for degradation[5].

Protein folding within the ER exists in a delicate balance, and the physiological states of increased protein synthesis can quickly overload the folding capacity of the ER leading to a buildup of unfolded or misfolded peptides in the ER lumen, termed ER stress. In an effort to combat this stress the cell activates the unfolded protein response (UPR), a highly conserved, multifaceted stress response aimed at restoring normal ER homeostasis[6]. Collectively the UPR attenuates normal protein synthesis, upregulates ERAD machinery, increases the size of the ER, and upregulates various chaperones, including protein disulfide isomerases (PDIs) a large family of proteins that assists in the oxidative folding of nascent peptides[6]. Failure of the UPR to restore normal ER homeostasis leads to cell death through the activation of apoptotic pathways[6].

ER stress and activation of the UPR are common in the progression of numerous diseases including various cancers[7], neurodegenerative disorders[8], and viral infections[9]. The individual etiology of these disorders is as diverse as the cell's response to each. And the exact intricacies of the molecular mechanisms underlying activation of the UPR and subsequent up-regulation of distinct PDIs remains poorly understood. A greater understanding of host pathways involved could potentially aid in the development of future treatments. The following review briefly discusses recent findings of ER stress, the UPR, and the role of individual PDIs in various respiratory disease states.

The Unfolded Protein Response

The unfolded protein response (UPR) is a highly conserved collection of pathways responsible for monitoring the status of the ER. In mammals the UPR consists of three pathways each controlled by a particular sensor, inositol-requiring protein 1 (IRE1), protein kinase RNA (PKR)-like ER kinase (PERK), and activating transcription factor-6 (ATF6) (Figure 1). IRE1 exists in two isoforms α and β, this review focuses on solely IRE1 α, as the role the β isoform in activation of the UPR and induction of PDIs during pulmonary disease is less well characterized. These three pathways work in concert to decrease the protein load of the ER while simultaneously increasing its folding capacity. If the ER stress is too pronounced, or prolonged, the UPR directs the cell towards apoptosis[10-12].

Generally, the PERK pathway limits protein synthesis, the IRE1 pathway increases mRNA and protein degradation and the size of the ER, while the ATF6 pathway

up-regulates chaperone proteins and protein degradation[10]. Each of these transducers are integral membrane proteins residing in the ER membrane. Under normal conditions are held in inactive conformations by GRP78, an ER resident chaperone. Under conditions of ER stress unfolded or misfolded protein builds up within the ER lumen, GRP78 dissociates from the sensors owing to higher affinity to exposed hydrophobic residues on the unfolded proteins[11]. While GRP78 is considered a master regulator of the UPR, the ER chaperone HSP47 has recently been shown as a selective regulator of the IRE1 arm of the UPR, displacing GRP78 and facilitating IRE1 oligomerization[13]. Additionally, there is evidence that the individual transducers can bind unfolded protein directly[14].

Following GRP78 disassociation IRE1 dimerizes and undergoes trans-autophosphorylation of cytosolic kinase domains[11]. Upon phosphorylation IRE1 displays endonucleolytic activity specifically targeting X-box binding protein 1 (XBP1) mRNA, this activity removes an intron and ultimately induces a frame shift by removing a stop codon. The spliced XBP1 (XBP1s) is translated and acts as a transcription factor driving the expression of ER chaperones, ERAD proteins, and lipid synthesis[15]. In mammals both spliced and un-spliced (XBP1u) are translated, interestingly while XBP1s acts a potent transcriptional activator of UPR effector genes, XBP1u acts as a repressor of the UPR[16] (Figure 1).

Tumor necrosis factor associated factor 2 (TRAF2) is known to interact with IRE1 under conditions of extended ER stress leading to activation of downstream inflammatory and apoptotic signaling[10].

IRE1 is also capable of forming higher order structures that utilize their endonucleolytic activity to degrade ER-localized mRNAs in a process called regulated IRE1 dependent degradation of mRNA (RIDD)[17].

Like IRE1, PERK also undergoes dimerization and transphosphorylation following release from GRP78. PERK then phosphorylates eukaryotic initiation factor 2a (eIF2a) attenuating cap dependent protein translation. This decrease in global translation leads to the cap independent translation of activating transcription factor 4 (ATF4). ATF4 leads to the expression of amino-acid transporters, genes important in protecting the cell against oxidative stress, and XBP1[10]. ATF4 also leads to the expression of C/EBP homologous protein (CHOP), another transcription factor that drives the cell towards apoptosis[10].

Unlike both IRE1 and PERK, ATF6 can exist as either a monomer or an oligomer, stabilized by disulfide bonds, while still bound to GRP78. ATF6 contains a Golgi localization signal that is masked by GRP78, upon disassociation ATF6 is translocated to the Golgi body where it is consecutively cleaved by two proteases SP1 and SP2[18]. Interactions with PDIs ensure that only reduced monomeric ATF6 is moved to the Golgi. The proteases liberate the N-terminal cytosolic domain of ATF6 (ATF6-N). ATF6-N is a transcription factor that moves to the nucleus and induces expression of various UPR target genes. Chaperone proteins are the primary targets of ATF6-N, including GRP78, GRP94, and PDIs[10].

While it is useful to separate UPR signaling pathways into discrete units, there exists a large amount of crosstalk between them. Genes under the control of the UPR often contain ER stress response elements (ERSEs) in their promoter regions that are responsible for transcriptional induction. Both XBP1s and ATF6-N can bind to these elements, though ATF6-N binding requires additional transcription factors[19]. Interestingly, XBP1s and ATF6-N can form heterodimers, which further complicates signaling[20]. Moreover, all three UPR pathways often involve the same proteins[10]. The PERK and ATF6 pathways lead to XBP1 expression, which is then processed by IRE1. And all three pathways converge on NF-κB activation, though each uses a distinct mechanism.

However, this does not mean the UPR exists in a binary state of either active or inactive. The individual pathways of the UPR can be activated independently of one another. For instance, numerous groups have shown differential pathway activation following influenza infection, suggesting distinct triggers for each signaling pathway[21, 22].

Furthermore, while UPR activation is classically thought to involve the accumulation of unfolded protein within the ER lumen, there are numerous studies demonstrating activation of the UPR in the absence of unfolded protein. Toll-like receptor (TLR)2 and 4 have been shown to activate IRE1 and subsequent XBP1 maturation in macrophages[23]. Notably this XBP1 activation did not induce expression of canonical ER stress genes, but was required for the continued production of proinflammatory

cytokines[23]. Similarly, it has been reported dendritic cells constitutively activate the IRE1 arm of the UPR in the absence of ER stress, and this activation was required for homeostasis of CD8α^+ dendritic cells[24, 25]. Additionally, mitochondrial reactive oxygen species have been shown to exacerbate TLR induced activation of the UPR[26]. These findings are particularly interesting as the utilization of TLRs as an alternative activation pathway would suggest that pathogens themselves or pattern-associated molecular patterns (PAMPs)[27] and damage-associated molecular patterns (DAMPs) are directly capable of activating the UPR[28].

Protein Disulfide Isomerases

The UPR upregulates a wide variety of chaperone proteins in an effort to restore normal proteostasis, among these are protein disulfide isomerases (PDIs) a large family of redox active chaperones that play important roles in the formation, reduction, and isomerization of disulfide bonds[29]. Currently there are over 20 members of the PDI family, each differentiated from one another by the number and organization of TRX domains[29, 30]. Individual TRX domains are classified as catalytically active (a) or protein binding (b) by the presence or absence of a largely conserved CXXC sequence. (Table 1). The CXXC motif allows for the oxidoreductase activity of PDIs by alternating between an oxidized form, where both cysteines are linked through a disulfide bond, and a reduced form containing two free sulfhydryl groups. This effectively transfers a disulfide to the client protein. While the individual catalytic sequences vary the overall mechanism remains the same, with the intervening residues modulating the pKa of the reactive residues. The N terminal Cys exists as a thiolate ($-S^-$) anion due to its lower pKa, which

mediates nucleophilic attack forming a mixed disulfide. The C terminal Cys is partially buried within the protein elevating its pKa relative to other thiols and preventing the reverse reaction from occurring. The pKa of this C terminal Cys is rapidly decreased by a conformational change in the protein itself, which brings the side chain of a highly conserved arginine in close proximity to the active site. This shift in pKa changes the C terminal Cys from a thiol to a thiolate anion, which acts to resolve the mixed disulfide through a subsequent nucleophilic attack. Additionally, salt bridges located beneath the active site also serve to modulate the pKa of the Cys residues. PDIs in the reduced dithiol state participate in isomerization reactions, shuffling disulfide bonds between Cys residues, whereas oxidized (-S-S-) PDIs introduce disulfide bonds into associated peptides. The introduction of a disulfide bond into the client protein leaves the oxidized PDI in the reduced state where it can be rapidly re-oxidized by an intricate network of enzymes. (Figure 2) The isomerization of disulfide bonds does not involve a net change in disulfides, so the enzyme remains in the reduced state following the reaction. The above process is extensively reviewed in the following references[31, 32].

The re-oxidation of PDIs falls primarily to ERO1, which using FAD as a cofactor, transfers electrons from PDIs to molecular oxygen reducing it to hydrogen peroxide[33]. Additional enzymes can oxidize PDIs: such as GPx7 and GPx8, two peroxidases that directly oxidize PDIs while reducing hydrogen peroxide[34]. Glutathione is the primary redox buffer in the ER, and reduced glutathione is known to oxidize PDI *in vitro*[35]. Interestingly oxidized glutathione has been shown to reduce PDIA3 *in vivo*, demonstrating its buffering role. The ability to reduce PDIA3 suggests glutathione possesses the ability

to reduce other PDIs *in vivo* as well[36]. While the non-catalytic b domains lack an active site, they nonetheless assist in the chaperone activity of PDIs by assisting in protein binding.

PDIs were originally characterized as ER resident proteins; most members of the family contain either a canonical KDEL sequence or a non-canonical retention sequence. Despite the near total presence of an ER retention sequence PDIs are commonly found throughout the cell, at the cell surface, or even preferentially secreted from the cell[37]. The dispersal throughout the cell despite the presence of a retention sequence may suggest unexplored roles for non-canonical retention sequences.

As one might expect, owing to the high degree of homology in the PDI family there exists a large amount of redundancy in terms of both functionality and client proteins. However, certain proteins appear to be clients of specific PDIs[38]. PDIA3 has enhanced specificity towards glycoproteins owing to its association with both calreticulin and calnexin, two lectin-based chaperones within the ER lumen[39].

UPR and PDIs in asthma and pulmonary fibrosis

The UPR is initiated to manage the ER stress, but intense ER stress can result in apoptosis. Excessive ER stress and unhindered UPR can lead to apoptosis, proinflammatory signaling, and epithelial-mesenchymal transition (EMT), features that have all been linked to lung fibrosis[40-43] and asthma [39, 44-46].

Although, evidence is emerging, that downstream of UPR, PDIs are upregulated in both asthma and pulmonary fibrosis, their function in the pathophysiology of lung diseases is not well understood. We have identified that various PDIs are upregulated in allergic asthma [39, 45], and their increases correlated with the higher bronchodilator response or blood eosinophilic counts in allergic asthmatics [39, 45]. Intriguingly our in-depth analysis of lung epithelial specific knockouts of PDIA3 demonstrated that PDIA3 specifically regulate, eosinophilic, and pro-fibrotic responses in lung epithelial cells by oxidizing cysteine sulfhydryl (-SH) groups in Eotaxin, Periostin, and EGF[45]. Furthermore, we also demonstrated that PDIA3 facilitates -S-S- mediated oligomerization of pro-apoptotic BAK to induce intrinsic apoptosis in allergic airway disease models[45, 46]. Ablation of *Pdia3* specifically in lung epithelial cells attenuated, apoptotic, inflammatory, and fibrotic responses in a model of allergic airway disease[45]. These and other literature have led to the hypothesis that heterogeneous severe asthma could potentially be classified as an endotype of asthma[47].

Although, there is very little known about the impact of PDIs in pulmonary fibrosis recent literature have highlighted that PDIs potentially regulate disulfide bonds in many pro-apoptotic and pro-fibrotic proteins including collagen crosslinking enzyme lysyl oxidase like 2 (LOXL2)[45, 46, 48]. Literature have also indicated that PDIA3 drives the trans-differentiation of murine alveolar epithelial cells and it is regulated by pro-fibrotic injury in mice[49]. We have also identified that PDIA3 as a regulator of -S-S- bonds in death receptor CD95 (FAS) and inhibition or downregulation of PDIA3 decreases -S-S-

bonds in FAS, lung epithelial apoptosis and ultimately attenuation of pulmonary fibrosis in murine models of pulmonary fibrosis[46].

So far there are no proven therapeutics available to inhibit PDIs in the clinic, however, decades of research from various laboratories have identified many inhibitors that have shown *in-vivo* and *in-vitro* efficacy in inhibiting PDIs. Interestingly, rutinosides (plant flavonoids) that are known to inhibit PDIs are now being used in different clinical studies [50], also it is interesting to note that Dr. Stockwell's group have identified LOC14 as a specific inhibitor of PDIA1 and –A3[51, 52]. This literature suggests that UPR and subsequent induction of PDIs regulate pathology of various diseases and inhibiting PDIs may be a potential therapeutic approach that would benefit patients with chronic diseases.

UPR and PDIs in respiratory viral infection

Approximately 40 viruses are known to interact with the UPR, with many of these ultimately causing the induction of ER chaperone proteins[53]. In this section we highlight a few common respiratory viruses that display significant morbidity and mortality while also being known to cause exacerbations of lung diseases, with a specific focus on influenza[54].

Influenza

There are four types of influenza virus: A, B, C, and D; all are segmented RNA viruses and similar in composition and structure[55-58]. Of these four types A and B are the most clinically relevant[55, 59], with each being capable of causing seasonal epidemics, while A is responsible for recurrent pandemics, most recently in 2009, and are associated

with significantly enhanced morbidity and mortality[60]. Influenza C is capable of infecting humans but causes extremely mild to asymptomatic infections[61].

Influenza A virus (IAV) circulates in a wide range of mammalian and avian hosts, and it is this wade host range along with the segmented genome that is responsible for the pandemic potential of the virus[55, 59]. The segmented genome allows for reassortment or exchange of genetic information during mixed infection of cells with different viruses[62], resulting in rapid unpredictable change to the proteins of the virion, termed antigenic shift. This is contrast to subtle changes brought about through errors in replication, termed antigenic drift[63]. While all strains of the virus can reassort the limited host range of B, C, and D prevents an extreme phenotypic change[60, 64, 65]. Influenza B and C primarily infect humans, though B can infect seals as well[66, 67]. Influenza D infects ruminants and are not known to cause disease in humans[56]. While antigenic shift can affect any viral protein, its effect is arguably most significant when it occurs on the viral surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) the two major antigenic determinants of the virus[68].

Influenza hemagglutinin

HA is largely responsible for viral binding and entry into the target cell, generally interacting with sialic acid residues on host proteins[69]. This interaction allows for clatherin mediated endocytosis ultimately leading to fusion of the viral and cellular membranes depositing the viral core in the cytoplasm of the cell[70]. HA is synthesized Into the ER as a roughly 70kD monomer (HA0), this monomer undergoes processing within the ER to remove an N terminal signal sequence as well as oxidative folding

utilizing host redox active chaperone proteins[38, 39]. Once fully processed the HA monomers noncovalently oligomerize into a homotrimer and are sent to the Golgi body[71, 72]. In the Golgi gylcosyl groups are added and modified and the processed trimer is sent to the plasma membrane where it aids in viral budding[39]. HA0 is inactive and requires cleavage by host proteases to become active[73]. This cleavage results in two fragments, 50kD HA1 which contains the globular head region of the protein responsible for binding to the target cell, and 20kD HA2 which contains the fusion peptide[69, 71]. HA1 and 2 are linked by disulfide bonds that help stabilize the unstable structure of the cleaved HA[59]. Regardless of the viral strain cleavage occurs after oligomerization[39].

Through the entry process HA is principally responsible for setting the host range and tissue tropism of the individual influenza strains[72]. IAV HA is currently has into 18 subtypes divided into two antigenically distinct groups[68, 74]. H1, 2, 5, 6, 8, 9, 11, 12, 13, 16, 17, and 18 comprise group 1 and H3, 4, 7, 10, 14, and 15 comprise group 2[55, 75, 76]. H17 and 18 have been recently isolated from bats and bind to MHCII receptors on target cells, which may have interesting and profound implications on the immune response and overall pathogenesis of these viruses[77]. Despite the rather low overall sequence homology between HA groups, roughly 40%, they share key important amino acid residues responsible for substrate binding and membrane fusion as well as overall structural homology[59, 76]. Nearly all HA subtypes can be isolated from waterfowl where influenza typically presents as an asymptomatic gut infection[78]. H1, 2, and 3 are the main subtypes circulating in humans[59]. Avian viruses typically bind to α2,3 linked sialic acid residues, while human viruses tend to bind to α2,6 linked residues[60]. Porcine viruses bind to both

types of linkages, these specificities are believed to reflect the abundance of these different linkages on tissues at different sites of infection, lung epithelial cells form human viruses and intestinal epithelial cells for avian viruses[60]. Swine lung epithelial cells contain both types of linkages and may explain why swine are viewed as the primary generators of pandemic influenza strains[55, 79]. Interaction with sialic acid residues are not the only factor involved in determining host range, the cleavage and stability of HA also play important roles[72].

The HA trimer is anchored to the surface of the virion and extends roughly 14 nm from the surface[59, 80]. The cleavage site is in a loop located towards the bottom of the trimer close to the interface with the viral membrane, typically comprised of a single arginine residue, termed a monobasic site[75]. H5 and H7 have the potential for significant modifications to this loop, adding additional basic amino acids, known as a polybasic site, as well as insertions of other amino acids[81]. These supplementary residues alter the cleavage specificity of HA and extend the loop easing interaction with proteases[59, 82]. HAs containing a monobasic site are typically cleaved by tissue specific proteases at the plasma membrane of the cell or after the virus has left the cell[83], while HAs containing a polybasic site can be cleaved by more ubiquitously expressed proteases like trypsin or plasmenogin, and can be cleaved inside the cell before viral budding[72, 84]. These changes can lead to a virus that results in fatal disease in birds and are called highly pathogenic avian influenza (HPAI) viruses[85]. These highly pathogenic viruses are distinct from pandemic strains and are potentially capable of jumping from birds directly to humans with no reassortment[59, 86]. These modifications of HA are also associated

with drastic alterations in overall stability of the trimer. Upon acidification of the endosome cleaved HA undergoes an irreversible conformational change exposing the fusion peptide, if this change occurs prematurely it can have profound effects on viral fusion and infection[80]. HAs containing a monobasic site are typically more stable being activated around pH 5, while ones containing polybasic sites can be activated at pH 6[75]. This is the pH of the upper respiratory track and nasal passages in humans[87] and may explain the capacity of HPAI for jumping directly to humans.

While HA is largely responsible for the host range and tropism it is important to remember that these are ultimately polygenic effects, for instance the pandemics of 1957 and 1968 had changes to their polymerase subunits and these changes have been demonstrated to modulate host range[88].

Despite the importance of HA, it is largely ignored as a therapeutic target, while it is an important antigenic determinant of the virus and as such will likely be a target of the immune system, there are no FDA approved compounds targeting HA[89]. Umifenovir is commonly used to treat IAV infection in both Russia and China, it is believed to prevent association between sialic acid residues and HA, blocking viral fusion[90]. Umifenovir also possesses some immunomodulatory effects, however given its primary target it is likely most effective when given during the first 48 hours of infection during peak viral replication[90, 91]. While not currently FDA approved in the US, there are currently a number of clinical trials being conducted in other countries, not only against influenza infection but also novel coronavirus infection as well.

Influenza neuraminidase

NA is the other major surface glycoprotein of the virion, accounting for up to 20% of the glycoproteins on the surface[92]. There are currently 11 NA subtypes, like HA it divided into two antigenically distinct groups[77]. Group 1 contains N1, 4, 5, and 8, while group 2 contains N2, 3, 6, 7, and 9[60, 77]. N10 and 11 are classified as NA like homologs and do not belong to either group and do not possess typical NA activity[77]. NA has exo-sialidase activity, cleaving terminal sialic acid residues, and plays a critical role in viral exit, preventing viral aggregation on the host cell[55, 60, 75]. Additionally, this sialidase activity plays a minor role in viral entry, cleaving sialic acid residues in mucus lining the epithelial cells of the airway[93]. NA maturation occurs much like HA, both are synthesized into the ER, require extensive oxidative folding through interaction with host redox active chaperone proteins, and undergo further processing in the Golgi before being transported to the plasma membrane[60, 94]. However, unlike HA, NA does not need to be cleaved in order to be active. While both HA and NA oligomerize, active NA is a homotetramer, and the individual monomers are held together by intermonomer disulfide bonds[94]. Previous work has demonstrated these bonds are required for NA activity[95]. Interestingly the stalk domain of NA can be variable in length resulting in an NA tetramer that can be either slightly above or slightly below HA trimer, this has implications for the replication and pathogenesis of the virus[96, 97].

Given NAs importance it is not surprising that it is a common target for antiviral therapies, NA inhibitors are currently one of three classes of FDA approved anti influenza drugs[89, 98]. Despite the difference between the NA groups the active sites are

remarkable similar showing a high degree of conservation among amino acids[75]. Though antigenic drift often rapidly decreases the effectiveness of these inhibitors. A single amino acid substitution H274Y is enough to significantly decrease the effectiveness of oseltamivir though it has comparatively little effect of the related compound zanamivir[99]. These oseltamivir resistant strains of IAV comprised the majority of influenza isolates 2007-2008 influenza season[100].

Despite being utilized mainly for viral egress NA may have additional strain specific functions. The WSN strain HA is cleaved by the enzyme plasminogen[101], this in and of itself is not unusual, however, researchers have found that the plasminogen is actively recruited by the WSN NA[102]. Thus, in addition to cleaving sialic acid residues the WSN NA concentrates plasminogen in an area where it is able to be utilized by the virus, either at the cell surface or after the virus has released.

Influenza A virus (IAV) is known to activate different arms of the UPR depending on the model[21, 22, 103]. Hassan *et al*. demonstrated in isolated primary human tracheobronchial epithelial (HTBE) cells that IAV infection activated the IRE1 branch of the UPR but not the PERK or ATF6 branches[21]. That same year Roberson *et al*. showed in isolated primary mouse tracheal epithelial cells IAV infection strongly activated the ATF6 branch, but not PERK or IRE1[22]. As indicated the models for these two studies are different which may account for the differences in UPR activation. However, one less explored difference between the two studies is the time post viral infection in which the activation status of the UPR was explored. Hassan *et al*. examined UPR activation shortly

after infection while Roberson *et al*. explored UPR activation at 24- and 48-hours post infection. This distinction may help explain the contradictory results as the two studies are examining the UPR at different points of the viral replication cycle where reproductive needs and thus utilization of host proteins may be quite different. A recent third study by Landeras-Bueno *et al*. using chemical genomics showed IAV infection leads to the attenuation of the PERK branch of the UPR in A549 cells[103]. Interestingly, in contrast to the previous studies they showed no down regulation of IRE1 or ATF6. Again, this may be due to differences in the time points examined.

The relationship between IAV and PDIs is more straightforward, in 2006 Solda *et al*. clearly demonstrated PDIA3 was required for efficient folding of IAV HA *in vitro[38]*. They also showed while other PDIs were able to act as surrogate chaperones for various proteins, efficient HA disulfide bond formation required PDIA3. Loss of IAV replication in cells treated with siRNA against PDIA3 supported this finding, though direct results on viral proteins were not examined[22]. More recently expanded siRNA screens identified PDIA1 and 4 in addition to PDIA3 as having a role in IAV replication[104]. The authors found substantial decreases in both IAV NP and M1 protein levels as well as significant decreases in viral transcript levels following siRNA treatment. Both M1 and NP lack disulfide bonds as part of their functional conformations[105], thus it is unclear whether the examined PDIs are interacting directly with the NP or M1.

Building on those results we have shown PDIA3 plays an important role in IAV replication *in vivo*. Utilizing conditional PDIA3 knockout mice we demonstrate PDIA3

deletion in lung epithelial cells significantly decreases levels of viral transcripts and proteins, as well as corresponding decreases in inflammatory cytokines and inflammatory and immune cells in the bronchoalveolar lavage fluid (BALF)[39]. Moreover, we show ablation of *Pdia3* in the lung epithelium diminishes IAV mediated methacholine induced AHR, providing a physiological readout illustrating the importance of PDIA3 in IAV replication[39].

Other common respiratory viruses

Rhinovirus (RV) has recently been shown to cause impaired UPR activation in primary Cystic Fibrosis (CF) bronchial cells[106]. However, this impairment of UPR activation does not appear to be cell intrinsic as activation with known chemical inducers of the UPR produces a robust UPR response as indicated by increased GRP78 and CHOP expression[106]. Moreover, chemical activation of the UPR in RV infected primary cells significantly impeded viral replication and subsequent release of the virus. Suggesting that like IAV, RV is capable of directly activating the UPR. This is confirmed by a recent study from Song *et al*. showing the human RV16 infection, specifically the non-structural protein 2B activates both PERK and ATF6 UPR pathways in H1-HeLa cells[107]. Interestingly this protein also simultaneously inactivates the IRE1 pathway by blocking phosphorylation and subsequent XBP1 splicing[107].

Like the above viruses Respiratory Syncytial Virus (RSV) infection activates the UPR. In both A549 cells and primary HTBE cells UPR activation is characterized by increased GRP78 levels, and activation of both the IRE1 and ATF6 pathways, though no

PERK activation was detected[108]. However, a study involving a mouse model exploring RSV's role in lung fibrosis found PERK activation 7 days post infection along with elevated ATF6, GRP78[109]. XBP1 levels were not elevated and IRE1 activation was not explored. Intriguingly, while RSV activates the IRE1 branch of the UPR in both cell lines and isolated primary cells, IRE1 inhibits RSV replication[108]. In both Ire1$^{-/-}$ mouse embryonic fibroblasts and A549 cells treated with an IRE1 inhibitor viral transcript and protein levels were significantly higher than in RSV infected control cells[108]. Though the exact mechanism for this is unclear, it may be related to IRE1 RIDD endonuclease activity.

Coronaviruses (CoV) are another virus family capable of causing respiratory exacerbation as well as significant illness on their own[110]. SARS-CoV and MERS-CoV in particular have high pandemic potential and are associated with significant mortality[110]. It is well established CoV can induce the UPR in culture, this UPR activation has been linked to the spike (S) protein and for certain CoV the exact amino acid domain responsible is known[111, 112]. The S protein of SARS-CoV and CoV-HKU1, both beta-coronaviruses, induce the transcription of GRP78, GRP94, and CHOP through activation of the PERK pathway *in vitro*[111]. Cells infected with MHV-A59, a model murine CoV in the same family as SARS and MERS, show activation of all three UPR branches, though ATF6 activation is limited later in infection[110]. Relative activation of UPR branches varies between individual CoV as cells infected with SARS-CoV show limited XBP1 slicing and ATF6 cleavage[113, 114]. Interestingly, an in vitro study using a selective PERK inhibitor found alleviating translational repression increased the levels

of viral proteins but decreased viral titers[110]. This was hypothesized to be due to increased translation of host anti-viral proteins.

There is currently little information on the relationship between RV, RSV, and CoV and host PDIs. Thus, any potential interactions remain to be characterized, and any prospective anti-viral pharmacological influence need to be explored.

While we have limited ourselves to a discussion of respiratory viruses known to interact with the UPR, there are numerous other non-respiratory viruses that do so[53]. Herpes Simplex Virus[115], Dengue Virus[116], Hepatitis B[117], as well as HIV[118] all interact with the UPR or PDIs despite all infecting drastically different cell types. These host-viral interactions may provide promising targets for the basis of the development of future antivirals

UPR and PDIs in immune signaling

Both the UPR and PDIs play important roles in immune signaling and activation. Increased expression of proinflammatory cytokines during ER stress has been firmly established[119]. And because it allows for ER expansion, increased protein production, and subsequent protein secretion, activation of the UPR is needed for the development and function of both secretory and immune cells[15, 120, 121]. Additionally, GRP94 has been shown to be involved in the maturation of Toll-like receptors[122].

PDIA3 is a core component of the peptide loading complex, required for antigen presentation through MHC class I[123]. In addition, PDIs play important roles in cytokine

folding and maturation. siRNA knockdown of PDIA3 in the lung epithelium has been shown to alter the oxidative folding of eotaxin and periostin in mice[45]. PDIA1 is found at high levels in the secretory granules of eosinophils, suggesting a direct role in cytokine secretion[124].

UPR and PDIs in feedback regulation

There are an increasing number of papers exploring the redox regulation of the UPR sensors through thiol-disulfide exchange[125]. In other words, modulation of the UPR itself through its downstream PDI effectors. A recent study determined PDIA1 and A3 are important in the regulation of PERK, utilizing lentiviral PDIA3 depletion in combination with small molecule based PDI inhibition the authors concluded oxidized PDIA1 was an important activator of PERK, while PDIA3 was critical to regulating the oxidation state of PDIA1[125].

One group found PDIA6 is important in modulating IRE1 signaling through interaction with Cys148 of the activated protein, facilitating its decay, thus acting as an attenuator of IRE1 signaling[126]. Another study by the same group exploring the UPR and glucose stimulated insulin secretion found PDIA6 regulates the RIDD activity of IRE1[127]. Utilizing shRNA against PDIA6, Eletto *et al.* found that during UPR activation through chemical stressors, PDIA6 modulates the kinase activity of PERK as well as the XBP1 splicing ability of IRE1. However, upon activation due to glucose concentration only RIDD activity of IRE1 is triggered, PDIA6 regulates this activity, and that this regulation was dependent of the enzymatic activity of PDIA6, rather than expression of PDIA6[127].

Another different study found PDIA5 is important for ATF6 activation and export during ER stress[128]. Higa *et al.* determined this activation was redox dependent and PDIA5 was involved in disulfide bond rearrangement of ATF6 which facilitated its transport to the Golgi[128]. A very recent study utilizing trap mutants of various PDIs found PDIA16 modulates trafficking of ATF6 to the Golgi and assists in proteolytic cleavage[129]. Another study using shRNA depletion of PDIs found PDIA4 regulates ATF6 activity, as increased GRP78 expression was detected specifically following PDIA4 deletion as opposed to other PDIs[130]. Though, the exact nature of this control was not explored.

This avenue of research is promising as it demonstrates the possibility of controlling aberrant or exuberant UPR activation, or modulating specific arms of the UPR, through inhibition of individual PDIs.

Small molecule inhibition of PDIs

Given the scope of PDIs it is not surprising there is plentiful investigation into modulating their activity. Numerous chemicals have been shown to inhibit PDI activity, from antibodies[131] and hormones[132], to antibiotics[133]. In the past few years there has been an influx of inhibitors identified from small molecule screen libraries with increasing specificity towards PDIs[134-139]. Most of these molecules are identified as potential chemotherapeutic agents and have been characterized against PDIA1, the prototypical member of the PDI family[140]. However, given the high degree of homology between PDI family members some inhibitory activity against other PDIs is often

predicted. A full description of currently available small molecule PDI inhibitors can be found in Table 2, the following section will highlight a few key inhibitors.
16F16 was the first of this new wave of inhibitors, it acts by covalently binding to reactive Cys residues in the active site of PDIs[52]. Hoffstrom et al. utilized it to explore mechanisms linking PDIs to apoptotic cell death[52]. E64FC26 is a novel PDI inhibitor based off of an indene moiety that acts as a pan inhibitor of numerous PDIs though the inhibitory mechanism in unclear[141]. The authors found E64FC26 improved survival in a mouse model of multiple myeloma, with little adverse effects. KSC-34 is a particularly interesting inhibitor, it displays enhanced specificity towards PDIA1, which is not unusual, but KSC-34 is selective towards the N-terminal CGHC active site of the protein[140]. This means KSC-34 can be used to explore specific functionalities and protein interactions of each individual active site. Additionally, the specificity of this inhibitor is encouraging in that is suggests the possibility of identifying novel inhibitors with the same level of specificity targeted towards other PDIs.

LOC14 is unique, it is a reversible PDI inhibitor that binds adjacent to the CGHC active site and locks the enzyme in an oxidized conformation, though the exact residues remain to be elucidated[51]. LOC14 has been found to be neuroprotective in both cell culture and animal models and displays high stability[51]. We have recently demonstrated LOC14 is also capable of inhibiting PDIA3 as well as PDIA1, and that treatment with LOC14 significantly decreased Influenza replication and the maturation of viral proteins[39]. Another group has demonstrated the redox modulating effects of flavonoid compounds on PDIA3[142]. Rather than directly interacting with the active site these

compounds bind to residues on the protein binding b and b' domain absent on other PDIs [142, 143]. These flavonoids are interesting as they may provide a structural basis for the design of specific PDIA3 inhibitors.

The above compounds represent an expansive range of mechanisms and targets within the PDI family, from E64FC26 acting as a pan PDI inhibitor[141], to KSC-34 acting toward a specific active site on PDIA1[140]. PDIs are being found to play increasingly important roles in a wide variety of conditions and cellular activities, though current techniques to elucidate their exact role remain limited. Conventional RNA interference techniques take time to ensure sufficient knockdown of a target gene and targeting multiple genes simultaneously can pose additional problems. Employing targeted inhibitors allows for rapid decreases in protein activity and could theoretically be titrated to achieve a desired activity level. We have already demonstrated the efficacy of these inhibitors by utilizing them to determine the role of PDIs in oxidative folding of influenza proteins[39], while Cole *et al.* explored the specific role of the PDIA1 A site on protein folding and secretion[140]. Current compounds are able to target some of the PDI family, though inhibitory action against all PDIs has yet to be explored, and highly specific inhibitors towards PDIs other than PDIA1 remain to be developed. Nonetheless, the development of increasingly specific inhibitors specifically targeted towards individual PDIs would provide an invaluable tool to determine the distinct role of unique PDIs during disease pathogenesis.

Conclusion

The UPR and PDIs play critical roles in numerous cellular processes. Much remains unknown regarding their role in normal responses and their impact on the development of disease. Investigation into the branches of the UPR and their downstream effectors remains challenging. However, small molecule PDI inhibitors provide an exciting opportunity to tease apart the molecular mechanisms of ER stress and provide potential platforms for the development of future therapeutics.

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Conflicts of Interest: Vikas Anathy hold patents: U.S. Patent No. 8,679,811, "Treatments Involving Glutaredoxins and Similar Agents" and U.S. Patent, 9,907,828, "Treatments of oxidative stress conditions". Vikas Anathy have received consulting fees and research funds (contracts) from Celdara Medical LLC, NH for his contributions with the commercialization of glutaredoxin for the treatment of pulmonary fibrosis.

Figure Legends

Figure 1: Representation of canonical UPR signaling pathways. The UPR is activated by a buildup of unfolded protein within the ER lumen. GRP78 dissociates from the three ER stress sensors IRE1, ATF6, and PERK. Dimerization of IRE1 leads to autophosphorylation activating ribonuclease activity specific to XBP1 mRNA. This splicing generates XBP1s which is transported to the nuclease and induces the expression of UPR target genes. IRE1 phosphorylation also activates TRAF2 which directs the cell towards apoptosis through JNK signaling. IRE1 is also capable of associating into higher order structures which allow for non-specific degradation of ER associated mRNAs (RIDD). PERK dimerization leads to autophosphorylation activating kinase activity specific to eIF2a, halting protein translation. This loss of translation drives expression of ATF4 which acts as a transcription factor and induces the expression of UPR target genes. eIF2a is regenerated by GADD34. Upon dissociation of GRP78 from ATF6, ATF6 is transported to the Golgi where it is cleaved by cellular proteases to produce a transcription factor which induces the expression of UPR target genes.

Figure 2: Functions of PDIs in oxidative folding. A. Oxidized PDIs catalyze disulfide bond formation of nascent peptides in the ER. Leading to proper oxidative folding or non-native disulfide bond formation. Reduced PDIs facilitate isomerization of disulfide bonds. B. PDIs are oxidized via interactions with ERO1. ERO1 uses FAD to transfer electrons to molecular oxygen generating hydrogen peroxide. PRX4 can also directly oxidize PDIs. PDIs transfer disulfides to client proteins. Glutathione contributes to disulfide bond reduction.

FIGURES

Figure 1: Representaion of canonical UPR signaling pathways

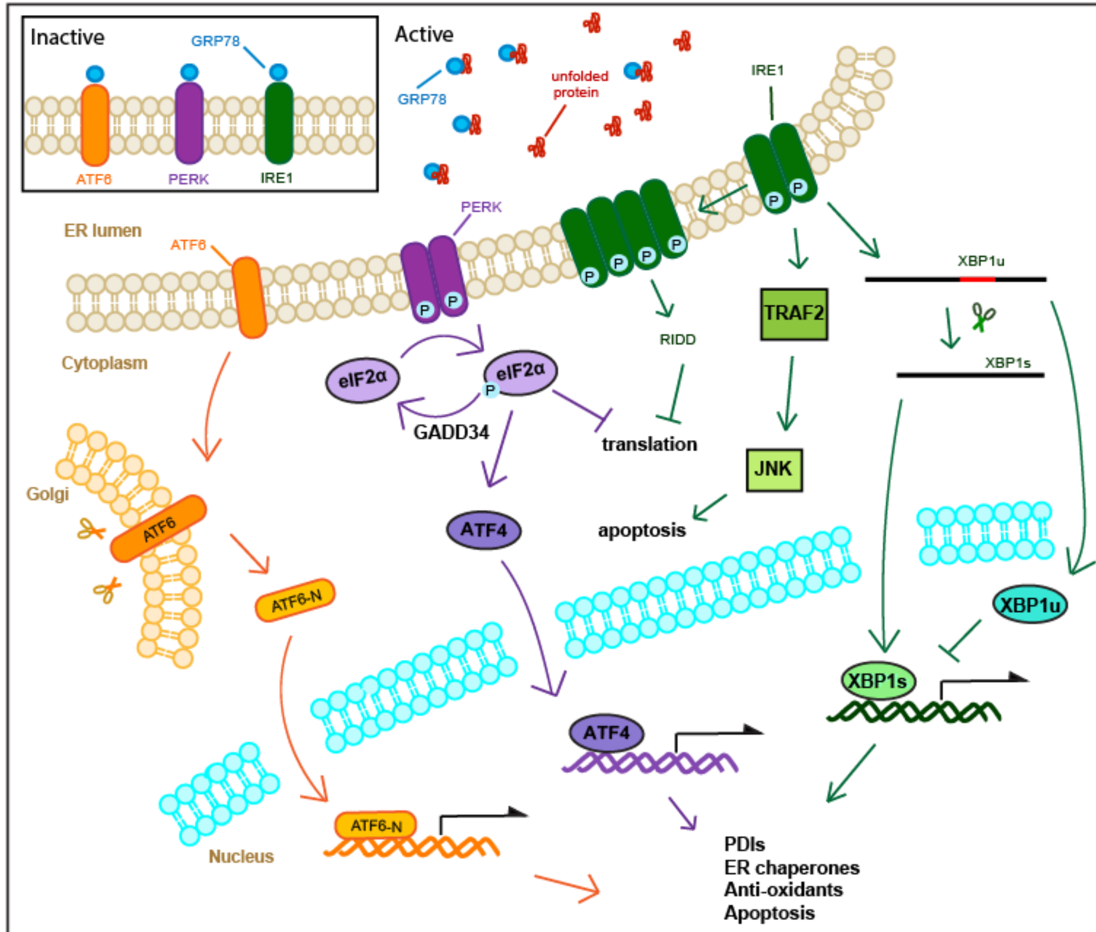

Figure 2: Functions of PDIs in oxidative folding

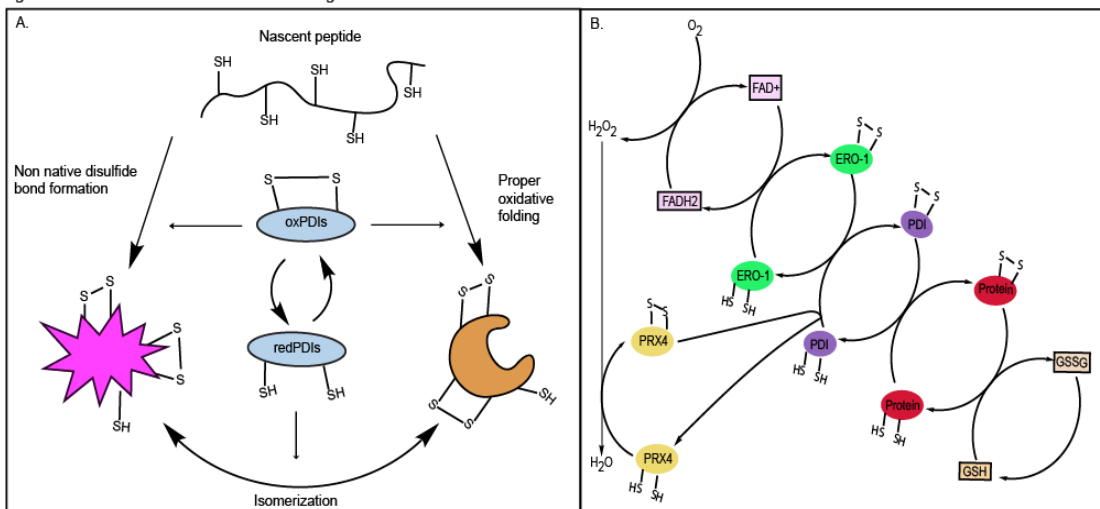

Table 1: Characteristics of human PDI gene family

Protein Name	Gene Name	Accession Number	Gene Location	TRX domain organization	Catalytic sequence	ER locatlization	ER retention sequence	Molecular Weight (Da)
PDIA1	*P4HB*	P07237	17q25.3	a-b-b'-a'	CGHC, CGHC	ER lumen	Yes	57116
PDIA2	*PDIA2*	Q13087	16p13.3	a-b-b'-a'	CGHC, CTHC	ER lumen	Yes	58206
PDIA3	*PDIA3*	P30101	15q15.3	a-b-b'-a'	CGHC, CGHC	ER lumen	Yes	56782
PDIA4	*PDIA4*	P13667	7q36.1	a'-a-b-b'-a'	CGHC, CGHC, CGHC	ER lumen	Yes	72932
PDIA5	*PDIA5*	Q14554	3q21.1	b-a'-a-a'	CSMC, CGHC, CPHC	ER lumen	Yes	59594
PDIA6	*PDIA6*	Q15084	2p25.1	a'-a-b	CGHC, CGHC	ER lumen	Yes	48121
PDIA7	*PDILT*	Q8N807	16p12.1	a-b-b'-a'	SKQS, SKKC	ER lumen	Yes	66657
PDIA8	*ERP27*	Q96DN0	12p12.3	b-b'	no catalytic site	ER lumen	Yes	30480
PDIA9	*EPR29*	P30040	12q24.13	b	no catalytic site	ER lumen	Yes	28993
PDIA10	*ERP44*	Q9BS26	9q31.1	a-b-b'	CRFS	ER lumen	Yes	46971
PDIA11	*TMX1*	Q9H3N1	14q22.1	a	CPAC	Membrane bound	No	31791
PDIA12	*TMX2*	Q9Y320	11q12.1	a	SNDC	Membrane bound	Yes	34038
PDIA13	*TMX3*	Q96JJ7	18q22.1	a-b-b'	CGHC	Membrane bound	Yes	51872
PDIA14	*TMX4*	Q9H1E5	20p12.3	a	CPSC	Membrane bound	Yes	38952
PDIA15	*TXNDC5*	Q8NBS9	6p24.3	a'-a-a'	CGHC, CGHC, CGHC	ER lumen	Yes	47629
PDIA16	*TXNDC12*	O95881	1p32.3	a	CGAC	ER lumen	Yes	19206
PDIA17	*AGR2*	O95994	7p21.1	a	CPHS	ER lumen	Yes	19979
PDIA18	*AGR3*	Q8TD06	7p21.1	a	CQYS	ER lumen	Yes	19171
PDIA19	*DNAJC10*	Q8IXB1	2q32.1	a'-b-a'-a-a'	CSHC, CPPC, CHPC, CGPC	ER lumen	Yes	91080
PDIB1	*CASQ1*	P31415	1q23.2	b-b-b'	no catalytic site	ER lumen	No	45160
PDIB2	*CASQ2*	O14958	1p13.1	b-b-b'	no catalytic site	ER lumen	No	46436

Table 2: Characteristics of various PDI inhibitors

Compound name	Mode of action	Clinical Trial *	Reference
Bacitracin	Competitive inhibitor, binds to free thiols in substrate binding region. Cell impermeable	Yes (57)	85
16F16	Irreversibly binds to cysteine residues in active site. Cell permeable	No	52
LOC14	Allosteric inhibitor. Binds adjacent to active site, forces protein to maintein oxidized conformation. Reversible. Cell permeable	No	39,51
PACMA31	Irreversibly binds to cysteine residues in active site. Cell permeable	No	86
CCF642	Allosteric inhibitor. irreversibly binds to conserved lysine directly adjacent to the active site. Cell permeable	No	7
P1	Irreversibly binds to cysteine residues in active site. Cell permeable	No	87
E64FC26	Pan-PDI inhibitor, mechanism unknown. cell permeable	No	93
KSC-34	PDIA1 inhibitor, selective for C53 in a doain active site. Cell permeable	No	92
ML359	PDIA1 inhibitor, mechanism unknown. Reversible. Cell permeable	No	88
RB-11-ca	PDIA1 inhibitor, selective for C53 in a doain active site. Cell permeable	No	89
Juniferdin	PDIA1 inhibitor, mechanism unknown. Cell permeable	No	88
Eupatorin	Flavanoid compound, binds to tryptophan residues near the active site of PDIA3	No	94,95
Eupatorin-5-methyl ether	Flavanoid compound, binds to tryptophan residues near the active site of PDIA3.	No	94,95
Quercetin-3-rutinoside	PDIA1 inhibitor, binds to b' domain. Reversible. Cell impermeable	No	50
T8	Allosteric inhibitor, binds near active site. Reversible. Cell permeable	No	90
RL90	Anti-PDIA1 antibody	No	83
17β-estradiol	Binds to bb' domain	Yes (2054)	84
35G8	Believed to bind with cysteine residues in active site	No	91

* Clinicaltrial.gov

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CHAPTER 2

LUNG EPITHELIAL PROTEIN DISULFIDE ISOMERASE A3 (PDIA3) PLAYS AN IMPORTANT ROLE IN INFLUENZA INFECTION, INFLAMMATION, AND AIRWAY MECHANICS

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ABSTRACT

Protein disulfide isomerases (PDI) are a family of redox chaperones that catalyze formation or isomerization of disulfide bonds in proteins. Previous studies have shown that one member, PDIA3, interacts with influenza A virus (IAV) hemagglutinin (HA), and this interaction is required for efficient oxidative folding of HA *in vitro*. However, it is unknown whether these host-viral protein interactions occur during active infection and whether such interactions represent a putative target for the treatment of influenza infection. Here we show that PDIA3 is specifically upregulated in IAV-infected mouse or human lung epithelial cells and PDIA3 directly interacts with IAV-HA. Treatment with a PDI inhibitor, LOC14 inhibited PDIA3 activity in lung epithelial cells, decreased intramolecular disulfide bonds and subsequent oligomerization (maturation) of HA in both H1N1 (A/PR8/34) and H3N2 (X31, A/Aichi/68) infected lung epithelial cells. This reduced disulfide bond formation significantly decreased viral burden, and also pro-inflammatory responses from lung epithelial cells. Lung epithelial-specific deletion of PDIA3 in mice resulted in a significant decrease in viral burden and lung inflammatory-immune markers upon IAV infection, as well as significantly improved airway mechanics. Taken together, these results indicate that PDIA3 is required for effective influenza pathogenesis *in vivo*, and pharmacological inhibition of PDIs represents a promising new anti-influenza therapeutic strategy during pandemic and severe influenza seasons.

AUTHOR SUMMARY

Current Influenza virus therapies target viral proteins and are limited in their effectiveness owing to rapid viral mutation. Understanding host cellular and molecular processes utilized by the virus during its replication may aid in the development of new therapeutic interventions to improve patient survival. Influenza proteins such as hemagglutinin (HA) rely on disulfide bonds for stability and activity and as such rely on host redox enzymes of the endoplasmic reticulum (ER) and the secretory pathway to express and assemble fully mature viral proteins. Past work has shown that one of these enzymes, protein disulfide isomerase (PDI) A3 is important for efficient folding of HA and viral replication *in vitro*. However, little is known about how PDIA3 mediates this effect or its role during active viral infection *in vivo*. Here, we demonstrate that PDIA3 is required for productive viral infection *in vivo* using conditional PDIA3 knockout mice. Moreover, we also show that small molecule mediated inhibition of PDIs with the reversible inhibitor LOC14 results in decreased viral replication, inflammation, and oxidative folding of HA. Thus, PDIA3 and other host redox active enzymes may provide targets for future therapies, and LOC14 may provide the basis for novel therapeutic interventions.

INTRODUCTION

Viruses harness their host cell transcriptional machinery and endoplasmic reticulum (ER) to express and properly fold and mature virus proteins, respectively [1]. Viral proteins often rely on the redox-dependent formation of disulfide bonds (-S-S-) for stability and function [2]. In general disulfide bonds within membrane and secretory

proteins are constructed in the ER and require ER-localized oxidoreductases [3-5]. One of these oxidoreductases is protein disulfide isomerase (PDI) A3, which belongs to a family of PDIs that catalyze formation and isomerization of disulfide bonds within nascent peptides in the ER [6, 7]. PDIA3 is unique among other PDIs in that it has an enhanced specificity towards glycoproteins owing to its interactions with calreticulin and calnexin, two lectin binding chaperone proteins within the ER [8]. In addition to numerous roles with in the cell, recent studies have linked PDIA3 with various cancers and the onset of neurodegenerative disorders [9, 10], though its role during viral infection is less well studied.

Influenza specifically infects and replicates in lung epithelial cells due to the presence of compatible sialic acid residues that can bind to influenza hemagglutinin (HA) [11, 12]. Previous work has shown that influenza HA contains several disulfide bonds and traverses through the ER to attain its final conformation via ER based folding and glycosylation[13, 14]. PDIA3 has been shown to interact with IAV-HA and to facilitate disulfide bond formation in HA during its passage through the ER [15]. It is also evident that these disulfide bonds formed by PDIA3 are essential for HA activity *in vitro* [15-17]. Despite *in vitro* evidence it is not well established whether PDIA3 is required for viral burden *in vivo*, nor is it known about the effect of PDIA3 deletion has on the disulfide status and maturation of HA during active IAV infection in primary lung epithelial cells and in mouse lungs.

In this study, we observed that PDIA3 is specifically upregulated in IAV-infected lung epithelial cells and mouse lungs as compared to other PDI family members. Additionally, the newly discovered small molecule inhibitor of PDI, [18] LOC14 exhibited the capacity to inhibit recombinant (r)PDIA3 at an IC50 of approximately 5 μM. Treatment of IAV-infected primary mouse tracheal epithelial cells (MTECs) with LOC14 resulted in decreased viral burden and pro-inflammatory cytokine production. IAV-HA showed a deficiency in disulfide bonds in cells treated with LOC14. Furthermore, this deficiency of disulfide bonds also decreased mature IAV-HA, indicating that PDI inhibition affects HA maturation within the host cell. Following influenza infection, mice with lung epithelium-specific knockout of *Pdia3* showed decreased overall viral burden, reduced inflammation and improved lung mechanics.

RESULTS

PDIA3 expression is increased following IAV infection

Wild type (C57BL/6NJ-*WT*) mice were infected with 2000 egg infectious units (EIU) of influenza A virus (IAV) strain H1N1 (PR8) and lung lysates were analyzed for PDI expression. Six days post infection a significant increase in *Pdia3* transcripts were observed when compared to other *Pdia* gene transcripts (Fig 1A). Mice infected with replication-deficient, UV irradiated (mock) virus, did not show any increases in *Pdia3*, suggesting the *Pdia3* induction depends on active viral replication. We next examined PDIA3 protein expression in the lung sections of PR8 infected *WT* mice, and observed a marked increase in PDIA3 levels predominantly in the bronchial epithelial cells in mice infected with PR8 compared to mock treated mice (Fig 1B). Next, we sought to determine

if this increase following IAV infection was exclusive to PR8 H1N1 or also prevalent in other influenza strains. Shoemaker et al. [19] have performed microarray using lung homogenates of mice infected with various strains of influenza virus, to examine gene expression over different time points. A retrospective analysis of this microarray data set (GSE63786) revealed *Pdia3* is the only *Pdi* significantly upregulated compared to uninfected controls at 72 hrs, 120 hrs, and 168 hrs post infection with various strains of IAV (Fig 1C & S1). A similar microarray experiment was performed by Gerlach et al. [20] in human lung epithelial cells 36 hrs post infection and a retrospective analysis of this data set (GSE48466) also revealed significantly upregulated *PDIA3* in response to pandemic influenza infection (Fig 1D). Despite increased expression of *PDIA3* in response to seasonal influenza infection, this was not found to be statistically significant (Fig 1D).

PDIA3 is known to interact with the hemagglutinin (HA) protein of IAV to construct disulfide bonds (-S-S-) in HA of IAV [15]. We performed immunoprecipitations (IPs) in human bronchial epithelial (HBE) cells after IAV infection. Upon analysis of the immunoprecipitated samples by western blot, we found both increased PDIA3 expression, as well as high levels of HA co-immunoprecipitating with PDIA3 (Fig 1E). Western blots of whole cell lysate (WCL) showed clear increases in HA and PDIA3, and no alterations in GAPDH were observed (Fig 1E). Next, we knocked down PDIA3 using siRNA and then lung epithelial cells were infected with IAV or mock virus. Subsequent Western blot analysis showed that knockdown of PDIA3 decreased levels of HA in IAV infected cells as compared to scrambled siRNA transfected cells (Fig 1F). These results suggest following IAV infection PDIA3 is predominantly induced in human and mouse lung

epithelial cells, and siRNA mediated decrease in PDIA3 expression diminish IAV-HA production in lung epithelial cells.

PDI inhibitors decrease PDIA3 activity and influenza protein production

PDIs play important roles in the folding of cellular proteins, and the prototypical PDI, PDIA1, has been found to be implicated in numerous diseases [21]. As such there are several commercially available small molecule inhibitors that target PDIA1, while little is known regarding the effect of these inhibitors on other PDIs. It is presumed that these inhibitors will have inhibitory effect on other PDIs owing to the high conservation of a thioredoxin-like (CXXC) active site and the high degree of homology between protein binding domains of the various PDI family members[18, 22-24]. We therefore tested four PDI inhibitors: 16F16, LOC14, PACMA31, and CCF 642, all extensively characterized as inhibitors of PDIA1, for their efficacy to inhibit PDIA3. Reduction of Dieosine-diglutathione (DiE-GSSG) has been used as an assay to determine PDI activity [25, 26]. We first determined that DiE-GSSG could also be used as a substrate for recombinant PDIA3 (rPDIA3) (Fig 2A). Using this assay, we found that 16F16 had minimal effect on the activity of rPDIA3, while both PACMA31 and CCF 642 showed inhibitory effects only at high concentrations (≥100 μM) (data not shown). However, reversible inhibitor LOC14 (Fig 2B) exhibited inhibition of rPDIA3 with an inhibitory capacity at 50% (IC50) of 4.97 μM (Fig 2C and D).

It has been previously shown that PDIA3 is required for IAV propagation [17]. We therefore next examined, if pharmacological PDI inhibition would alter IAV

propagation. We treated primary mouse tracheal epithelial cells (MTECs) with LOC14 or vehicle control (VC) prior to IAV infection, during infection, and post infection (Fig 2E), to avoid excessive load of inhibitors and cytotoxicity at a given time. Examination of the cells 24 hours post infection via flow cytometry for the presence of influenza nucleoprotein (NP) [27] (Fig 2F), detected no NP in the mock virus infected VC or 300 μM LOC14 treated cells. IAV infected VC group showed an increase in NP and we observed a dose dependent decrease in the NP+ cells following LOC14 treatment (Fig 2F). Using these data, we were able to calculate an effective concentration 50% (EC50) of 9.952 μM for LOC14 based on the percentage of NP+ cells (Fig 2G).

To determine if LOC14 treatment altered PDI activity within the cell we monitored the reduction of DiE-GSSG by lysates from IAV-infected cells treated with either LOC14 or vehicle control. We found LOC14 treatment (Fig 2E), with repeated doses of 10 μM (total 30 μM), decreased PDI activity in infected MTECs compared to controls (Fig 2H). DiE-GSSG is reported to be a substrate for PDIs [26]. Taken together these results indicate that LOC14 inhibits the *oxidoreductase* activity of PDIA3 and LOC14 can be used to block viral replication.

LOC14 treatment in primary mouse tracheal epithelial cells (MTECs) alters disulfide (-S-S-) bonds of HA

Influenza-HA is synthesized as a 70 kDa (HA0) precursor that is cleaved in the Golgi to yield two fragments of approximately 50 kDa (HA1) and 20 kDa (HA2) following oligomerization [28]. To determine if LOC14 alters -S-S- of HA, we treated primary

MTECs with LOC14 (Fig 3A). Briefly, MTECs were treated with 10 μM LOC14 prior to IAV infection, during infection, and post IAV infection; resulting in a total of 30 μM LOC14. Western blot (WB) analysis showed that LOC14 treatment no alterations in HA0 levels compared to vehicle treated cells in IAV-infected MTECs (Fig 3B, WCL). However, levels of cleaved HA1 showed a significant decrease following LOC14 treatment (Fig 3B, WCL-WB, Fig S2A). PDIA3 is unique compared to other PDIs in that it predominantly facilitates -S-S- bonds in glycoproteins that are being processed and traversing through the ER [29]. Therefore, we next determined whether inhibition of PDIA3 would alter -S-S- bonds of HA. We used a biotin switch assay to assess disulfides by blocking free cysteine sulfhydryl (-SH) groups using N-ethyl maleimide (NEM) and reducing -S-S- bonds with DTT, and then newly revealed -SH groups are labeled with biotin-conjugated alkylating agent (MPB) (Fig 3C). This technique reveals high labeling of proteins with -S-S- bonds and low labeling of proteins with -SH groups. Subsequent neutravidin pull-down and Western blot analysis showed drastic decrease in -S-S- bond formation in IAV-HA following LOC14 treatment compared to vehicle control treated cells infected with IAV (Fig 3D). We then examined oligomerization of HA using non-reducing SDS-PAGE. Mature HA trimers are known to be resistant to dissociation by SDS [30, 31]. LOC14 treatment increased monomeric HA and correspondingly decreased high molecular weight HA (150-200 kDa) as compared to vehicle treated controls (Fig 3E, right lanes, -DTT). Samples reduced using DTT showed no high molecular weight HA (Fig 3E, left lanes, +DTT). Indicating LOC14 induces defects in HA oligomerization by altering oxidative folding of HA. Interestingly, we observed a decrease in monomeric forms of HA0 in LOC14 treated samples compared to vehicle control samples infected with IAV

(Fig 3E, left lanes, +DTT). Potentially suggesting that a decrease in overall HA production following LOC14 treatment

Our analysis of IAV burden in these cells showed a decrease in IAV-*PA* transcripts following LOC14 treatment, but this was not statistically significant (Fig 3F). However, we observed a significant decrease in the number of MTECs positive for IAV NP in LOC14 treated IAV infected cells as compared to vehicle control treated cells (Figs 3G & S2B). Next, reinfection of MTECs with supernatants isolated from earlier infected MTECs treated with either vehicle or LOC14 showed a marked decrease in the number of cells positive for IAV NP in LOC14 treated supernatants as compared to vehicle treated supernatants (Figs 3H & S2C), suggesting a decreased production of infectious virus following LOC14 treatment. Quantification of mRNAs for *Ccl20*, and *Irf7*, an important transcriptional regulator of the type I interferon response by RT-qPCR showed a significant decrease in transcript levels following LOC14 treatment, suggesting decreased viral burden (Fig 3I & J).

LOC14 is known to be a potent activator of the unfolded protein response (UPR)[23]. In order to assess cytotoxic effects of LOC14 treatment we examined UPR activation, by monitoring ATF6 cleavage and GRP78 protein expression, two transducers of the UPR, and caspase assays (Fig S2D & E). No significant changes in either readout was observed, indicating LOC14 treatment had no effect on cellular stress levels of either mock or IAV infected cells 24 hrs post infection.

Taken together these results suggest that LOC14 alters oxidative folding of HA, and that deficit in the formation of disulfide bonds has significant implications for oligomerization of HA and subsequent influenza burden in MTECs.

LOC14 treatment alters oxidative folding of different HA in H3N2 serotype

Owing to remarkable conservation of cysteine residues between various strains of influenza (Fig S3) we sought to determine if alterations in oxidative folding also affected other hemagglutinin serotypes e.g. H3. To determine the effects of LOC14 treatment on H3 HA, we infected MTECs with X31 which contains the HA and NA genes of A/Aichi/1/1968 (H3N2)[32]. Primary MTECs were treated with LOC14 pre- and post-infection with X31 (Fig 3A). LOC14 treatment did not alter HA0 levels compared to vehicle treated cells (Fig 4A, WCL-WB). However, levels of cleaved HA1 showed a significant decrease following LOC14 treatment (Fig 4A, WCL-WB, top panel, Fig S4A). The biotin switch assay followed by neutravidin pull-down showed considerably less disulfide bonds in IAV-HA (H3) following LOC14 treatment (Fig 4A, neutravidin-WB, bottom panel). We then examined oligomerization of HA using non-reducing SDS-PAGE. LOC14 treatment decreased high molecular weight HA (150-200 kDa) as compared to vehicle treated controls (Fig 4B, right lanes, -DTT), while samples reduced using DTT showed no high molecular weight HA (Fig 4B, left lanes, +DTT), though no corresponding increase in monomeric HA was observed. These results suggest that a decrease in oxidative folding in the form of -S-S- bonds of HA leads to impaired oligomerization in H3-HA of X31. Again, we observed a decrease in monomeric forms of HA0 in LOC14 treated samples compared to vehicle control samples infected with IAV (Fig 4B, left lanes, +DTT).

Potentially suggesting that a decrease in overall HA production following LOC14 treatment.

Subsequent analysis of IAV burden in these cells showed a significant decrease in IAV-*PA* transcripts following LOC14 treatment (Fig 4C), and a significant decrease in the number of MTECs positive for IAV-NP in LOC14 treated IAV infected cells as compared to IAV infected vehicle control treated cells (Figs 4D, S4B). Next, reinfection of MTECs with supernatants isolated from earlier infected MTECs treated with either vehicle or LOC14 showed a marked decrease in the number of cells positive for IAV NP, suggesting decreased production of infectious virus following LOC14 treatment (Figs 4E, S4C). Quantification of *Ccl20*, and *Irf7*, by RT-qPCR showed a significant decrease in transcript levels following LOC14 treatment (Fig 4F and G). Taken together these results suggest that LOC14 decreases -S-S- bonds of H1 as well as H3 HA, and that these variations in disulfide bonds have significant implications for oligomerization of HA and subsequent inflammatory response independent of influenza strain.

Ablation of *Pdia3* in the lung epithelium attenuates viral burden in mice

To determine whether PDIA3 in airway epithelial cells contributes to the establishment of viral burden, lung inflammation and airway mechanics, we generated a doxycycline inducible transgenic *CCSP-rTetA/TetOP-Cre/Pdia3*$^{loxp/loxp}$ (*ΔEpi-Pdia3*) mouse to delete *Pdia3* specifically in lung epithelial cells. Mice carrying *TetOP-Cre/Pdia3*$^{loxp/loxp}$ or *CCSP-rTetA/TetOP-Cre* were used as littermate controls (*Ctr*). Characterizations of these mice are described elsewhere [6]. All mice were kept on

doxycycline for the length of the experiment beginning seven days prior to IAV infection to ensure ablation of *Pdia3* throughout the infection protocol (Fig 5A). Our previous work with these transgenic mice showed clear decrease of PDIA3 following doxycycline exposure in the lung epithelial cells [6]. RT-qPCR analysis of viral transcripts (IAV-*PA*, -*HA*, and –*NA*) in lung extracts showed significantly decreased viral load as evidenced from the influenza markers that are examined in *ΔEpi-Pdia3 mice compared to Ctr mice (Fig 5B-D).* Strikingly, Western blots showed that HA protein levels were significantly decreased following lung epithelial *Pdia3* ablation (Fig 5E, Fig S5). Accordingly, these decreases in HA also resulted in decreased HA oligomers (150-200 kDa) in- *ΔEpi-Pdia3* mice compared to *Ctr* mice (Fig 5F). Taken together these results suggest lung epithelial PDIA3 plays an important role in efficient HA production and IAV burden *in vivo.*

We next determined if this decrease in viral burden due to *Pdia3* deletion led to a concomitant decrease in influenza-induced inflammation. Analysis of chemokines in the lung tissues showed that production of neutrophil chemoattractant CXCL1 and dendritic/macrophage chemoattractant CCL20 were decreased in IAV-infected ΔEpi-*Pdia3* mice when compared with IAV-infected Ctr mice. (Fig 5G and H). *Analysis of* total cells in bronchoalveolar lavage fluid (BALF) also showed a significant decrease in inflammatory cell numbers in ΔEpi-*Pdia3* mice infected with IAV as compared to Ctr mice infected with IAV (Fig 5I). Quantitation of specific inflammatory cell types revealed significant attenuation of total macrophages, neutrophils, and lymphocytes in IAV infected ΔEpi-*Pdia3* mice when compared with IAV infected Ctr mice (Fig 5J-L).

Ablation of Pdia3 in the lung epithelium attenuates influenza-induced airway hyperresponsiveness (AHR)

Viral infection is known to increase methacholine sensitivity and AHR in mice and humans [33-35]. Therefore, we examined the consequence of epithelial specific deletion of Pdia3 on AHR to increasing doses of inhaled methacholine. Following the saline (vehicle control for methacholine) challenge, peripheral airway resistance (G), but not central airway resistance (Rn) or parenchymal tissue elasticity (H), was elevated in control (Ctr) mice infected with IAV compared to mock infected Ctr mice, suggesting that IAV caused dysfunction of the small airways and/or lung parenchyma [36] (Fig 6 A-C). However, there was no increase in G in ΔEpi-Pdia3 mice infected with IAV. Furthermore, methacholine challenges showed that IAV increased AHR as measured by changes in both Rn and G; however, the severity of the AHR measured by both G and Rn was significantly decreased in IAV-infected ΔEpi-Pdia3 mice (Figure 6D & E). This suggests that due to IAV infection, the presence of PDIA3 increases responsiveness to methacholine and results in constriction of the central and peripheral airways. Taken together these results suggest that lung epithelial PDIA3 plays an important role in the establishment of IAV burden, inflammation, and AHR

DISCUSSION

The pathogenesis of IAV involves a complex interplay between numerous host and viral factors [37]. Additional understanding of the influenza virus as well as the host cellular pathways utilized by IAV would provide valuable insight toward the development

of novel therapeutic and preventative modalities less dependent on viral antigenic shift and drift.

Recent studies have suggested that several PDIs play important roles in influenza pathogenesis [17, 38]. However only the calnexin/calreticulin associated, glycoprotein specific PDIA3 is known to be directly involved in the processing of IAV hemagglutinin (HA), one of the major antigenic determinants of the virus [15, 17, 39]. Disrupting this PDIA3-HA interaction provides an intriguing therapeutic target. While there is a large amount of diversity in IAV hemagglutinin [40], the cysteine residues involved in disulfide bonding are highly conserved (Fig S3), highlighting their importance in the structure and function of HA.

Herein, we show that PDIA3 levels are increased following IAV infection, and PDIA3 transcript levels are upregulated compared with other PDIs. Treatment with a reversible PDI inhibitor LOC14 results in altered -S-S- bonds in both H1 and H3 HA along with associated alterations of HA maturation and subsequent decreases in markers of viral infection, demonstrating potent anti-influenza activity independent of viral strain. While LOC14 is known to be a potent UPR activator[23], 24 hours post LOC14 treatment dosed cells do not show signs of exacerbated cellular stress or enhanced apoptosis. Furthermore, genetic ablation of *Pdia3* in the airway epithelium, the primary site of influenza infection, results in a similar phenotype of diminished overall viral load. Finally, we also show restoration of normal lung mechanics following *Pdia3* ablation in IAV infected mice. Taken together our results suggest the importance of lung epithelial PDIA3 in formation

of -S-S- bonds of IAV HA and subsequent establishment of IAV infection, airway inflammation and AHR.

PDIs play critical roles in oxidative folding of proteins, catalyzing and isomerizing disulfide bonds [7]. PDIA3 is required for the efficient folding of HA by catalyzing and isomerizing disulfide bonds and ensuring proper cysteine pairings [15]. Past work has demonstrated the necessity of these disulfides in the proper folding of HA, as well as in the formation of the HA trimer and its transport through the secretory system of the cell to the plasma membrane [14, 41], potentially linking oxidative folding to viral assembly. LOC14 treatment led to significant alterations of the disulfide bonds of both H1 and H3 HA. Regardless of IAV strain, HA cleavage occurs after it has left the ER, either in the Golgi or at the cell surface [42, 43]. Following LOC14 treatment we observe a marked decrease in HA1, which suggests that altered oxidative folding prevents the normal proteolytic processing of HA. Studies involving HA mutants lacking the ability to form proper disulfide bonds demonstrate retention of the mutant misfolded HA in the ER and enhanced proteasome-mediated degradation [14, 44-47].

HA is known to oligomerize to its SDS resistant trimeric conformation within the ER regardless of ATP concentration, blocked transport from the ER, temperature alterations, and the trimming of carbohydrate side chains[31]. This suggests that trimerization is largely dependent on the acquisition of the proper tertiary structure of the HA0 monomers. Impairment of proper disulfide bond formation has been shown to hinder detection of correctly folded trimeric HA; moreover, *Pdia3* deletion has shown to cause a

decreased number of HA molecules acquiring its trimeric structure [14, 15]. We found that PDI inhibition with LOC14 resulted in a similar phenotype, causing a marked decrease in the appearance of high molecular weight SDS resistant HA species combined with concomitant increases in monomeric H1-HA, though no increase in monomeric H3-HA was observed. Collectively, these results suggest that alteration in HA maturation (oligomerization) is driven by intramolecular -S-S- bonds rather than other protein modifications.

Interestingly we observe variable decreases in HA0 levels following LOC14 treatment for both H1-HA and H3-HA on Western blots. These variations may be due to differences in the amount of protein used as well as different gel-running time for the various Western blots.

While LOC14 treatment has an effect on the folding of HA, at this juncture, it is not possible to determine which PDIs are being affected. LOC14 has been extensively characterized against PDIA1, the prototypical member of the PDI family. It has been proposed in the literature that LOC14 would be effective against other PDI family members owing to high sequence homology and conservation of the active site, though this has yet to be verified [18, 22]. siRNA experiments have shown PDIA1, 3, and 4 all play some role in influenza infection, leading to significant decreases in viral replication, though no viral proteins that reach the plasma membrane through the secretory pathway were examined [38]. Thus the effects we see could be the result of LOC14 inhibiting multiple PDIs, though PDIA3 is the only PDI directly involved in HA maturation [15]. However, a lack of

specificity would theoretically result in substantial loss of PDI activity in cell lysates treated with LOC14; while we observe significant decreases in PDI activity there is nonetheless high background signal in lysates of cells treated with LOC14. This could suggest that DiE-GSSG might be able to act as a substrate for other redox active cellular proteins such as thioredoxins or glutaredoxins. Another possibility is an alteration of the equilibrium between the bound and unbound inhibitor, the inhibitory capacity of LOC14 may be altered under diluted assay conditions. Further refinement and experimentation are needed to accurately assess the biological capacity of LOC14 on PDI activity in influenza-infected cells.

LOC14's lack of specificity toward a single PDI may also explain differences in chemokine levels following treatment. Various PDIs might be involved more directly in not only the production, but also the secretion of these chemokines or other cytokines, for example, PDIA1 is found at high levels in the secretory granules of eosinophils [48], and we have previously shown siRNA knockdown or ablation of *Pdia3* in lung epithelial cells decreases various cytokine levels, and alters oxidative folding of Eotaxin, EGF and Periostin in mice [6].

The lack of specificity of LOC14 also leaves open the possibility of inhibiting not only PDIs but also other oxidoreductases with thioredoxin fold domains[18, 49]. Thioredoxin reductases utilize selenocysteine to mediate their effects[50], as such the potential interactions with LOC14 are harder to predict. Direct interaction between these proteins and viral HA is unlikely, as they are not found in high abundance within the ER[49,

50]. Previous work has demonstrated treatment with glutathione derivatives significantly decreases influenza replication, which was attributed to an alteration in the redox state of PDIs[51], suggesting the importance of the redox buffering capacity of the cell for influenza replication. We predict that if LOC14 is able to interact with these redox-buffering enzymes it would allow for the possibility of additional indirect inhibition of PDIs, though extensive experimentation would be required to accurately determine additional LOC14 targets.

To investigate the role of PDIA3 during IAV infection *in vivo* we deleted *Pdia3* specifically in the lung epithelium. Our results show deletion of *Pdia3* in the lung epithelium significantly decreased overall viral load. Moreover, we show *Pdia3* deletion significantly attenuates IAV mediated airway inflammation and AHR. *Pdia3* deletion strikingly ablates increases in peripheral airway resistance found in control mice following IAV infection. Viral infection is known to induce or exacerbate AHR in humans and mice [33, 34], and present data provides strong evidence that deletion of PDIA3 in the lung epithelium leads to relevant improvements of lung mechanics. The protection against changes in Rn and G during methacholine challenge suggest the changes are due to reduced airway narrowing of both large and small airways[52, 53]. While the lack of effect on H suggests that is does not change the severity of airway closure during methacholine challenge [54]. Though it remains possible the reduction in AHR is simply due to decrease in viral burden following ablation of *Pdia3*

Interestingly HA levels were severely decreased after ablation of PDIA3, pointing

to a potential role for PDIA3 in influenza entry into the cell. In certain cells PDIA3 found on the plasma membrane is known to play an important role in gamete fusion, act as a receptor for certain vitamins, and facilitate HIV entry [55-57]. However experiments using cell impermeable PDI inhibitors showed little impact on influenza replication, suggesting PDIA3's role in influenza propagation may be limited to protein maturation in the ER [38].

In conclusion, our findings support that PDIA3 activity is involved in IAV replication, specifically in the oxidative folding of IAV HA. Interestingly this effect appears to occur independent of influenza strain. Lung epithelial specific ablation of *Pdia3* suggested that IAV infection and spread may require *Pdia3 in vivo* and ablation of *Pdia3* also decreased inflammatory markers as well as virus load in the lung. These results suggest that inhibiting PDIA3 during influenza infection may decrease viral load and improve lung mechanics. Furthermore, we have shown that a reversible, small molecule PDI inhibitor LOC14 has anti-influenza activity and may form a potential basis for future anti influenza therapeutics by inhibiting PDIs.

MATERIALS AND METHODS

Ethics statement

All animal studies were approved by the University of Vermont Institutional Animal Care and Use Committee and carried out in accordance with the Guide for the Care and Use of Animals of the National Institutes of Health. The University of Vermont adheres to the "U.S. Government Principles for the Utilization and Care of Vertebrate Animals

Used in Testing, Research, and Training", "PHS Policy on Humane Care and Use of Laboratory Animals", "USDA: Animal Welfare Act & Regulations", and "the Guide for the Care and Use of Laboratory Animals". The University is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). University of Vermont's PHS Assurance Number: A3301-01, expiration date: October 31, 2021. University of Vermont IACUC was approved on July 30, 2013, and the animal protocol number is 13-063. The de novo renewal was approved on July 17, 2018, and the animal protocol number is 13-063.

Viruses

Influenza A virus Puerto Rico 8/34 (H1N1) (10100374) and A X-31, A/Aichi/68 (H3N2) (10100375) were purchased from Charles River.

Cells and treatments

Primary MTECs were isolated and cultured from age and sex matched wild type (WT) C57BL/6NJ mice as previously described [6]. Cells were plated at 2×10^6 cells/dish and when greater than 90% confluent, infected with mouse-adapted H1N1 influenza A virus Puerto Rico 8/34 (PR8) or H3N2 A X-31, A/Aichi/68 (X31) at 2.5 Egg infectious units (EIU)/cell in a DMEM/F12 (Gibco) growth factor–free medium. Ultraviolet light (UV)–irradiated virus that was replication-deficient (mock) was used as a control. Following infection the cells were incubated for 1 h at 37 ºC, the plates were then washed twice with 2 mL PBS to remove unbound virus, and supplemented with growth factor–free medium. MTECs were pretreated for 2 h with 10 µM LOC14 (Tocris, 5606), during viral

infection, and 1 h post viral infection, DMSO was used as a control. All treatments were performed in growth factor–free medium.

Transgenic Mice

Bitransgenic mice carrying the rat club cell secretory protein (CCSP) promoter 5′ to the open reading frame for the reverse tetracycline transactivator (*CCSP-rtTA*; line 1, which in adult lungs is expressed in bronchiolar and type II epithelial cells) [58] plus 7 tetracycline operon 5′ to the open reading frame for Cre recombinase *(TetOP-Cre)* mice were provided by Dr. Whitsett (Cincinnati Children's Hospital) [59]. *CCSP-rtTA*$^{+}$*, TetOP-Cre*$^{+}$ mice were bred with mice carrying the *Pdia3*$^{loxp/loxp}$ alleles [60]. Mice expressing *CCSP-rtTA/TetOP-Cre*/*Pdia3*$^{loxp/loxp}$ were used to ablate Pdia3 from lung epithelial cells (denoted as *ΔEpi-Pdia3*) by feeding doxycycline-containing chow (6 g/kg; Purina Diet Tech, St Louis, Mo) 7 days before exposure to virus. Mice were maintained on doxycycline-containing food until completion of the experiment. Double-transgenic littermates containing either *CCSP-rtTA/TetOP-Cre* or *CCSP-rtTA/Pdia3*$^{loxp/loxp}$ (*Ctr* mice) and fed doxycycline-containing food were used as controls in the experiments.

Assessment of AHR

Mice were anesthetized by IP injection of pentobarbital solution (90 mg/kg), tracheotomized with an 18-gauge cannula, then mechanically ventilated at a rate of 200 breaths/min using a FlexiVent computer controlled small animal ventilator (SCIREQ). While on ventilators mice received the paralytic, pancuronium bromide. Parameters of Newtonian resistance (Rn), tissue dampening (G), and elastance (H) were calculated as

previously described [61]. Airway hyperresponsiveness is represented as the average of the three peak measurements for each animal, obtained at increasing methacholine doses.

Bronchoalveolar lavage processing

Bronchoalveolar lavage fluid (BALF) was collected by lavaging lungs with 1.0 mL of sterile PBS. Cells were isolated by centrifugation, and total cell counts were determined using a hemocytometer (Hausser Scientific) on an Inverted Infinity and Phase Contrast Microscope (Fisher Scientific). Differential cell counts were obtained via cytospins using Hema3 stained (Fisher Scientific) total cells. Differentials were performed on a minimum of 300 cells/animal.

Analysis of mRNA expression

Right lung lobes were flash-frozen and pulverized, and total RNA was isolated using Qiazol Lysis Reagent (Qiagen) and purified using the RNeasy kit (Qiagen). One microgram of RNA was reverse transcribed to cDNA for quantitative assessment of gene expression using SYBR green (Bio-Rad). Expression values were normalized to indicated housekeeping gene(s). The primers used in this study are listed in Supplemental table 1.

Western blot analysis

Following dissection, right lung lobes were flash-frozen for protein or mRNA analysis. Lungs were pulverized and lysed in buffer containing 20 mM Tris·HCl (pH 8.0), 100 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, and 1% protease inhibitor cocktail (Sigma-Aldrich, P8340) (v/v), 1% phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich,

P5726, P0044) (v/v). Proteins from cell lysates were prepared in the same buffer. Insoluble proteins were pelleted via centrifugation. Following protein quantitation of the supernatant, samples were resuspended in loading buffer with dithiothrietol and resolved by SDS-PAGE. Proteins were transferred to PVDF, and membranes were probed using a standard immunoblotting protocol. Protein quantification of supernatant was determined using DC Protein Assay (Bio-Rad, 5000116). Samples were resuspended in loading buffer with dithiothrietol and resolved by SDS-PAGE. The quantification of protein expression was performed by densitometry using Image Studio Lite software (LI-COR Biosciences). Antibodies used for western blots can be found Supplemental table 2

Image processing

Digital images were acquired using an Amersham Imager 600RGB (GE). Photoshop (CC 2018; Adobe) and Illustrator (CC 2018; Adobe) were used to assemble the figures. Samples were run on the same gel. When required, brightness and contrast were adjusted equally in all lanes.

ELISA

Lung protein samples were assayed for CXCL1 (DY453, R&D) and CCL20 (DY760, R&D) by ELISA according to the manufacturer's instructions.

Non-reducing gel electrophoresis

Lung homogenates were resuspended in loading buffer without the reducing agent dithiothrietol (DTT). A separate set of samples were resuspended in loading buffer with

DTT and incubated at 95°C for 10 minutes to reduce the disulfide bonds. The samples were resolved by SDS-PAGE and subjected to western blot analysis as described.

Immunofluorescence

Following euthanization, left lobes were fixed with 4% paraformaldehyde, stored at 4°C overnight for fixation of the tissue, mounted in paraffin, and 5 µm sections were affixed to glass microscope slides for histopathology as previously described [61]. Sections were prepared for immunofluorescence by deparaffinizing with xylene and rehydrating through a series of ethanols. For antigen retrieval, slides were heated for 20 min in 95°C pH 6.0 sodium citrate buffer with 0.05% TWEEN-20 then rinsed in dH_2O. Sections were then blocked for 1 hr in 1% BSA in PBS, followed by incubation with primary antibody for PDIA3 (LSBio, LS-B9768) at 1:300, overnight at 4°C. Slides were then washed 3x5min in PBS, incubated with Alexafluor 647 at 1:1000 in 1% BSA, and counterstained with DAPI in PBS at 1:4000 for nuclear localization. Sections were imaged using a Zeiss 510-META confocal laser-scanning microscope. Images were captured at x40 magnification in oil immersion. The image files were converted to Tiff format. Brightness and contrast were adjusted equally in all images.

PDIA3 activity assay

PDI disulfide reduction activity was monitored in PDI assay buffer containing 0.1 M potassium phosphate buffer pH 7.0 and 2 mM EDTA by adding 10ng recombinant human PDIA3 (Prospec, ENZ-474) or 2µg MTEC lysate to 150mM DiE-GSSG (Cayman, *11547*) in the presence of 5 µM DTT. DiE-GSSG is comprised of two eosin molecules

attached to oxidized glutathione, resulting in proximity quenching of the eosin molecules. Upon reduction of the -S-S- bond (i.e. PDIs, DTT), fluorescence emission of eosin increases dramatically [25]. The increase in fluorescence signal was monitored at 528 nm with excitation at 485 nm using a Synergy HTX plate reader (Biotek). The total reaction volume was 100 μL. For inhibition of PDIA3 the reaction mixture was incubated with LOC14 (Tocris, 5606) at the indicated concentrations for 30 minutes on ice prior to the addition of DiE-GSSG. IC50s were calculated using GraphPad Prism (version 7.0, GraphPad); briefly initial rates of fluorescence over time were determined over the first 10 minutes for indicated inhibitor concentrations, IC50s were then determined using three-parameter non-linear regression.

Biotin Switch Assay

To block free sulfhydryls, cells were lysed in buffer containing 20 mM Tris·HCl (pH 8.0), 100 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, 1% protease inhibitor cocktail (Sigma-Aldrich, P8340) (v/v), 1% phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich, P5726, P0044) (v/v), and 1 mM N-Ethylmaleimide (NEM) for 1 hour at ambient temperature. Excess NEM was then removed via acetone precipitation. Briefly, Acetone was cooled to -20ºC. Four times the sample volume was added to protein samples. Samples were then vortexed and incubated overnight at -20 ºC. Precipitated protein was pelleted by centrifugation at 14,000 x *g* for 10 minutes. The supernatant was aspirated and the resulting pellet was suspended in buffer containing 20 mM Tris·HCl (pH 8.0), 100 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, 1% SDS, and 1% protease inhibitor cocktail (Sigma-Aldrich, P8340) (v/v), 1% phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich, P5726, P0044)

(v/v). Upon resuspension disulfides were reduced with 20 μM DTT and newly formed sulfhydryl groups were labeled with 1 mM 3-(N-maleimido-propionyl) biocytin (MPB) (Invitrogen, M1602) for 1 hour at ambient temperature. Excess DTT and MPB were removed via acetone precipitation. The labeled lysate was precipitated using NeutrAvidin agarose resin (Thermo Scientific, 29200) and subsequently probed using an anti-HA antibody. As a reagent control, lysates from cells were incubated with DMSO and subjected to the same procedures.

Flow Cytometry

The following antibodies were used for flow cytometric staining: anti-IAV NP (Abcam, ab20921), Mouse IgG1 isotype control (Abcam, ab91356). Intracellular staining was performed using the BD Cytofix/Cytoperm kit (Becton Dickinson, 554714), according to the manufacturer's instructions. All samples were run on a Guava easyCyte HT cytometer (Millipore) and analyzed using Flowjo (version 10.4.2, TreeStar)

Statistics

Data were analyzed by one-way analysis of variance (ANOVA) and a Tukey's post-hoc test to adjust for multiple comparisons, or student's t test where appropriate. Statistical analysis was performed using Graph Pad Prism (version 7.0, Graph Pad). A p value ≤ 0.05 was considered significant. Data from multiple experiments were averaged and expressed as mean values ± SEM.

Microarray analysis

GEO2R (www.ncbi.nlm.nih.gov/geo/info/geo2r.html) was used to perform comparisons on differentially expressed genes between influenza-infected samples and normal controls on GSE63786 and GSE48466. GEO2R automatically performs a base 2 log transformation.

Caspase Assay

25ug of treated MTEC lysate was brought to 25ul with dH2O and 25ul Caspase-Glo 3/7-assay reagent (Promega, G8091) were mixed and incubated in the dark at ambient temperature for 20 minutes. Total luminescence was monitored using a Synergy HTX plate reader (Biotek). Values were expressed as relative luminescence units.

siRNA

Mouse type II epithelial cells (C10) were cultured as describe previously[17]. Cells were treated with Accell control small interfering (si)RNA (Dharmacon, D-001920-02-05) or PDIA3 siRNA (Dharmacon, E-045187-00) as per manufactuers instructions for 24 hours prior to infection with influenza virus or UV-irradiated mock virus.

Immunoprecipitation

HBE cells were lysed in cells were lysed in buffer containing 20 mM Tris·HCl (pH 8.0), 100 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, 1% protease inhibitor cocktail (Sigma-Aldrich, P8340) (v/v), 1% phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich, P5726, P0044) (v/v). PDIA3 was precipitated using anti-PDIA3 antibody (Enzo Life

Sciences, ADI-SPA-585-F) and Protein G agarose beads (Invitrogen, 15920010). As a control, lysate from cells were incubated non-specific rabbit gamma globulin (Jackson ImmunoResearch, 011-000-002) and subjected to the same procedures. Samples were run on reducing gels.

Acknowledgments

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Funding Sources

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V. Anathy and N. Chamberlain have a pending patent application on Protein disulfide isomerase A3 inhibitory compounds and methods of use US APP. NO. 62/661,601.

Author Contributions

V. Anathy initiated the collaborations with A. van der Vliet, B. Suratt, O. Dienz, and J. Alcorn, who provided reagents, critical review of the experimental design and edits

in the manuscript. N. Chamberlain and V. Anathy designed the experiments. N. Chamberlain, B. Mihavics, E. Nakada, S. Bruno, and S. Hoffman performed the experiments. N. Chamberlain, D. Heppner, D. Chapman, and V. Anathy analyzed and interpreted the data. N. Chamberlain, and V. Anathy wrote the manuscript. All authors reviewed and provided editorial input for the manuscript.

FIGURE LEGENDS

Figure 1: PDIA3 expression is increased following IAV infection.

A. Mice were infected intranasally with 2000 EIU of IAV or mock virus. *Pdia* mRNA levels were measured in whole lung lysates 6 days post infection (n=4-5 mice/group). B. Confocal immunofluorescence microscopy of mouse lung tissue sections 6 days post infection with either IAV or mock virus stained with anti-PDIA3 antibody followed by secondary antibody conjugated to Alexa 647. Nuclei were stained with DAPI. C. Retrospective analysis of microarray data of *Pdia1*, *3*, and *4* mRNA transcript levels (GEO data set GSE63786) Data expressed as Log_2 normalized counts. D. Retrospective analysis of microarray data of *PDIA3* mRNA transcript levels from (GEO data set GSE48466). Data expressed as Log_2 normalized counts, Data are expressed as means (± SEM) * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.001$ by ANOVA. E. Western blot analysis of IAV HA: PDIA3 interaction in HBE cells following PDIA3 immunoprecipitation. Numbers below indicate average densitometry per group of WCL PDIA3 normalized to GAPDH F. Western blot analysis of IAV HA and PDIA3 in mouse lung epithelial cells transfected

with scrambled (-) or *Pdia3*-specific siRNA (+). Numbers below indicate densitometry of IAV HA normalized to β actin.

Figure 2: PDI inhibitor LOC14 inhibits PDIA3 and decreases influenza protein production.

A. Kinetics of PDIA3 catalyzed reduction of DiE-GSSG in the presence of increasing concentrations of recombinant PDIA3. B. Chemical structure of reversible PDI inhibitor LOC14. C. Dose dependent inhibition of PDIA3 reductase activity with LOC14. Heat-treated rPDIA3, alkylated (NEM) rPDIA3, and LOC14 without PDIA3 were included as controls. D. IC50 of LOC14 for PDIA3 determined based on the initial rate of fluorescence formation from C. E. Treatment regimen for LOC14 in MTEC cells. F. Percentage of IAV NP+ primary mouse tracheal epithelial cells 24 hours post infection with IAV or mock virus following treatment with LOC14 or vehicle control. G. EC50 of LOC14 determined from percentage of infected cells from F. H. Significant decreases in PDI activity in the cells treated with LOC14. ** $p<0.01$ by t test.

Figure 3: LOC14 treatment in primary mouse tracheal epithelial cells (MTECs) decreases -S-S- of HA and viral burden.

A. Schematic representing the time points of IAV infection and LOC14 treatment. B. Western blot analysis of PR8 HA0/HA1 of cells treated as in A. GRP94 was used as a loading control. Duplicates (mock) and triplicates (IAV) of identical conditions are shown. C. Schematic depicting biotin switch assay and subsequent labeling of reduced sulfhydryl groups by MPB. D. Western blot analysis of thiol content of IAV HA following LOC14

treatment by MPB labeling and neutravidin pulldown. E. Western blot analysis of HA oligomerization following LOC14 treatment of IAV infected cells, samples analyzed by reducing (+DTT) and non-reducing (-DTT) run on a SDS-PAGE. F. mRNA expression of IAV *PA* from cell lysates of mock infected and IAV infected primary MTECs analyzed by RT-qPCR. G. Percentage of IAV NP+ MTECs following LOC14 treatment, *p<0.05 compared to mock groups, #p<0.05 compared to IAV-VC group by ANOVA. H. Percentage of IAV NP+ MTECs following exposure to LOC14 or vehicle control treated supernatant from F, *p<0.05 compared to VC group by t test. I and J. mRNA expression of *Irf7* and *Ccl20* from cell lysates of mock infected and IAV infected primary MTECs analyzed by RT-qPCR. PCR results were normalized to housekeeping gene *Gapdh*. *p<0.05 compared to mock groups, #p<0.05 compared to IAV-VC group by ANOVA. Data are expressed as means (± SEM) of 3 samples per group.

Figure 4: LOC14 treatment alters oxidative folding of different HA in H3N2 serotype. A. Western blot analysis of X31 HA0/HA1, GAPDH was used as a loading control (top panel), Western blot analysis of thiol content of IAV HA following LOC14 treatment by MPB labeling and neutravidin pulldown (bottom panel). B. Western blot analysis of HA oligomerization following LOC14 treatment of IAV infected cells, samples analyzed by reducing (+DTT) and non-reducing (-DTT) run on a SDS-PAGE. C. mRNA expression of IAV *PA* from cell lysates of mock infected and IAV infected primary MTECs analyzed by RT-qPCR, *p<0.05 compared to mock groups, #p<0.05 compared to IAV-VC group by ANOVA. D. Percentage of IAV NP+ MTECs following LOC14 treatment by flow cytometry, *p<0.05 compared to mock groups, #p<0.05 compared to IAV-VC group by

ANOVA. E. Percentage of IAV NP+ MTECs following exposure to LOC14 or vehicle control treated supernatant from E by flow cytometry, *p<0.05 compared to VC group by t test. F and G. mRNA expression of *Irf7* and *Ccl20* from cell lysates of mock infected and IAV infected primary MTECs analyzed by RT-qPCR. PCR results were normalized to housekeeping gene *Gapdh*. *p<0.05 compared to mock groups, #p<0.05 compared to IAV-VC group by ANOVA. Data are expressed as means (± SEM) of 3 samples per group.

Figure 5: Ablation of *Pdia3* in the lung epithelium attenuates viral burden and inflammation in mice.

A. Schematic representing the time points of IAV infection and doxycycline treatment and euthanasia of mice. B-D. Analysis of mRNA for influenza proteins (*PA*, *HA* and *NA*) in whole lung lysate by RT-qPCR, results normalized to the geometric mean of housekeeping genes *Pp1b* and *Gapdh,* *p<0.05 compared to mock groups, #p<0.05 compared to IAV-*Ctr* group by ANOVA. E. Western blot analysis of IAV HA in *Ctr* and *ΔEpi-Pdia3* mice, GRP94 was used as a loading control. * Indicates nonspecific band. F. Western blot analysis of HA oligomerization following ablation of PDIA3 in the airway epithelium, samples analyzed by reducing (+DTT) and non-reducing (-DTT) SDS-PAGE. G and H. ELISA for CXCL1 and CCL20 from whole lung homogenates, *p<0.05 compared to mock groups, #p<0.05 compared to IAV-*Ctr* group by ANOVA. I-L. Analysis of inflammatory and immune cells in the BALF by hema 3 stain and differential counting, *p<0.05 compared to mock groups, #p<0.05 compared to IAV-*Ctr* group by ANOVA. Data are expressed as means (± SEM) of 6–10 mice/group.

Figure 6: Ablation of *Pdia3* in the lung epithelium attenuates influenza-mediated methacholine induced AHR.

A-C. Assessment of AHR via a forced oscillation technique in *Ctr* and Δ*Epi-Pdia3* mice. Saline was administered to determine baseline Newtonian resistance (Rn), tissue resistance (G), and tissue elastance (H), *$p<0.05$ compared to mock groups, #$p<0.05$ compared to IAV-Δ*Epi-Pdia3* group by ANOVA. D-F. Methacholine (12.5-50 mg/mL) induced AHR represented as percent increase from saline following exposure to increasing doses of methacholine. *$p<0.05$ compared to mock groups, #$p<0.05$ compared to IAV-Δ*Epi-Pdia3* group by ANOVA. Data are expressed as means (± SEM) of 6–10 mice/group.

SUPPORTING INFORMATION

Figure Legends

S1 Fig. GSE63786 PDI transcript levels.

A. Retrospective analysis of microarray data of *Pdia* mRNA transcripts from GEO data set GSE63786. Data expressed as Log_2 normalized counts. * $p\leq0.05$, ** $p\leq0.01$, *** $p\leq0.001$, **** $p<0.001$ by ANOVA. B. Quantification of PDIA3 expression from Fig 1E WCL. Protein bands were quantitated by densitometry, expressed relative to GAPDH, and normalized to mock-infected cells. Data are expressed as means (± SEM) of 3 samples per group. C. Quantification of IAV H1 HA expression from Fig 1F. Protein bands were quantitated by densitometry, expressed relative to β actin, and normalized to siControl treated IAV infected cells.

S2 Fig. Flow cytometric dot plots.

A. Quantification of HA expression from Fig 3B. Protein bands were quantitated by densitometry, expressed relative to GRP94, and normalized to vehicle control treated mock-infected cells. Data are expressed as means (± SEM) of 2-3 samples per group.

B. Representative flow cytometric dot plots corresponding to PR8 NP+ cells in figure 3H.

C. Representative flow cytometric dot plots corresponding to PR8 NP+ cells in figure 3I.

D. Western blot analysis of cellular stress markers following LOC14 treatment. E. Caspase 3/7 activity, measured by a luminescence assay, following LOC14 treatment. $^*p<0.05$ compared to mock groups by ANOVA

S3 Fig. BLAST alignment of IAV hemagglutinin sequences.

A. Alignment of H1N1 HA sequences from 1934 to 2009. B. Alignment of HA sequences from various influenza serotypes. Boxes indicate conserved Cysteine residues. (Clustal Omega v1.2.1)

S4 Fig. Flow cytometric dot plots.

A. Quantification of HA expression from Fig 4A. Protein bands were quantitated by densitometry, expressed relative to GRP94, and normalized to vehicle control treated mock-infected cells. Data are expressed as means (± SEM) of 2-3 samples per group. $^*p<0.05$ compared to mock groups, #p<0.05 compared to IAV-VC group by ANOVA.

A. Representative flow cytometric dot plots corresponding to X31 NP+ cells in figure 4E.

B. Representative flow cytometric dot plots corresponding to X31 NP+ cells in figure 4F.

S5 Fig. Quantification of HA-western blots

Quantification of HA expression from Fig 5E. Protein bands were quantitated by densitometry, expressed relative to GRP94, and normalized to mock infected *Ctr* mice. Data are expressed as means (± SEM) of 3-5 samples per group. *$p<0.05$ compared to mock groups, #$p<0.05$ compared to IAV-*Ctr* group by ANOVA.

S1 Table. List of oligonucleotides used in this study

S2 Table. List and origin of antibodies used in this study

FIGURES AND TABLES

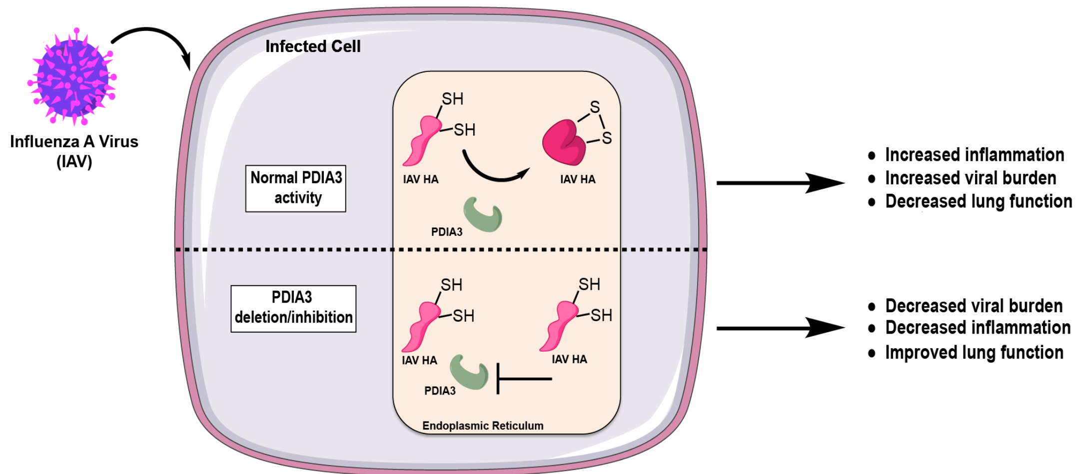


Figure 1

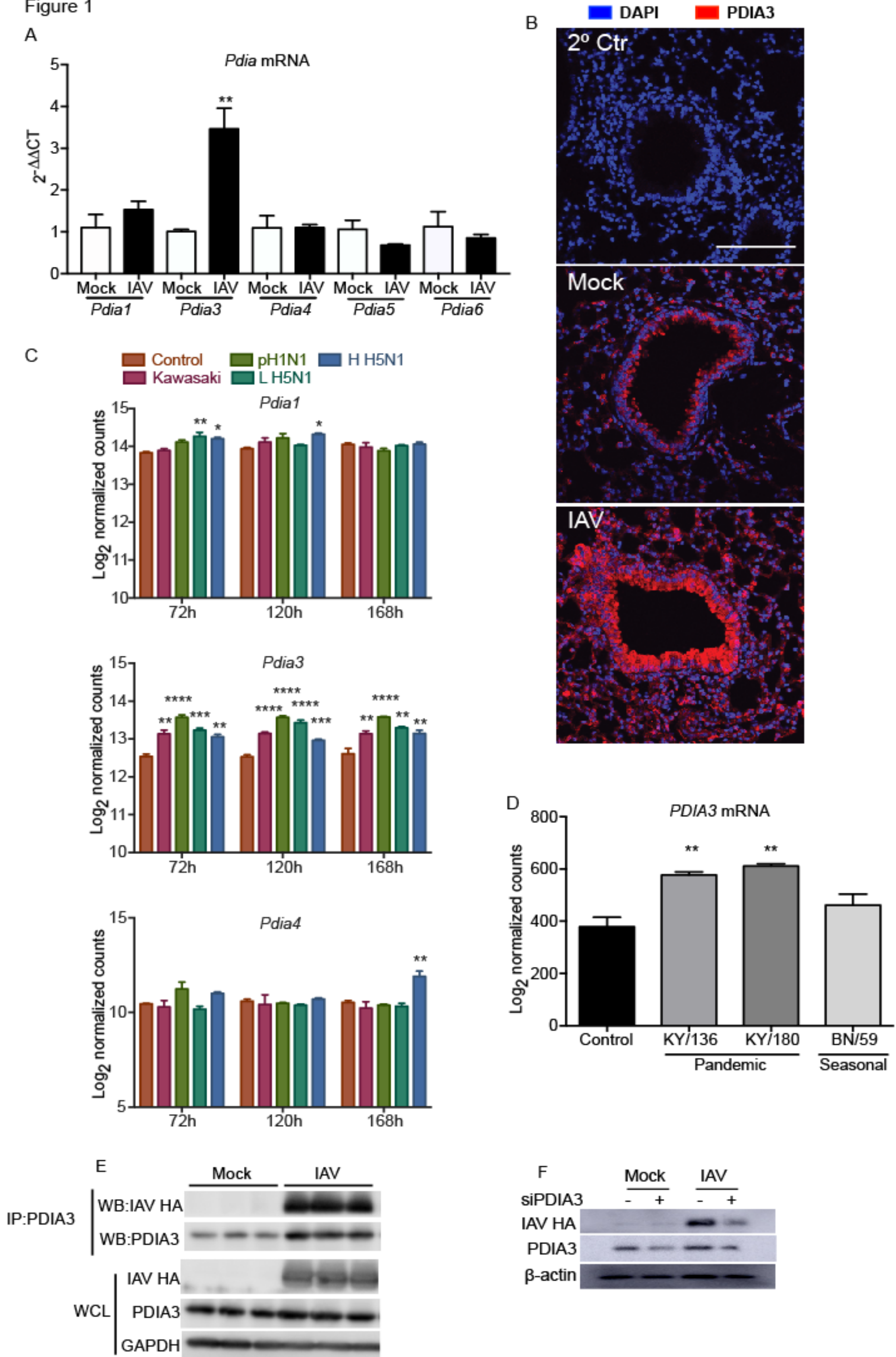
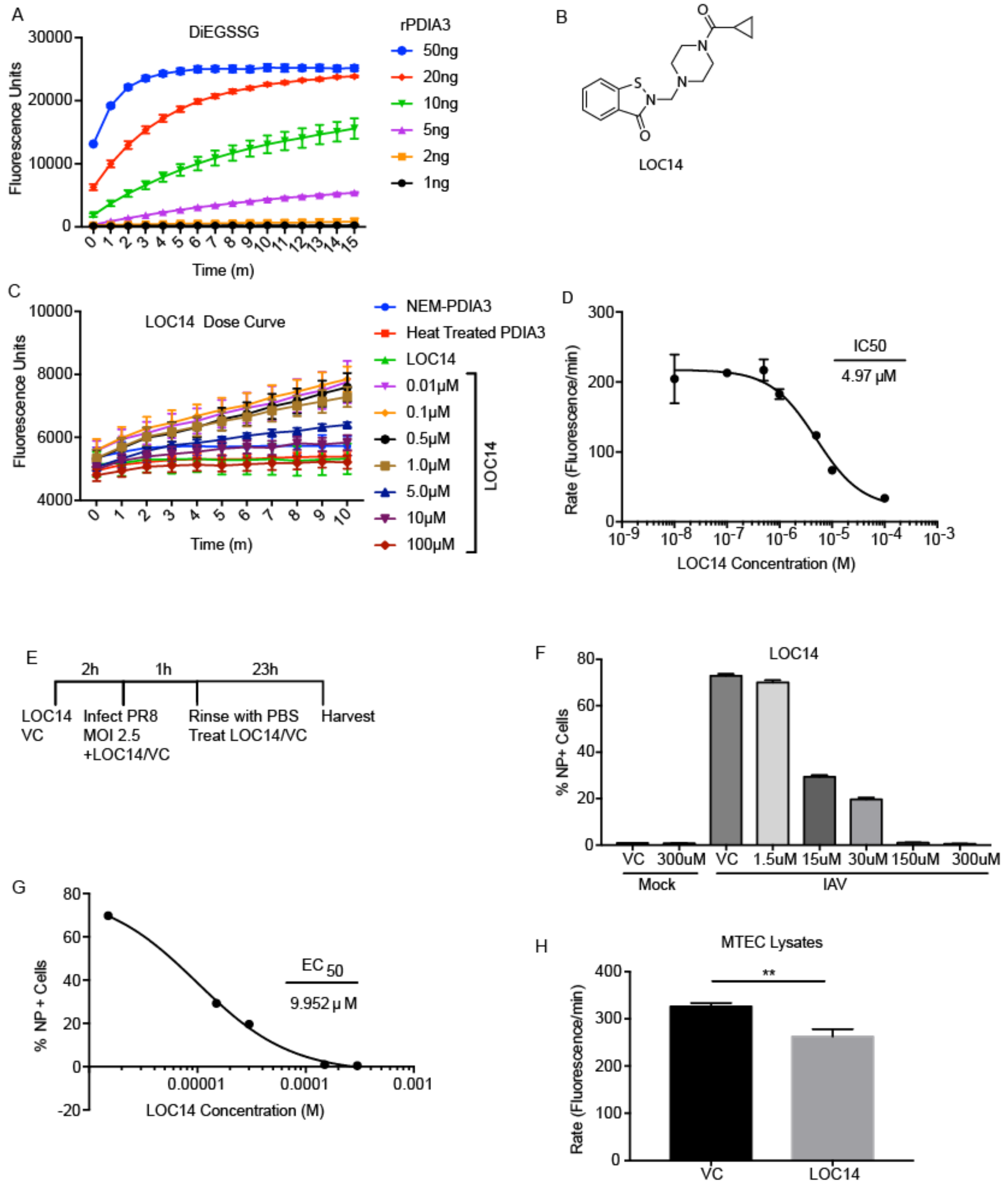


Figure 2



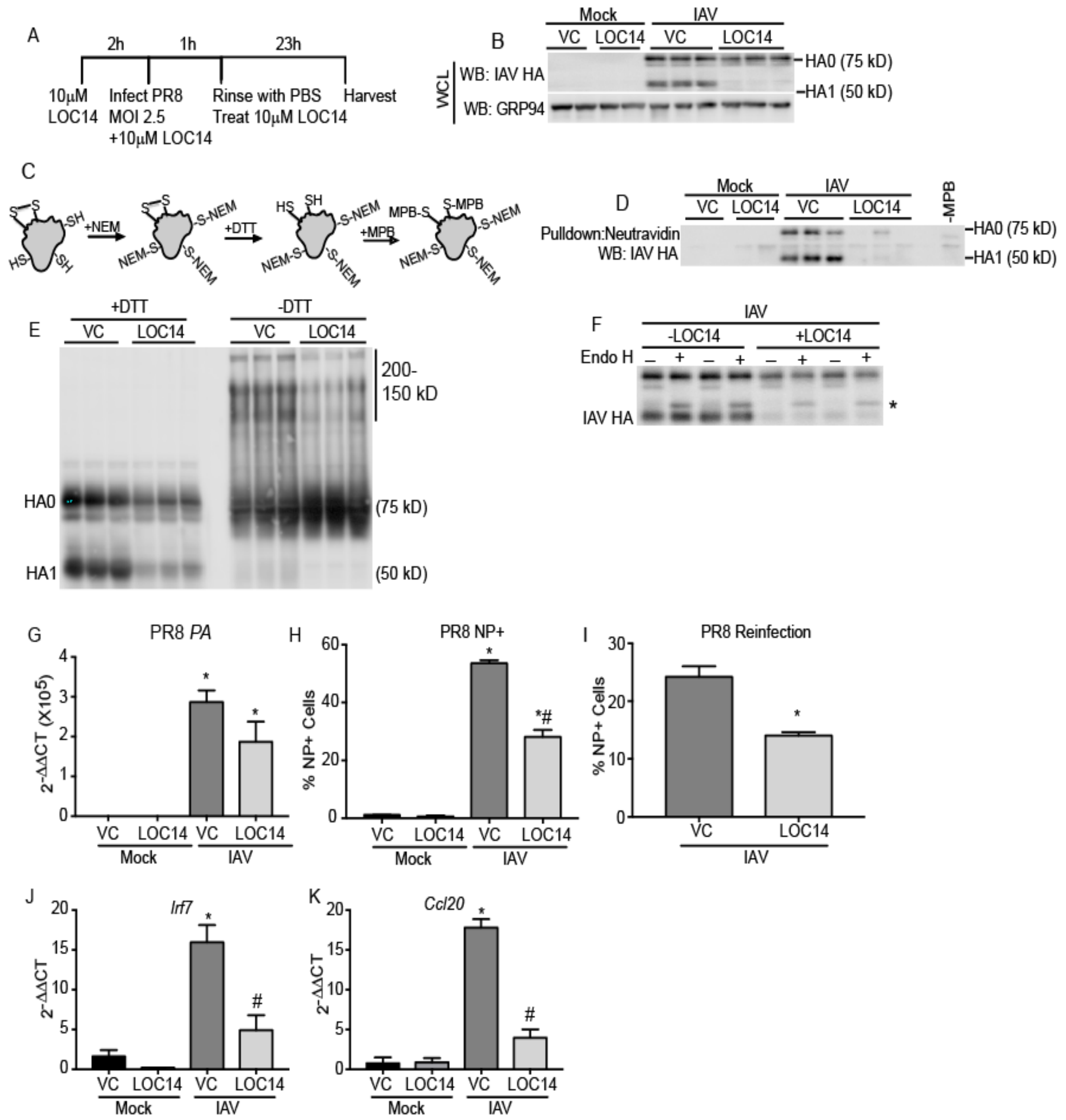
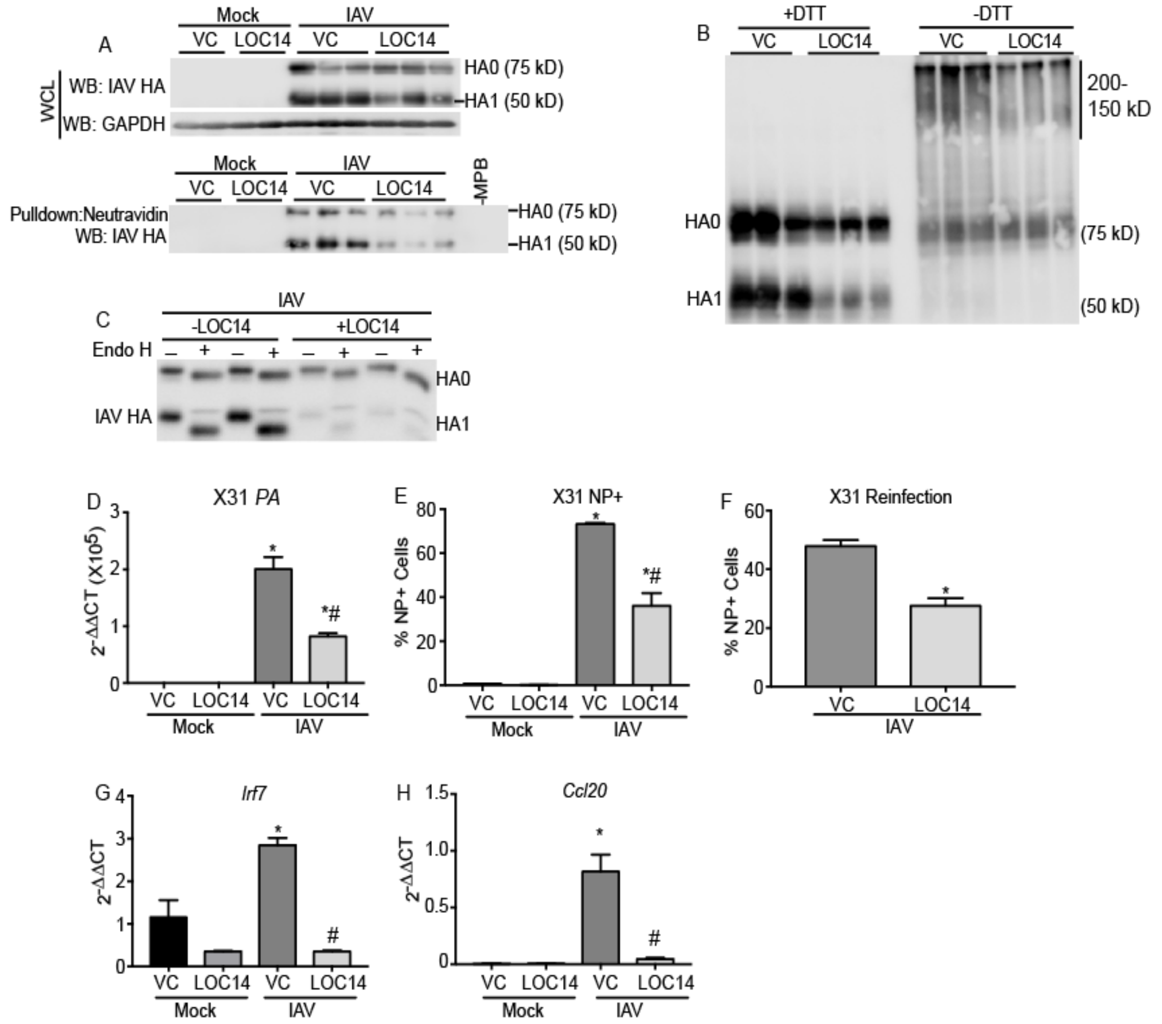


Figure 3
A
2h
1h
23h
10μM LOC14
Infect PR8 MOI 2.5 +10μM LOC14
Rinse with PBS Treat 10μM LOC14
Harvest
B
Mock
IAV
VC
LOC14
WCL
WB: IAV HA
WB: GRP94
HA0 (75 kD)
HA1 (50 kD)
C
+NEM
+DTT
+MPB
D
-MPB
Pulldown:Neutravidin
WB: IAV HA
E
+DTT
-DTT
200-150 kD
HA0
HA1
(75 kD)
(50 kD)
F
-LOC14
+LOC14
Endo H
IAV HA
G
PR8 PA
2-ΔΔCT (X10^5)
H
PR8 NP+
% NP+ Cells
I
PR8 Reinfection
J
Irf7
K
Ccl20
2-ΔΔCT

Figure 4



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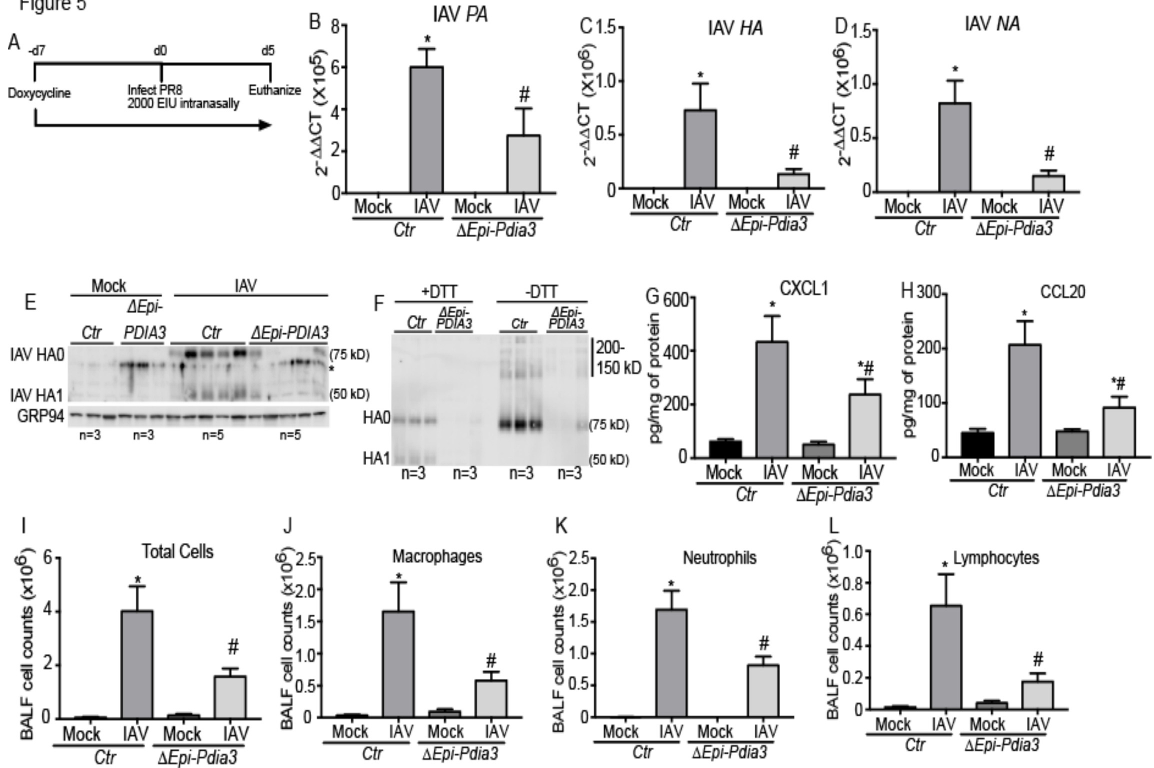

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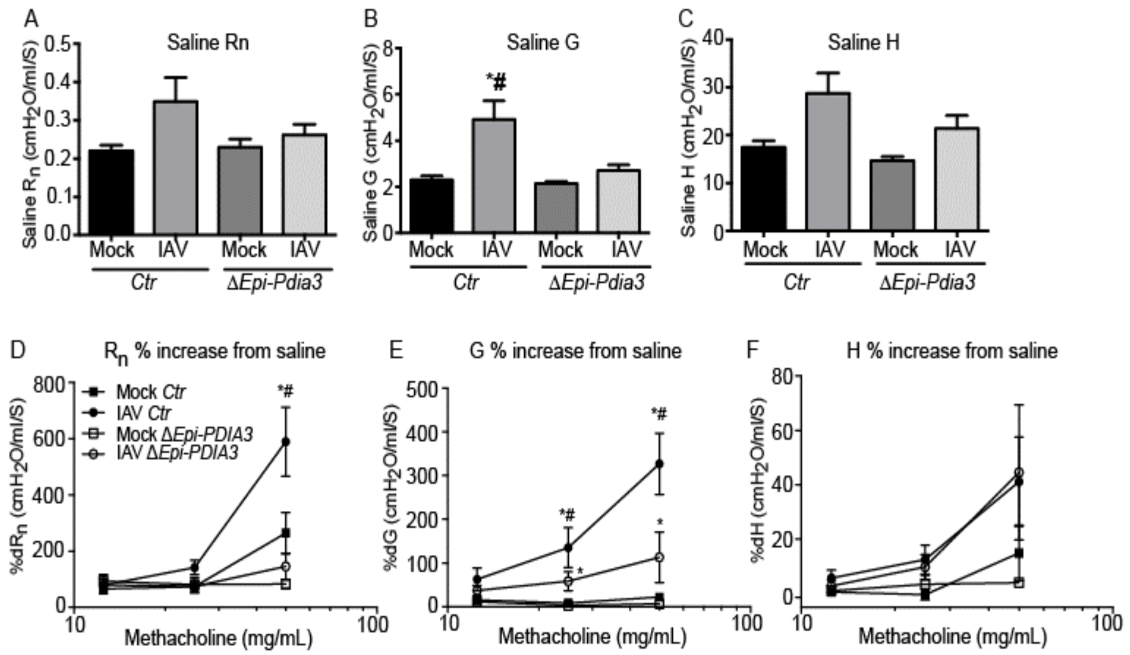

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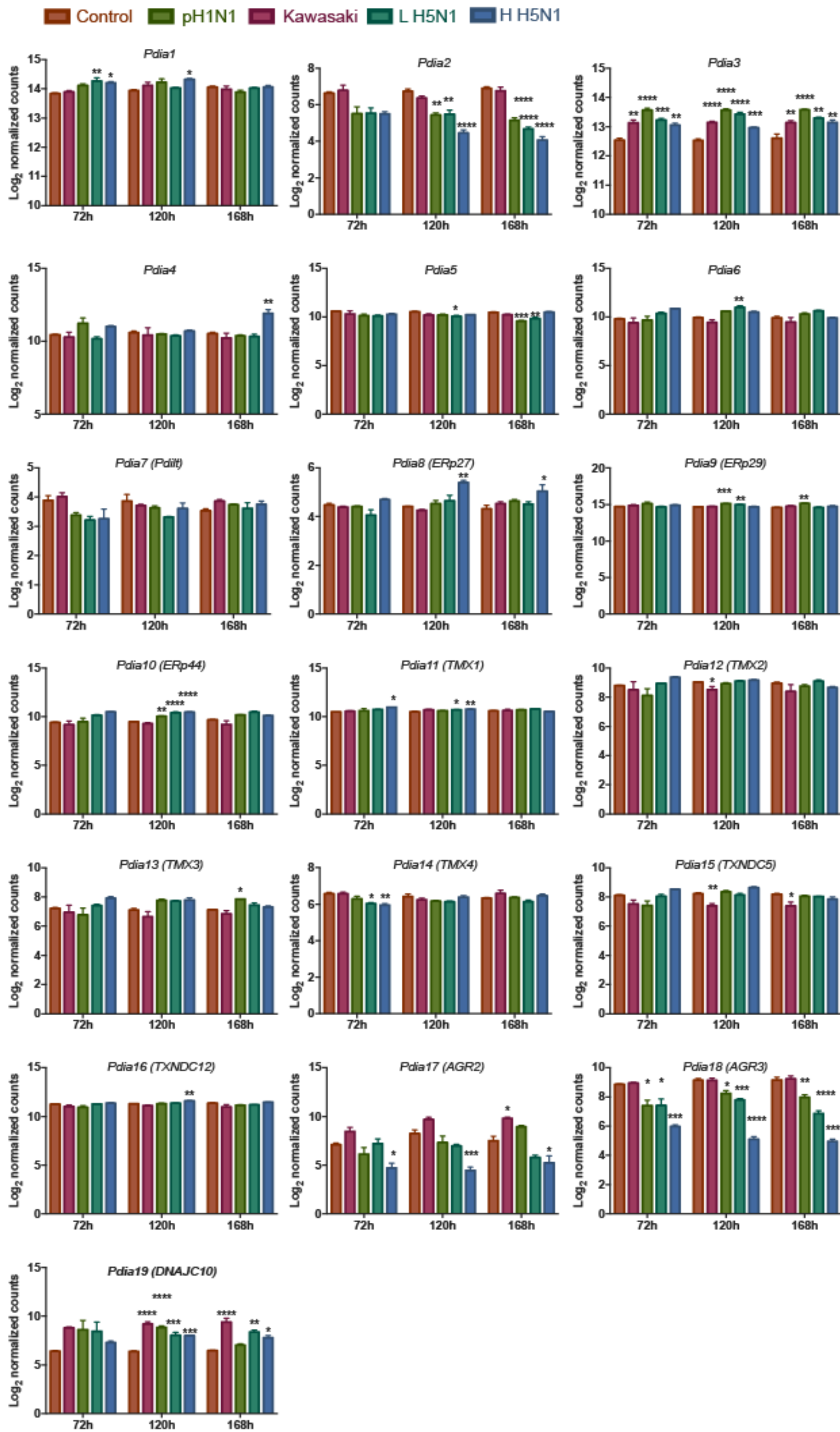

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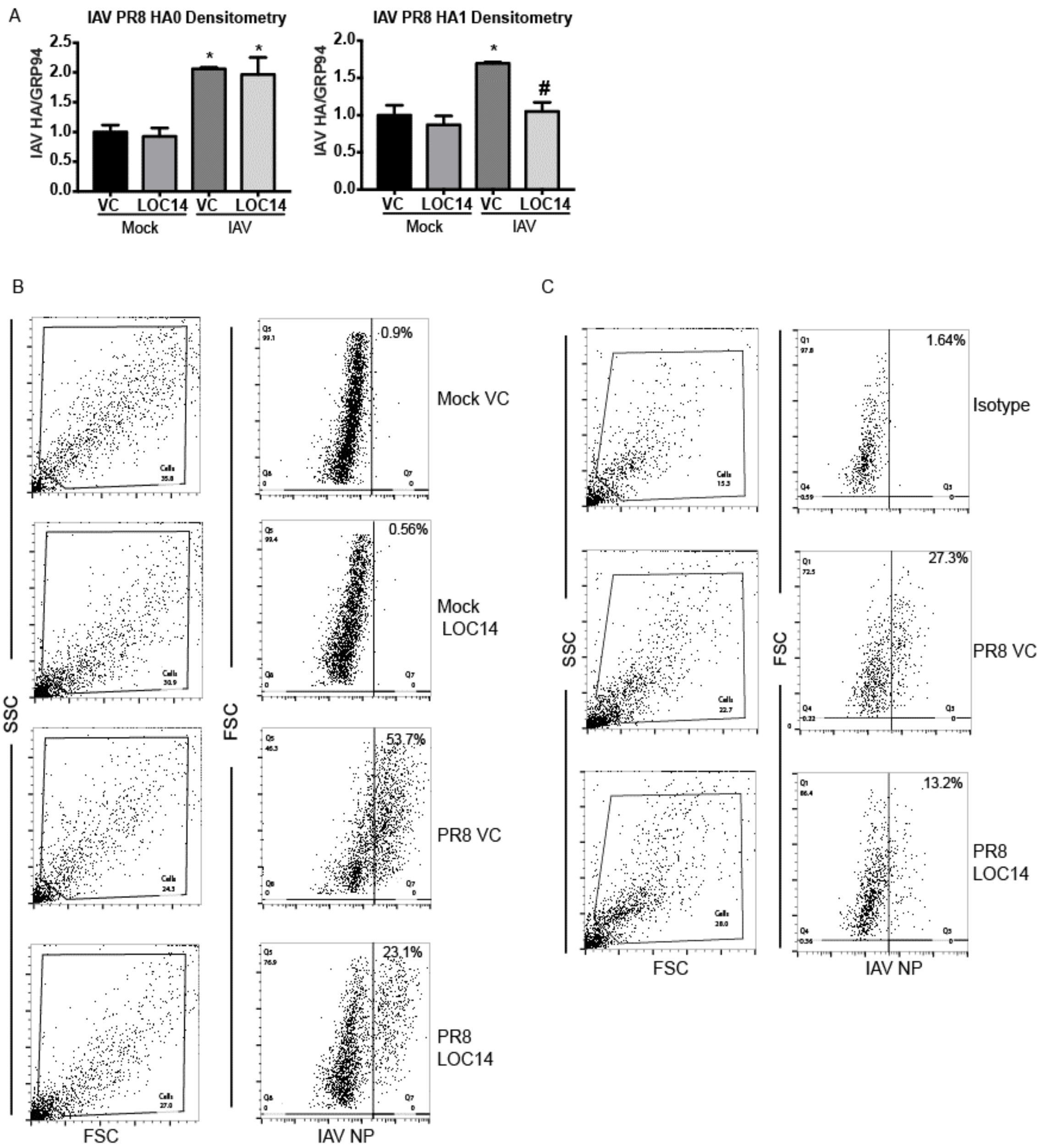

Figure S3

A

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1934    MKANLLVLLCALAAADADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDSHNGKLCR
1992    MKAKLLVLLYAFVATDADTICIGYHANNSTDTVDTIFEKNVAVTHSVNLLEDSHNGKLCL
2009    MKVKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDSHNGKLCL

1999    LGGIAPLHLGKCNIAGWLLGNPECESLSTISSWSYIVETSNSDNGTCYPGDFINYEELRE
2004    LRGVAPLHLGKCNIAGWILGNPECESLSTASSWSYIVETSSSDNGTCYPGDFIDYEELRE
1934    LKGIAPLQLGKCNIAGWLLGNPECDPLLPVRSWSYIVETPNSENGICYPGDFIDYEELRE
1992    LKGIAPLQLGNCSVAGWILGNPECELLISKESWSYIVETPNPENGTCYPGYFADYEELRE
2009    LKGIAPLQLGNCSVAGWILGNPECELLISKESWSYIVETPNPENGTCYPGYFADYEELRE

1999    QLSSVSSFERFEIFPKASSWPNHETNGGVTTACPYAGAKSFYRNLIWLVKKGNSYPKLSK
2004    QLSSVSSFERFEIFPKTSSWPNHDSNKGVTAACPHAGAKSFYKNLIWLVKKGNSYPKLSK
1934    QLSSVSSFERFEIFPKESSWPKHNTN-GVTAACSHEGKSSFYRNLLWLTEKEGSYPKLKN
1992    QLSSVSSFERFEIFPKESSWPNHTVT-GVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSK
2009    QLSSVSSFERFEIFPKESSWPNHTVT-GVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSK

1999    TGRMNYYWTLVEPGDTITFEATGNLVVPRYAFAMNRGSESGIIISDTPVHDCNTTCQTPK
2004    EGRMNYYWTLVEPGDKITFEATGNLVVPRYAFAMERNAGSGIIISDTPVHDCNTTCQTPK
1934    AGRMNYYWTLLKPGDTIIFEANGNLIAPMYAFALSRGFGSGIITSNASMHECNTKCQTPL
1992    HGRMNYYWTLLEPGDTIIFEATGNLIAPWYAFALSRGFESGIITSNASMHECNTKCQTPQ
2009    EGRINYYRTLLEPGDTIIFEANGNLIAPRYAFALSRGFGSGIITSNAPMDKCDAKCQTPQ

1999    GAINTSLPFQNVHPATIGECPKYVKSTKLRMATGLRNIPSIQSRGLFGAIAGFIEGGWTG
2004    GAINTSLPFQNIHPITIGKCPKYVKSTKLRLATGLRNVPSIQSRGLFGAIAGFIEGGWTG
1934    GAINSSLPYQNIHPVTIGECPKYVRSAKLRMVTGLRNIPSIQSRGLFGAIAGFIEGGWTG
1992    GSINSNLPFQNIHPVTIGECPKYVRSAKLRMVTGLRNIPSIQSRGLFGAIAGFIEGGWTG
2009    GAINSSLPFQNVHPVTIGECPKYVRSAKLRMVTGLRNIPSIQSRGLFGAIAGFIEGGWTG

1999    CIEFYHHCDDTCMESVKNGTYDYSKYSEESKLNREVIDGVKLDSTRIYQILAIYSTVASS
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1934    CIEFYHHCDNECMESVRNGTYDYPKYSEESKLNREKVDGVKLESMGIYQILAIYSTVASS
1992    CIEFYHHCNNECMESVKNGTYDYPKYSEESKLNREKIDGVKLESMGVYQILAIYSTVASS
2009    CIEFYHHCNDECMESVKNGTYDYPKYSEESKLNREKIDGVKLESMGVYQILAIYSTVASS
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B

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H5N1         ----------MEEIVLLLAIVSLVKSDQICIGYHANNSTEQVDTIMEKNVTVTHAQDILE
H1N1 (PR8)   ---------MKANLLVLLCALAAADADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLE
H8N7         ---------MEKFIAIAMLLASTNAYDRICIGYQSNNSTDTVDTLIEQNVPVTQTMELVE
H3N2         MKTIIALSYILCLVFAQKLPGNDNSTATLCLGHHAVPNGTIVKTITNDQIEVTNATELVQ
H7N7         -----MNTQILVFALVAIIPTN---ADKICLGHHAVSNGTKVNTLTERGVEVVNATETVE

H5N1         KTHNGKLCDLDGVKPLILRDCSVAGWLLGNPMCDEFINVPEWSYIVEKANPANDLCYPGD
H1N1         DSHNGKLCRLKGIAPLQLGKCNIAGWLLGNPECDPLLPVRSWSYIVETPNSENGICYPGD
H8N7         TEKHSAYCNTDLGAPLELRDCKIEAVIYGNPKCDIHLKDQGWSYIVERPSAPEGMCYPGS
H3N2         SSSTGGICDSP-HQILDGENCTLIDALLGDPQCDGFQN-KKWDLFVER-SKAYSNCYPYD
H7N7         RTSIPRICSKG-KRAVDLGQCGLLGTITGPPQCDQFLE-FSADLIIER-REGSDVCYPGK

H5N1         FNDYEELKHLLSRINHFEKIQIIPKSSWSDHEASSGVSSACPYNGKSSFFRNVVWLIK-K
H1N1         FIDYEELREQLSSVSSFERFEIFPKESSWPKHNTNGVTAACSHEGKSSFYRNLLWLTE-K
H8N7         VENLEELRFVFSNAASYKRIRLFDYSR--WNVTSSGTSKACNASTGGQSFYRSINWLTK-K
H3N2         VPDYASLRSLVASSGTLEFNNES---FNWTGVTQNGTSSACKRRSNKSFFSRLNWLTH--
H7N7         FANEEALRQILRESGGIDKETMG---FTYSGIRTNGATSACRR-SGSSFYAEMKWLLSNT

H5N1         GNCNTKCQTPMGAINSSMPFHNIHPLTIGECPKYVKSNRLVLATGLRNTPQRERRRKKRG
H1N1         HECNTKCQTPLGAINSSLPYQNIHPVTIGECPKYVRSAKLRMVTGLRNIPSIQ----SRG
H8N7         GNCKTKCQTYAGAINSSKPFQNASRHYMGECPKYVKKASLRLAVGLRNTPSVE----PKG
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H8N7         VKRRLSANAIDAGNGCFDILHKCDNECMETIKNGTYDHKEYEEEAKLERSKINGVKLEEN
H3N2         TKKQLRENAEDMGNGCFKIYHKCDNACIGSIRNGTYDHDVYRDEALNNRFQIKGVELKSG
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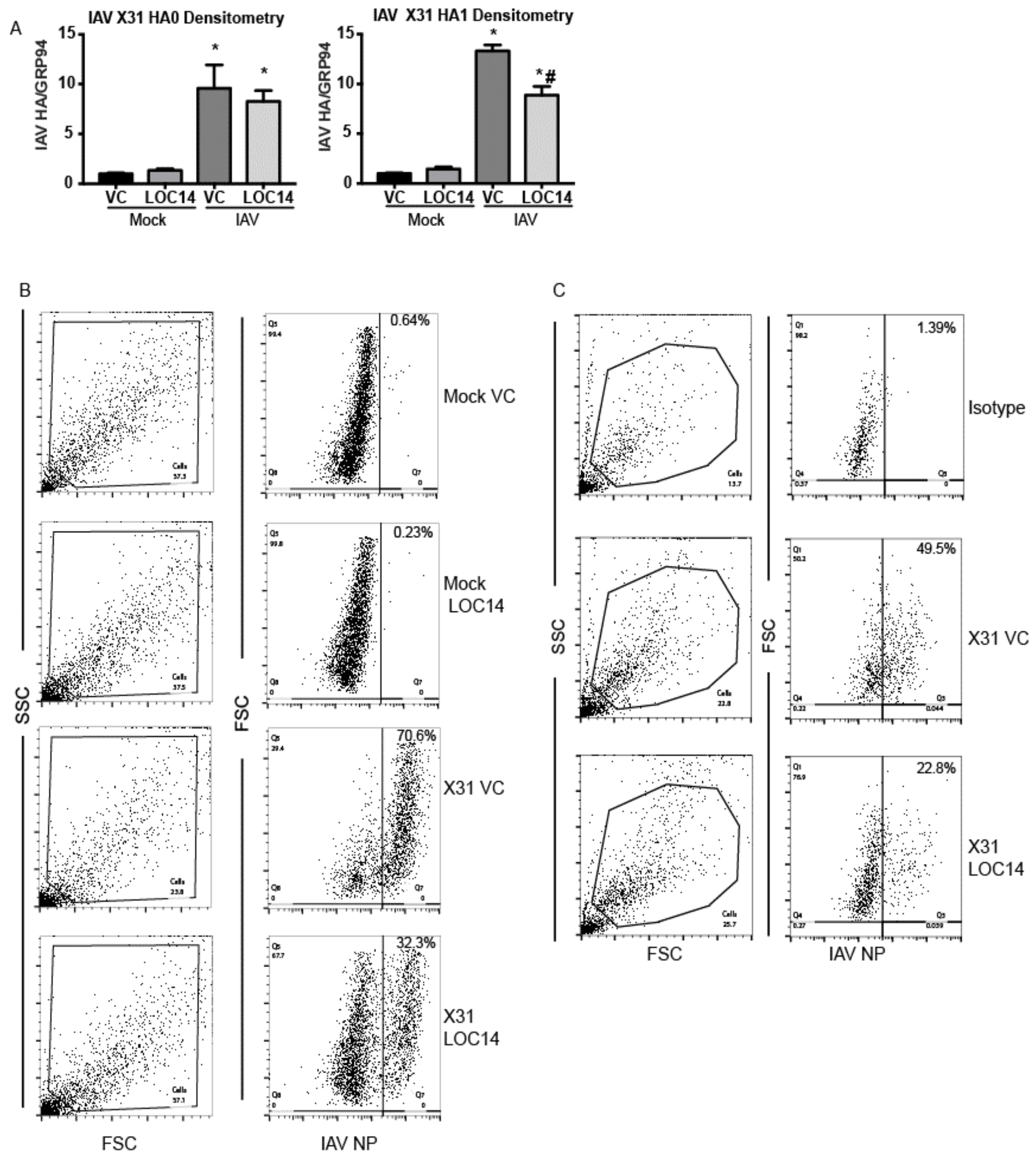
Figure S4



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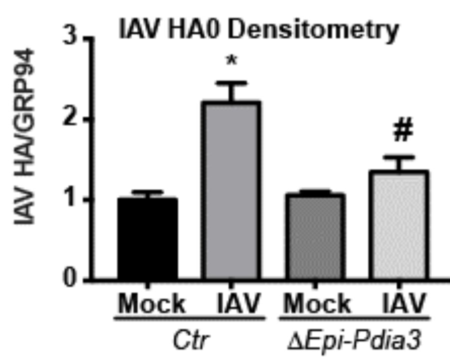

Supplemental Table 1: Oligonucleotide primers used in this study

Primer Name	Primer Sequence (orientation 5'-3')
Hemagglutinin-FW	TTGCTAAAACCCGGACACAC
Hemagglutinin-REV	CCTGACGTATTTTGGGCACT
Polymerase acidic-FW	CGGTCCAAATTCCTGCTGA
Polymerase acidic-REV	CATTTGGGTTCCTTCCATCC
Neuraminidase-FW	GTTGATGGAGCAAACGGAGT
Neuraminidase-REV	CAGCTCAGGATGTTGAACGA
mPP1B-FW	TTTTCATCTGCACTGCCAAG
mPP1B-REV	TCGAGTTGTCCACAGTCAGC
mRP2-FW	TTGCCAGCAATTTCGTGTGA
mRP2-REV	CCAGTTGACCTCTTCTGACA
mGAPDH-FW	AGGTCGGTGTGAACGGATTTG
mGAPDH-REV	TGTAGACCATGTAGTTGACCTCA
mIRF7-FW	GAAGACCCTGATCCTGGTGA
mIRF7-REV	CCAGGTCCATGAGGAAGTGT
mCCL20-FW	AGCAGCAAGCAACTACGACT
mCCL20-REV	TGACTCTTAGGCTGAGGAGGT
mPDIA1-FW	TTTGATGAAGGCCGCAACAA
mPDIA1-REV	TCAGCTTGCCGTCATAGTCA
mPDIA3-FW	TATGATGGGCCTAGGACTGC
mPDIA3-REV	TGCTGGCTGCTTTTAGGAAT
mPDIA4-FW	GTGGTCATCATTGGGCTCTT
mPDIA4-REV	CTTCTCAGGGTGTGTCAGCA
mPDIA5-FW	GGGAAGAACAGCAGACAAGC
mPDIA5-REV	TCTTACAGTGTGGGCACCAA
mPDIA6-FW	GGTGAGCTGCACCTTCTTTC
mPDIA6-REV	GCTGCTTTCTTCCATTCTGG

Supplemental Table 2: Western blot antibodies used in this study

Antibody	Species	Company	Product Number
IAV H1 HA	Rb	Sino Biological	11684-RP01
IAV H3 HA	Rb	Sino Biological	11707-T38
GAPDH	Mouse	Biolegend	919501
GRP94	Rat	Enzo LifeSciences	ADI-SPA-850-F

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CHAPTER 3

PDI INHIBITION ALTERS INFLUENZA NEURAMINIDASE ACTIVITY AND SUBSEQUENT VIRAL PATHOGENESIS *IN VIVO*

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ABSTRACT

Influenza (IAV) neuraminidase (NA) is a viral glycoprotein that plays a critical role in viral exit from the cell. NA, like other IAV proteins, undergoes extensive disulfide bond formation and these bonds are required for proper function. We have recently demonstrated the significance protein disulfide isomerase (PDI)A3 plays in proper oxidative folding of IAV hemagglutinin, another major viral glycoprotein stabilized by numerous disulfide bonds. However, it not known if PDIs play a role in NA maturation or if these interactions represent a putative target for the treatment of influenza infection. Here we show PDIA3 directly interacts with IAV NA and a cell line with CRISPR based knock out of PDIA3 showed decreased NA levels. Treatment with the reversible PDI inhibitor LOC14 alters oxidative folding and oligomerization of NA in both H1N1 and H3N2 infected lung epithelial cells. Moreover, we demonstrate LOC14 treatment is non-toxic and significantly attenuates the influenza induced pro-inflammatory response decreasing both inflammatory infiltrates in the lung as well as inflammatory cytokine levels in IAV infected mice despite strain specific effects on overall viral burden. These results suggest PDI activity is needed for effective influenza pathogenesis, via the NA activity and in vivo inhibition of PDI activity possibly represents a novel platform for the development of future host based antiviral therapies.

INTRODUCTION

The influenza A virus (IAV) causes severe respiratory illness and has a worldwide impact[1]. While vaccination and therapeutics are available, they are often rendered ineffective due to accumulation of mutations in the viral genome[1]. One potential strategy to circumvent this type of mutational resistance is targeting the host proteins or post-translational processes utilized by the virus during propagation. The relationship between IAV and host protein disulfide isomerases (PDIs), a family of proteins involved in disulfide bond formation, is well established[2-4]. Disulfide bonds play an important role in protein stability[5, 6] and viruses often utilize redox active host chaperone proteins to assist in their protein folding[7].

PDIs are major disulfide catalyzing family of enzymes in mammalian cells[8]. One isoform PDIA3 is known to be required for efficient folding of IAV hemagglutinin (HA) *in vitro*[2], and we have recently demonstrated PDIA3s importance in active infection *in vivo*[3]. A recent study has also shown specific overexpression of PDIA3, rather than other PDI isoforms, yields improved stability of HA[9]. While other PDI isoforms are known to play a role in IAV protein expression, the exact nature of that role remains unknown[4].

We have shown treatment with the reversible PDI inhibitor LOC14[10] *in vitro* significantly alters the oxidative folding of IAV HA leading to disruptions in HA maturation and also virus-induced inflammatory cytokine production[3]. However, whether these disruptions in disulfide bond formation are limited to HA remain unknown.

In order to further gain insight of this phenomenon, we investigated IAV Neuraminidase (NA) of Influenza A Virus (IAV). NA is an homotetrameric viral enzyme found on the surface of the virion[11]. NA is a glycoprotein stabilized by 32 disulfide bonds once fully matured and is known to traffic through the endoplasmic reticulum (ER), the primary site of localization of PDI [8, 12].

Active NA is required for viral release, cleaving sialic acid residues from cell surface proteins and lipids to prevent viral aggregation on the cell[11, 12]. Given the role of NA in the viral life cycle it is unsurprising that NA is a prominent target for anti-influenza therapies. NA inhibitors are currently front-line therapeutic options for influenza treatment[12]; however, their effectiveness is often limited by rapid viral mutation[13] and a limited window of application. While NA has been extensively studied an increased understanding of NA post-translational oxidative modifications may provide additional avenues for potential therapeutic targets

The structure of NA is largely conserved across multiple influenza strains[12]. individual NA monomers as well as the tetramers are stabilized by numerous conserved disulfide bonds[14]. The maturation of NA and HA are similar, both are surface viral glycoprotein that traffic through the secretory pathway of the infected cell[11]. While the role of PDIs, specifically PDIA3, in HA maturation are well established, it is unclear what role, if any, PDIs play during NA disulfide bond formation.

In this study, we provide evidence that PDIA3 and IAV-NA interact with one another. We also show ΔPDIA3 CRISPR lines alters NA oligomerization. Additionally, we demonstrate treatment of IAV-infected primary mouse tracheal epithelial cells (MTECs) with the reversible PDI inhibitor LOC14[10] resulted in the alteration NA disulfide bonds. This deficiency in disulfide bond formation decreased tetrameric NA and subsequent NA activity. Treatment of IAV infected mice with LOC14 decreased viral burden, inflammation, and pro-inflammatory cytokine production *in vivo*. These results demonstrate that PDIA3 is a modulator of IAV-NA activity and inhibiting PDIs *in vivo* decreases both IAV burden and subsequent pro-inflammatory responses, that leads to acute lung injury.

RESULTS

Comparison of novel PDI inhibitors on PDIA3

We have recently demonstrated the reversible PDI inhibitor LOC14[10] has inhibitory action against PDIA3[3]. Since this report, numerous other compounds have been characterized as PDIA3 inhibitors, however none have been compared against LOC14(Fig 1A). We compared three compounds: Eupatorin, Eupatorin-5-methyl ether, and Isoquercitin (Fig 1A), all plant flavonoids believed to interact with the b' domain of PDIA3[15]. Reduction of Dieosine-diglutathione (DiE-GSSG) has been previously used as a substrate to determine PDI activity[16, 17]. This assay shows Eupatorin and Eupatorin-5-methyl ether show inhibitor effects at high concentrations (IC50 values of 66μM and 133μM respectively), while Isoquercitin has an IC50 of approximately 29 μM (Fig 1B-D, Table 1). We then performed a direct comparison of the inhibitors on PDIA3,

20μM of each inhibitor was incubated with 10ng of PDIA3(Fig 1E). Relative activity was determined by comparison against PDIA3 incubated with the appropriate vehicle control for each inhibitor. Taken together these results indicate the reversible PDI inhibitor LOC14 is the most effective inhibitor of PDIA3.

PDIA3 interacts with IAV NA

NA is known to be stabilized by numerous disulfide bonds [12, 14] and a BLAST alignment demonstrates the cysteine residues involved in disulfide bonds show complete conservation across examined influenza strains (Sup Fig. 1). In order to characterize any PDIA3:NA interaction we performed co-immunoprecipitation experiments in human A549 adenocarcinoma alveolar basal epithelial cells after IAV infection or transfection with a GFP tagged IAV NA (NA-GFP). Analysis of the immunoprecipitations revealed that PDIA3 interacted with NA in both the H1N1 infection model as well as the transfection system as evidenced by immunoprecipitation and subsequent western blot analysis of immunoprecipitated samples as well as whole cell lysates (WCL) (Fig 2A and B). To determine whether PDIA3 expression impacts IAV- NA a stable CRISPR/Cas9 A549 cells deficient in PDIA3 were infected with either H1N1 PR8 or H3N2 X-31 or respective mock control. We found significant decreases in IAV-NA expression via western blot in both viral strains following PDIA3 ablated cell line (Fig 2C). Taken together these results indicated that PDIA3 and IAV NA interact and proper expression of IAV NA is dependent on the presence of PDIA3.

LOC14 treatment alters oxidative folding of IAV NA

IAV NA is a viral enzyme responsible for cleaving sialic acid residues of glycoproteins, enabling viral exit from the infected cell[11, 12]. We monitored recombinant-NA (rNA) activity by monitoring cleavage of (4-Methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA) to methylumbelliferone (4MU)[18] in the presence or absence of disulfide reducing agent . Cleavage of MUNANA by neuraminidase yields the fluorescent product 4-methylumbelliferone (4MU) enabling rapid assessment of NA function. This assay showed that rNA alone yields high fluorescence activity, while rNA incubated with the reductant DTT shows a loss of fluorescence activity. DTT itself had no effect on the substrate (Fig 3A). These results indicated that disulfide bonds are potentially required for NA activity.

We next examined the NA activity of acetone precipitated supernatants of IAV infected isolated primary mouse tracheal epithelial cells (mTECs). Following LOC14 treatment we observed a significant decrease in the NA activity of the supernants, suggesting the oxidative folding of NA was potentially altered (Fig 3B). We also observed LOC14 has no direct effect on NA activity (Fig 3A). Mature functional NA is known to exist as a homotetramer on the surface of the virion and the monomers and the tetramers are stabilized by disulfide bonds. In order to examine the impact of LOC14 treatment on NA oligomerization we utilized non-reducing SDS-PAGE, LOC14 treatment decreased in high molecular weight X31 N2 NA species as compared to vehicle treated controls (Fig 3C). In order to more fully explore the effect of PDIA3 on the oxidative folding of NA we utilized PDIA3 deficient human A549 CRISPR cells in both an infection and transfection

model. Western blot (WB) analysis of WCL showed that in the absence of PDIA3 levels of NA in the infection model were decreased, while in transfection model levels were more or less constant (Fig 3D). We next determined whether the absence of PDIA3 would alter -S-S- bonds of NA. We used a biotin switch assay to assess disulfides, briefly, by blocking free cysteine sulfhydryl (-SH) groups using N-ethyl maleimide (NEM) and reducing -S-S- bonds with DTT, and then newly revealed -SH groups are labeled with biotin-conjugated alkylating agent (MPB). This technique reveals high labeling of proteins with -S-S- bonds and low labeling of proteins with -SH groups. Subsequent neutravidin pull-down and Western blot analysis showed clear decreases in -S-S- bond formation in IAV-NA in both models in PDIA3 deficient cells as compared to wild type A549 cells infected with IAV or transfected with NA (Fig 3F). These results suggest that a decrease in oxidative folding in the form of disulfide bonds of NA leads to impaired oligomerization.

LOC14 treatment decreases influenza viral burden and subsequent inflammation *in vivo*

Wild type C57BL6/JN mice were treated three times with LOC14 via intraperitoneal injection one-hour pre infection and 24- and 48-hours post infection with H1N1 PR8 (Fig 4A). HA and ATF6 50 protein levels were decreased in a following LOC14 injection (Fig 4B-C). RT-qPCR analysis of viral transcripts in isolated lungs showed significantly decreased viral load in LOC14 treated mice (Fig 4D).

Analysis of total cells in BALF showed significant decrease in inflammatory cell profiles in LOC14 dosed mice infected with IAV as compared to DMSO mice infected

with IAV (Fig 4E) Quantitation of specific inflammatory cell types revealed significant attenuation of macrophages, neutrophils, and lymphocytes (Fig 4E). Moreover, quantification of *Irf7* transcripts also show significant decreases following LOC14 treatment (Fig 4F). Analysis of cytokines in the BALF showed that levels of the pro-inflammatory cytokines IL-6 and IL-1β, neutrophil maturation stimulator GCSF, neutrophil chemo attractant CXCL1 (KC), and proinflammatory chemokine CCL20 showed largely dose dependent decreases following LOC14 treatment (Fig 4G-K). Furthermore, no significant alterations in the serum alanine transaminase (ALT) levels indicated that LOC14 did not induce any systemic toxicity (Fig 4L). Moreover, total protein content of the BAL fluid of treated mice was decreased following LOC14 treatment indicating more intact epithelial barrier as compared to vehicle treated mice. Owing to complete conservation of cysteine residues between various strains of influenza (Sup Table 2) we sought to determine whether LOC14 also affected other neuraminidases e.g. N2. We found similar decreases in inflammatory markers and immune infiltrates as H1N1 infection, though we observed less substantial effects on viral levels (Sup Fig 1). Taken together these results suggested that LOC14 treatment decreases viral burden and subsequent inflammatory response *in vivo*.

DISCUSSION

PDIs are known to play important roles in influenza pathogenesis[3, 4, 9, 19], we have recently shown inhibition of PDI activity *in vitro* alters the maturation of IAV HA [3]. Moreover, specific deletion of PDIA3 in airway epithelial cells results in leading to disruptions of subsequent viral pathogenesis[3]. HA is not the only influenza protein

containing disulfide bonds[11, 12], though the extent PDIs play in the their maturation is not well known.

Herein, we demonstrate novel flavonoid-based PDI inhibitors[15] are not as effective against PDIA3 as the reversible PDI inhibitor LOC14[10]. We show the calnexin/calreticulin associated glycoprotein specific PDIA3 interacts with IAV NA, and genetic ablation of PDIA3 results in decreases in NA protein production during viral infection. LOC14 treatment alters the oxidative folding and oligomerization of NA resulting in a loss of activity. Previous work has demonstrated the importance of disulfide bonds in NA activity[14]. Furthermore, *in vivo* treatment of IAV infected mice significantly altered viral pathogenesis, decreasing both viral load and subsequent inflammatory response. Taken together our results suggest the importance of PDIA3 in the oxidative folding of IAV NA and demonstrate the significance of host PDIs in viral maturation and subsequent pathology.

The influenza virus causes yearly seasonal outbreaks and occasional recurrent pandemics resulting in significant morbidity and mortality[20, 21], especially in certain at-risk groups such as asthmatics or the elderly[22, 23]. However, despite the impact of the virus treatment options are largely limited. Besides vaccination there currently only three classes of FDA approved influenza anti-viral drugs; M2 [24], NA [25], and PA inhibitors[26]. Circulating influenza strains are largely resistant to M2 inhibitors[24, 27] and resistance to NA inhibitors is increasingly common[25, 28]. The PA inhibitor baloxavir marboxil represents the newest class of anti-influenza drugs, the first approved

in nearly 20 years[29], though resistance to baloxavir emerged has quickly emerged[26, 30]. Each of these drugs targets a specific IAV protein, as such resistance can emerge rapidly, a single amino acid substitution in the active site of IAV NA, H257Y, is enough to reduce the effectiveness of oseltamivir by over 400-fold[31].

All viruses are obligate intracellular parasites and rely almost entirely on host biochemical processes to propagate themselves. The PDI family plays an essential role in the oxidative folding of proteins[8], and PDIA3 is known to be required for the efficient folding of IAV HA catalyzing and isomerizing disulfide bonds and facilitating proper cysteine pairings[2]. The lab has previously demonstrated a significant decrease in viral burden in primary murine lung epithelial cells following siRNA mediated knockdown of PDIA3 [19]. Previous work has shown proper disulfide bond formation is required for IAV NA activity and proper formation of the protein[12, 14]. Our data shows PDIA3 also interacts with IAV NA and its absence impacts NA levels in the infected cell. Furthermore, PDI inhibition by LOC14 treatment potentially alters oxidative folding of NA and significantly decreases NA activity. This suggests PDIA3 could play a similar role in NA maturation as it does in HA maturation and demonstrates PDI inhibition impacts multiple influenza proteins simultaneously. HA and NA both traffic through the secretory pathway of the cell, going from the ER, the primary site of PDI expression, to the plasma membrane via the Golgi complex[11]. The M2 protein is known to follow the same pathway and is also stabilized by disulfide bonds, though whether these bonds involve interactions with PDIA3 or any PDI are not currently known[32]. A recent study involving siRNA screens demonstrated PDIA1 and 4, in addition to PDIA3 play a role in influenza infection[4].

While no proteins that traffic through the secretory pathway were examined, the authors found significant decreases in levels of IAV NP and M1. IAV NP is stabilized by transient disulfide bonds, thought to prevent protein aggregation[33], during its maturation the M1 protein is not stabilized by disulfide bonds[34]. The exact nature of the protein-PDI interactions and subcellular location is unclear and needs further exploration. The authors also recapitulated these results with two PDIA1 specific inhibitors, PACMA31[35] and Juniferidin[36]. LOC14 was first characterized as a PDIA1 inhibitor[10] and thus may have a similar effect on NP and M2, though further experimentation is required. Exactly what is happening to the proteins in question remains unknown. Production on either a transcriptional or translational level may be affected, as PDIs have been demonstrated to act as transcription factors[37]. Or perhaps more likely protein production occurs in a more or less normal fashion, but maturation and oxidative folding are impacted, HA mutants lacking the ability to form proper disulfide bonds are known to be retained in the ER and show enhanced proteasomal degradation[38, 39].

While PDIs are expressed at numerous locations within the cell we believe it is likely that protein maturation is impacted. PDI activity is required for HIV entry into the cell, oxidatively modifying surface proteins of the virion to allow for viral entry[40], however no such requirements are known for IAV entry. Previous studies have shown treatment with Bacitracin a broad-spectrum cell impermeable PDI inhibitor does not impact viral replication[4].

Two independent groups were able to impact multiple IAV proteins with single compounds targeting host PDIs[3, 4], this represents an improvement over current IAV therapeutics which each target individual viral proteins[24-26]. While these therapeutics can affect multiple IAV proteins by disrupting the viral replicative cycle, their effectiveness is largely limited following the first 48 hours after infection[41], when the majority of viral replication takes place[42]. As most viral protein production would occur during this time frame the direct anti-viral effect of LOC14 treatment may also be governed by similar restrictions.

Numerous cytokines are typically produced in response to viral infection including proinflammatory IL-6, IL-1β, and IL-33, each having broad ranging effects in coordinating and effectuating in the antiviral response[42-44]. We show dose dependent decreases in all of these cytokines in the BAL fluid of treated mice, along with similar decreases in the chemokine CXCL1, important in neutrophil recruitment[45]. These decreases suggest the infection is more contained following LOC14 treatment as we see concomitant decreases in viral readouts. However, we cannot rule out a direct immunomodulatory effect of PDI inhibition. Cytokines and chemokines are often stabilized by disulfide bonds[46, 47], and these bonds have been shown to be important for their function[48]. PDIs are known to assist in the folding and maturation of various chemokines[49, 50], though their direct role in the cytokines examined in this paper need to be determined. Still, given that PDIs are a major disulfide mechanism in the cell[8] it is likely they play some role in cytokine maturation. We do observe a difference in KC levels between BAL fluid and tissue lysate of infected mice, which again may suggest a role for PDI inhibition in modulation cytokine

release (Sup Fig 3), though other cytokines mirror the BAL fluid. Treatment with NA inhibitors can also yield similar decreases in immune activation[51], this is likely due to lower viral levels, as NA inhibitors are not known to directly modulate the immune system[52] and their limited effectiveness when used after the period of initial viral replication[51, 53].

Aberrant or over exuberant immune responses following viral infection, termed cytokine storm, lead to excess level of proinflammatory cytokines and widespread tissue damage and is often associated with worse clinical outcome[54]. The exact causes of cytokine storm are not currently known but it commonly follows infection with pandemic IAV strains and influenza strains of avian origin, though it can also occur during normal seasonal outbreaks[54]. Treatments for cytokine storm include inhibitors of the elevated cytokines in combination with anti-inflammatory drugs and displays conditional effectiveness based on stratified patient groups[55]. Given the immunomodulatory effect of LOC14 in both infectious and non-infectious models (Sup Fig 4), its effectiveness against cytokine storm warrants further investigation.

LOC14 treatment has a direct anti-influenza effect and an apparent anti-inflammatory effect, potentially altering oxidative folding of proinflammatory cytokines. Suggesting PDI inhibition may be effective beyond the initial stages of infection predominated by viral replication[41]. Additionally, given this methods reliance on host proteins it opens up the possibility of utilizing PDI inhibitors as a broad spectrum anti-

viral/anti-inflammatory treatment that can be utilized against novel infectious agents that lack treatment options.

MATERIALS AND METHODS

Ethics statement

All animal studies were approved by the University of Vermont Institutional Animal Care and Use Committee and carried out in accordance with the Guide for the Care and Use of Animals of the National Institutes of Health. The University of Vermont adheres to the "U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training", "PHS Policy on Humane Care and Use of Laboratory Animals", "USDA: Animal Welfare Act & Regulations", and "the Guide for the Care and Use of Laboratory Animals". The University is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). University of Vermont's PHS Assurance Number: A3301-01, expiration date: October 31, 2021. University of Vermont IACUC was approved on October 12, 2018, and the animal protocol number is PROTO202000102, IACUC Legacy number 19-005. The de novo renewal was approved on June 4, 2020, and the animal protocol number is PROTO202000102, IACUC Legacy number 19-005.

Viruses

Influenza A virus Puerto Rico 8/34 (H1N1) (10100374) and A X-31, A/Aichi/68 (H3N2) (10100375) were purchased from Charles River.

Cells and treatments

Primary MTECs were isolated and cultured from age and sex matched wild type (WT) C57BL/6NJ mice as previously described[49]. Cells were plated at 2×10^6 cells/dish and when greater than 90% confluent, infected with mouse-adapted H1N1 influenza A virus Puerto Rico 8/34 (PR8) or H3N2 A X-31, A/Aichi/68 (X31) at 2.5 Egg infectious units (EIU)/cell in a DMEM/F12 (Gibco, 21041025) growth factor–free medium. Ultraviolet light (UV)–irradiated virus that was replication-deficient (mock) was used as a control. Following infection, the cells were incubated for 1 h at 37 ºC, the plates were then washed twice with 2 mL PBS to remove unbound virus and supplemented with growth factor–free medium. MTECs were pretreated for 2 h with 10 μM LOC14 (Tocris, 5606), during viral infection, and 1 h post viral infection, DMSO was used as a control. All treatments were performed in growth factor–free medium.

Transfection

Cells were transfected using Lipofectamine 3000 transfection kit (Thermo Fisher L3000015) as per manufactures instructions with the following modification; 2.5µg of plasmid per plate, optional p3000 reagent was utilized, transfections were incubated for 4 hours. Human A549 adenocarcinoma alveolar basal epithelial cells were cultured as suggested by the ATCC. Cells were plated at 4×10^5 cells/dish 24h prior to transfection.

Immediately prior to transfection cells were washed with 1mL of PBS, and media replaced with 1mL Opti-MEM (Thermo Fisher 31985070). After 4 hours transfection media was aspirated and replaced with 2 mL Opti-MEM.

NA activity assay

NA activity assay was adapted from Leang *et al*[18]. The assay is based on NA enzyme activity cleaving the 2′-(4-Methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA) substrate (Cayman 16620) to release the fluorescent product 4-methylumbelliferone (4-MU). NA activity was monitored in NA activity buffer containing 33.3 mM 2-(N-morpholino)ethanesulfonic acid (MES) and 4 mM $CaCl_2$, pH 6.5 by adding 50µL infected cell supernatant or 5µg of infected cell lysate to 300µM MUNANA. The increase in fluorescence signal was monitored at 460 nm with excitation at 355 nm using a Synergy HTX plate reader (Biotek). The total reaction volume was 100 µL. Rates were calculated using GraphPad Prism (version 8.4.1, GraphPad).

CRISPR lines

PDIA3 deficient A549 cells were purchased from Synthego (Synthego Corporation, Menlo Park, CA). Cells were screened by plating 1 cell/well in a 96 well flat bottom plate. Single cell wells were marked and monitored for growth. After approximately 4 doublings cells were trypsinized and transferred to 6 well plates. Upon reaching ~70% confluency cells were trypsinized and transferred to T75 flasks. PDIA3 levels were ascertained by western blot.

Bronchoalveolar lavage processing

Bronchoalveolar lavage fluid (BALF) was collected by lavaging lungs with 1.0 mL of sterile PBS. Cells were isolated by centrifugation, and total cell counts were determined using a Guava easyCyte HT cytometer (Millipore) and analyzed using Flowjo (version 10.4.2, Ashland, OR: Becton, Dickinson and Company). Differential cell counts were obtained via cytospins using Hema3 stained (Fisher Scientific) total cells, on a minimum of 300 cells/animal, or using a Guava easyCyte HT cytometer with a protocol adapted from van Rijt *et al*[56]. Briefly, isolated cells were incubated with TruStain fcX (BioLegend 101320) to reduce background staining, then incubated in a mix containing 3μL CD3 PerCP/Cy5.5 (BioLegend 100218), 3μL B220 PerCP/Cy5.5 (BioLegend 103236), 2μL CD11c APC (BioLegend 117310), 2μL I-A/I-E FITC (BioLegend 107616), and 1μL CCR3 PE (BioLegend 144506) per sample. Cells were identified based on forward and side scatter characteristics and differential staining for macrophages (CD11c+), lymphocytes (CD11c-, CD3/B220+), eosinophils (CD11c-, CD3/B220-, CCR3+) and neutrophils (CD11c-, CD3/B220-, CCR3-). Data was analyzed using Flowjo. Validity was tested against differential counts based on cytospins.

Analysis of mRNA expression

Right lung lobes were flash-frozen and pulverized, and total RNA was isolated using Qiazol Lysis Reagent (Qiagen) and purified using the RNeasy kit (Qiagen). One microgram of RNA was reverse transcribed to cDNA using M-MHV Reverse Transcriptase (Promega) for quantitative assessment of gene expression using SYBR green (Bio-Rad).

Expression values were normalized to indicated housekeeping genes. The primers used in this study are listed in Supplemental table 5.

Western blot analysis

Following dissection, right lung lobes were flash-frozen for protein or mRNA analysis. Lungs were pulverized and lysed in buffer containing 20 mM Tris·HCl (pH 8.0), 100 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, and 1% protease inhibitor cocktail (Sigma-Aldrich, P8340) (v/v), 1% phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich, P5726, P0044) (v/v). Proteins from cell lysates were prepared in the same buffer. Insoluble proteins were pelleted via centrifugation. Following protein quantitation of the supernatant, samples were resuspended in loading buffer with DTT and resolved by SDS-PAGE. Proteins were transferred to PVDF, and membranes were probed using a standard immunoblotting protocol. Protein quantification of supernatant was determined using DC Protein Assay (Bio-Rad, 5000116). Samples were resuspended in loading buffer with DTT and resolved by SDS-PAGE. The quantification of protein expression was performed by densitometry using Image Studio Lite software (LI-COR Biosciences). Antibodies used for western blots can be found Supplemental table 6.

Image processing

Digital images were acquired using an Amersham Imager 600RGB (GE). Photoshop (CC 2020; Adobe) and Illustrator (CC 2020; Adobe) were used to assemble the figures. Samples were run on the same gel. When required, brightness and contrast were adjusted equally in all lanes.

ELISA

Lung protein samples were assayed for IL-6 (DY406, R&D), GCSF (DY414, R&D), IL-1β (DY401, R&D), KC (DY453, R&D), and CCL20 (DY760, R&D) by ELISA according to the manufacturer's instructions.

Non-reducing gel electrophoresis

Lung homogenates were resuspended in loading buffer without the reducing agent DTT. A separate set of samples were resuspended in loading buffer with DTT and incubated at 95°C for 5 minutes to reduce the disulfide bonds. The samples were resolved by SDS-PAGE and subjected to western blot analysis as described.

PDIA3 activity assay

PDI disulfide reduction activity was monitored as previously described[3], the reaction mixture contained 2μg MTEC lysate, 150mM DiE-GSSG (Cayman, 11547) in the presence of 5 μM DTT. The increase in fluorescence signal was monitored at 528 nm with excitation at 485 nm using a Synergy HTX plate reader (Biotek). The total reaction volume was 100 μL. For inhibition of PDIA3 10ng recombinant human PDIA3 (Prospec, ENZ-474) was used, the reaction mixture was incubated with LOC14 (Tocris, 5606), Eupatorin, Eupatorin 5 Methyl Ether, or Isoquercitin (Sigma Aldrich, E4660, CDS007351) at the indicated concentrations for 30 minutes on ice prior to the addition of DiE-GSSG. IC50s

were calculated using GraphPad Prism (version 8.4.1, GraphPad); briefly initial rates of fluorescence over time were determined over the first 15 minutes for indicated inhibitor concentrations, IC50s were then determined using three-parameter non-linear regression.

Biotin Switch Assay

To block free sulfhydryls, cells were lysed in buffer containing 20 mM Tris·HCl (pH 8.0), 100 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, 1% protease inhibitor cocktail (Sigma-Aldrich, P8340) (v/v), 1% phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich, P5726, P0044) (v/v), and 1 mM N-Ethylmaleimide (NEM) for 1 hour at ambient temperature. Excess NEM was then removed via acetone precipitation. Briefly, Acetone was cooled to -20ºC. Four times the sample volume was added to protein samples. Samples were then vortexed and incubated overnight at -20 ºC. Precipitated protein was pelleted by centrifugation at 14,000 x *g* for 10 minutes. The supernatant was aspirated and the resulting pellet was suspended in buffer containing 20 mM Tris·HCl (pH 8.0), 100 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, 1% SDS, and 1% protease inhibitor cocktail (Sigma-Aldrich, P8340) (v/v), 1% phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich, P5726, P0044) (v/v). Upon resuspension disulfides were reduced with 20 μM DTT and newly formed sulfhydryl groups were labeled with 1 mM 3-(N-maleimido-propionyl) biocytin (MPB) (Invitrogen, M1602) for 1 hour at ambient temperature. Excess DTT and MPB were removed via acetone precipitation. The labeled lysate was precipitated using NeutrAvidin agarose resin (Thermo Scientific, 29200) and subsequently probed using an anti-HA antibody. As a reagent control, lysates from cells were incubated with DMSO and subjected to the same procedures.

Serum ALT determination

Serum ALT levels were determined using an Alanine Transaminase Colorimetric Activity Assay Kit (Cayman, 700260). Assay was run according to manufactures instructions

House dust mite treatment

Human Bronchial Epithelial cells (HBEs) were plated at 4 x 10^5 cells and allowed to reach 70% confluency. Cells were staved in serum free media with LOC14 for 2h. Media was then changed dosed LOC14 and 50ug/mL HDM (Greer Laboratories, XPB70D3A2.5). Supernatants were harvested at 24h and IL8 levels determined by ELISA. MTECs were grown to 70% confluency then starved in serum free media dosed with 10uM LOC14 and 25ug/mL HDM for 48h. Media was then changed containing the same supplementation. Lysates were harvested 24h later and cytokine and chemokine levels were determined by ELISA. Mice were anesthetized using isoflurane and treated with 25ug of HDM intranasally on day 1, 8, and consecutively on days 15-19. Mice were given a single dose of LOC14 via interperitoneal injection. Lungs were harvested on day 20 and cytokine levels determined by ELISA.

Statistics

Data were analyzed by two-way analysis of variance (ANOVA) and a Benjamini, Kreiger, and Yekutieli procedure for controlling false discovery rate, one-way analysis of variance (ANOVA) and a Tukey's post-hoc test to adjust for multiple comparisons, or student's t test where appropriate. Statistical analysis was performed using Graph Pad Prism (version 8.4.1, GraphPad Software, La Jolla California USA, www.graphpad.com). A p value ≤ 0.05 was considered significant. Data from multiple experiments were averaged and expressed as mean values ± SEM.

Immunoprecipitation

HBE cells were lysed in cells were lysed in buffer containing 20 mM Tris·HCl (pH 8.0), 100 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, 1% protease inhibitor cocktail (Sigma-Aldrich, P8340) (v/v), 1% phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich, P5726, P0044) (v/v). PDIA3 was precipitated using anti-PDIA3 antibody (Enzo Life Sciences, ADI-SPA-585-F) and Protein G agarose beads (Invitrogen, 15920010). As a control, lysates from cells were incubated non-specific rabbit gamma globulin (Jackson ImmunoResearch, 011-000-002) and subjected to the same procedures. Samples were run on reducing gels.

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FIGURE LEGENDS

Figure 1: Comparison of novel PDI inhibitors on PDIA3

A. Chemical structure of PDI inhibitors LOC14, Euparotin, Eupatorin-5-Methyl ether, and Isoquercitin. B-D. IC50 of Euparotin, Eupatorin-5-Methyl ether, and Isoquercitin for PDIA3 determined based on the initial rate of fluorescence formation. E. Comparison of novel PDI inhibitors at 20μM concentration, represented as comparison to vehicle.

Figure 2: PDIA3 interacts with IAV NA

A. Western blot analysis of IAV NA:PDIA3 interaction in IAV H1N1 PR8 infected A549 cells following PDIA3 immunoprecipitation. B. Western blot analysis of IAV NA-GFP:PDIA3 interaction in NA-GFP transfected A549 cells following PDIA3 immunoprecipitation. C. Western blot analysis of IAV NA levels in PDIA3 deficient A549 CRISPR cells following infection with H1N1 PR8 or H3N2 X31, (+) and (-) indicate presence or absence of CRISPR.

Figure 3: LOC14 treatment alters oxidative folding of IAV NA

A. Kinetics of NA catalyzed cleavage of MUNANA in the presence of recombinant NA (rNA) and DTT treated rNA. DTT, LOC14, and LOC14 treated rNA were used as controls.

B. Kinetics of NA catalyzed cleavage of MUNANA from acetone precipitated supernatants of H1N1 PR8 infected MTECs. C. Western blot analysis of NA oligomerization following LOC14 treatment of H3N2 X31 infected cells, samples analyzed by reducing (+DTT) and non-reducing (-DTT) run on an SDS-PAGE. GAPDH was used as a loading control. D. Western blot analysis of PR8 NA infected and NA-GFP transfected PDIA3 deficient A549 cells. GAPDH was used as a loading control. E. Western blot analysis of thiol content of PR8 NA and NA-GFP transfected PDIA3 deficient A549 cells by MPB labeling and neutravidin pulldown.

Figure 4: LOC14 treatment attenuates viral burden and inflammation in H1N1 infected mice.

A. Schematic representing the time points of IAV infection and LOC14 treatment and euthanasia of mice. B. Western blot analysis of IAV HA, ATF6 50, GRP78, and GRP94 in treated mice, β-actin was as a loading control. C. Densitometry of WCL HA and ATF6 50 normalized to β-actin. D. Analysis of mRNA for influenza *PA* in whole lung lysate by RT-qPCR, results normalized to the geometric mean of housekeeping genes *Pp1b, Rp2,* and *Gapdh.* E. Analysis of inflammatory and immune cells in the BALF by hema 3 stain and differential counting. F. Analysis of mRNA for *Irf7* in whole lung lysate by RT-qPCR, results normalized to the geometric mean of housekeeping genes *Pp1b, RP2,* and *Gapdh.* G-K. ELISA for inflammatory cytokines and chemokines from BAL fluid of infected mice. L. Serum alanine aminotransferase levels. M. Total protein content in BAL fluid of treated mice. *$p<0.05$ compared to mock groups, #$p<0.05$ compared to IAV-DMSO group by two-way ANOVA. Data are expressed as means (± SEM)

Table 1: List of calculated IC50s in Figure 1

SUPPORTING INFORMATION

S1 TABLE: BLAST alignment of IAV neuraminidase sequences.

A. Alignment of NA sequences from various influenza serotypes. Blue color indicates conserved Cysteine residues. (Clustal Omega v1.2.1)

S2 Fig: LOC14 treatment attenuates viral induced inflammation in H3N2 infected mice.

Mice were treated as in Figure 4. A. Western blot analysis of IAV HA. * Indicates nonspecific band. B-C. Analysis of mRNA for *Irf7* and influenza *PA* in whole lung lysate by RT-qPCR, results normalized to the geometric mean of housekeeping genes *Pp1b, Rp2,* and *Gapdh.* D-G. Analysis of inflammatory and immune cells in the BALF by hema 3 stain and differential counting. H-K. ELISA for inflammatory cytokines and chemokines from BAL fluid of infected mice. $^*p<0.05$ compared to mock groups, $\#p<0.05$ compared to IAV-DMSO group by one-way ANOVA. Data are expressed as means (± SEM)

S3 Fig: LOC14 may have an effect on cytokine release.

A. KC concentrations by ELISA of H1N1 infected Vehicle and LOC14 treated lung lysate and BAL fluid. LOC14 concentration was 50mg/kg. B. KC concentrations by ELISA of H3N2 infected Vehicle and LOC14 treated lung lysate and BAL fluid. LOC14

concentration was 50mg/kg. *p<0.05 compared to control groups. Data are expressed as means (± SEM)

S4 Fig: LOC14 alters cytokine levels in non-infectious HDM inflammatory models

A. IL8 concentrations by ELISA of HDM treated human bronchial epithelial cell supernatant following treatment with LOC14. B. Cytokine and chemokine concentrations by ELISA of lung lysate from HDM treated mice treated with LOC14. *p<0.05 compared to control groups, **p<0.01 compared to control groups. Data are expressed as means (± SEM)

S5 Table. List of oligonucleotides used in this study

S6 Table. List and origin of antibodies used in this study

FIGURES

Figure 1: Comparision of novel PDI inhibitors on PDIA3

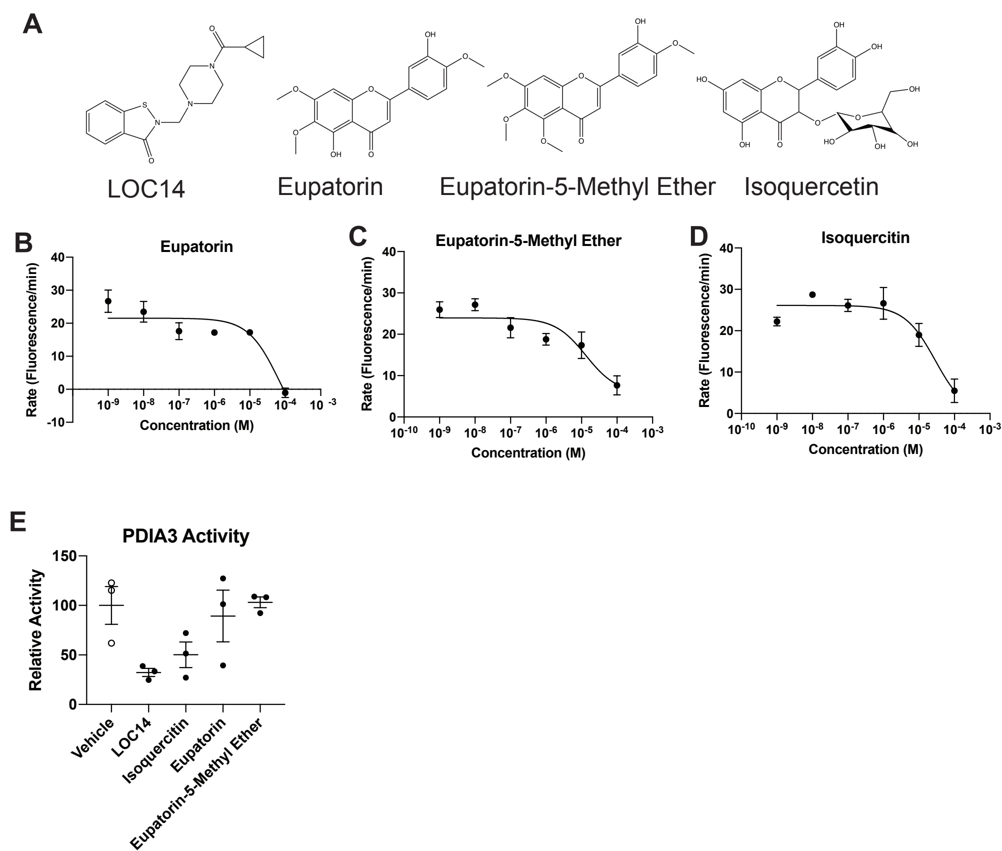


Figure 2: PDIA3 interacts with NA

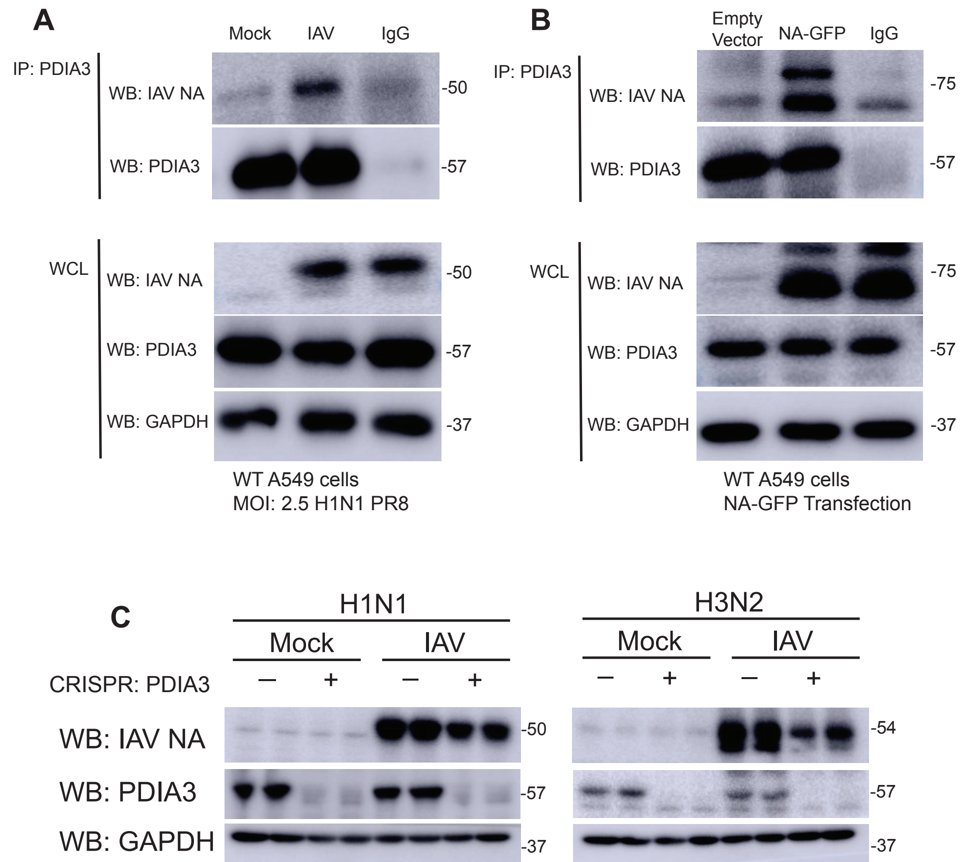


Figure 3: LOC14 alters NA oxidative folding

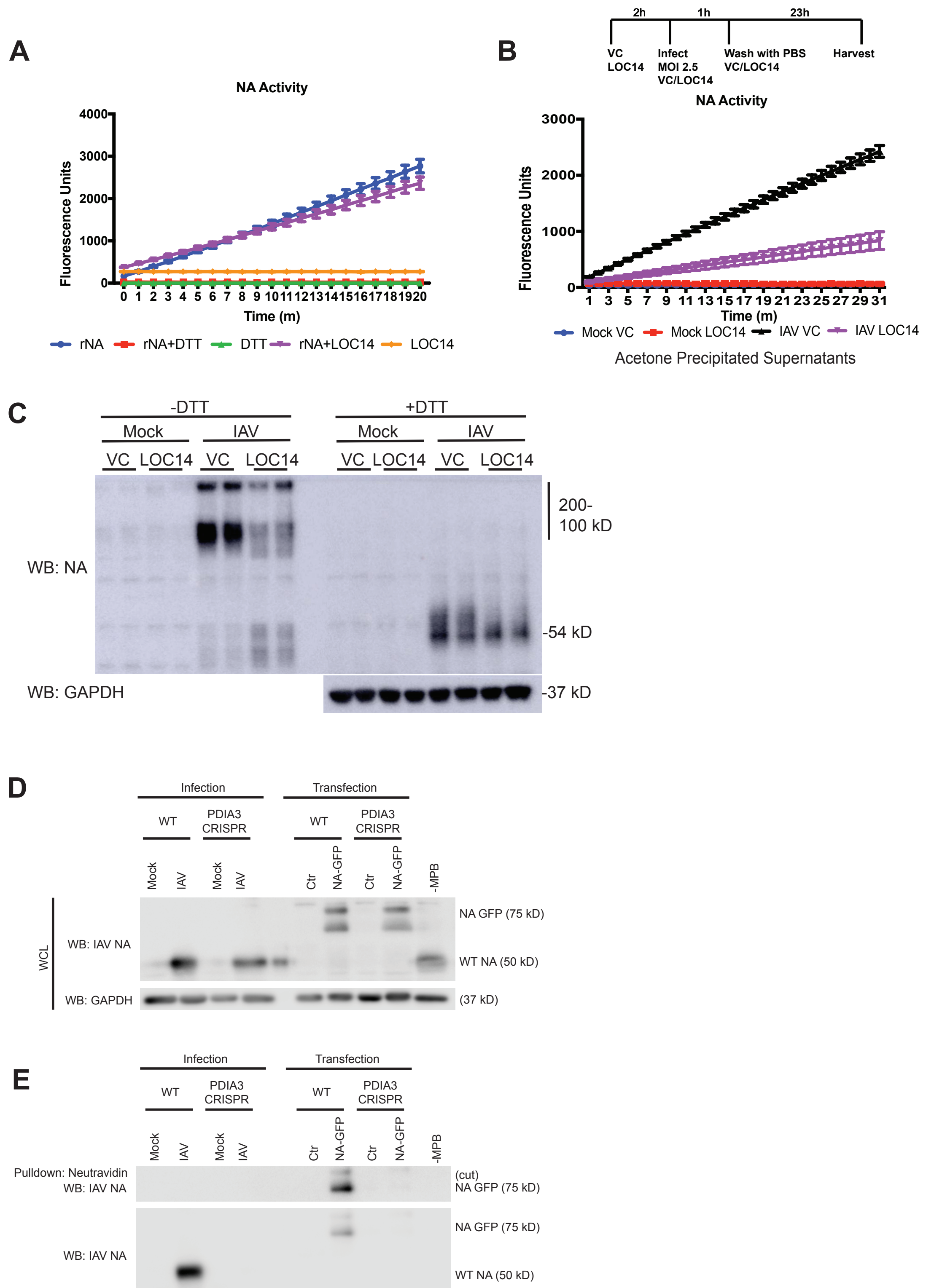


Figure 4: LOC14 treatment decreases H1N1 viral burden and inflammation *in vivo*

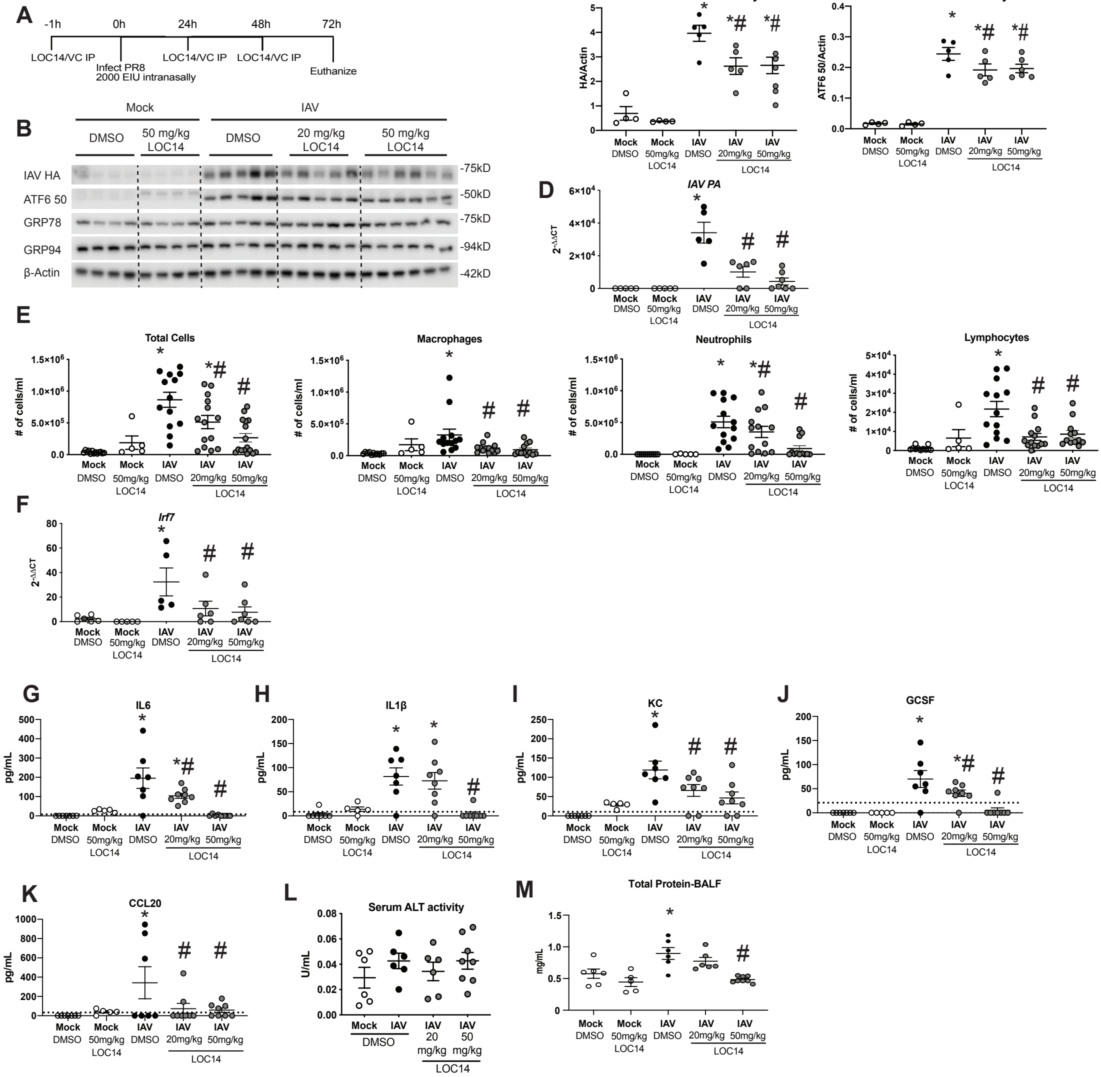


Table 1: List of calculated IC50s in Figure 1

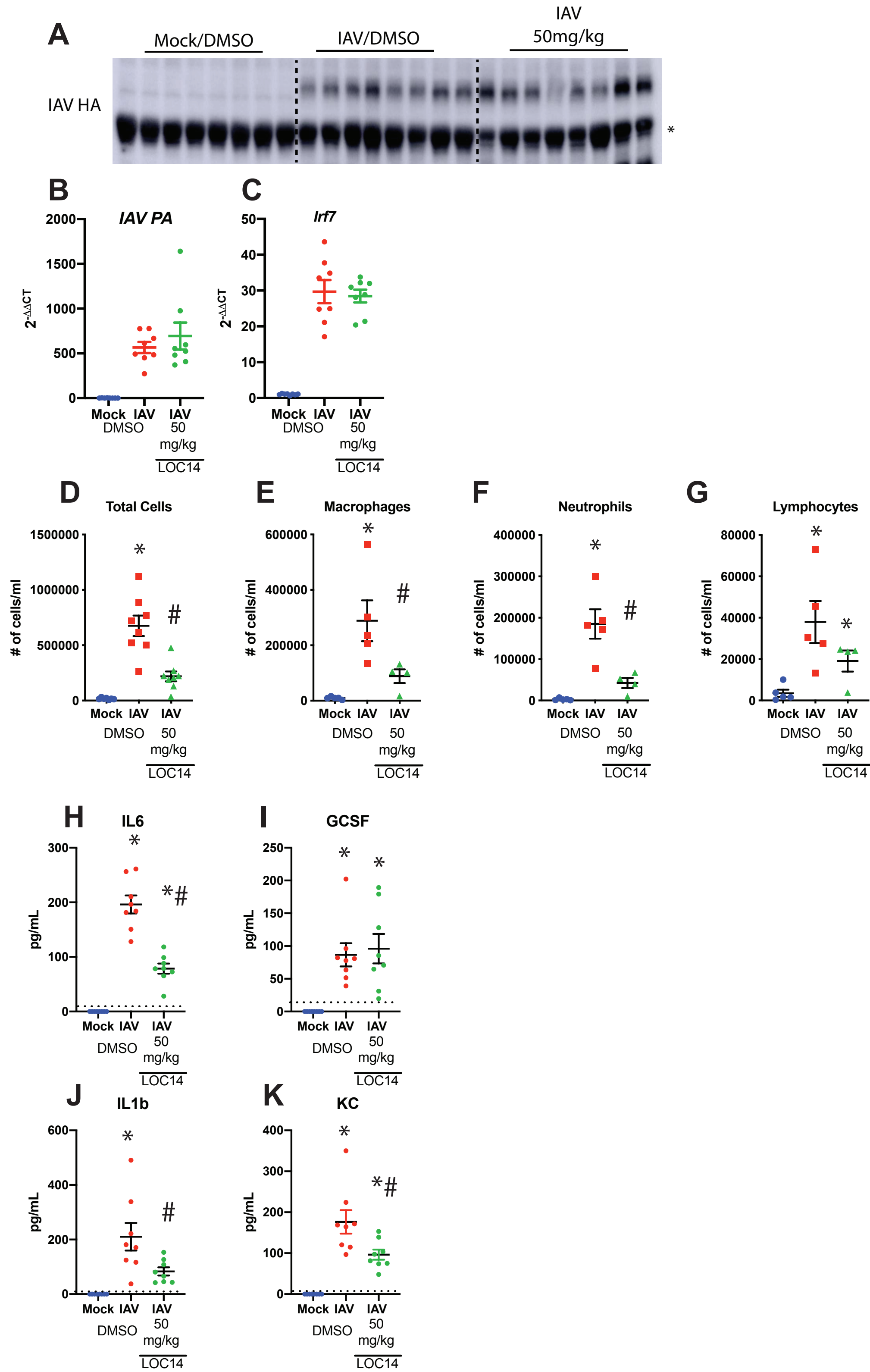
A

Compound	IC50
LOC14	4.96µM *Ref 3*
Eupatorin	66.7µM
Eupatorin-5-Methyl Ether	133µM
Isoquercetin	28.8µM

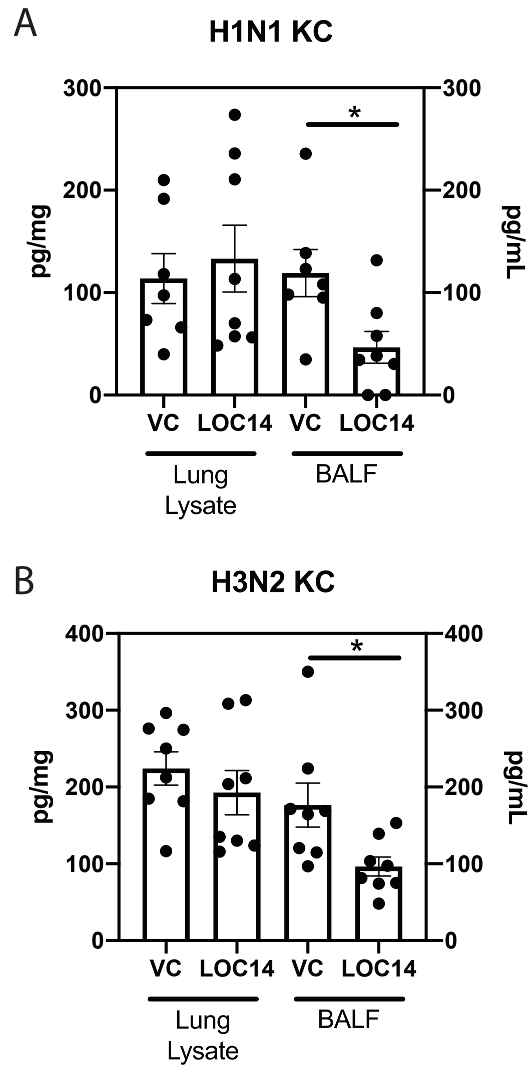
S1 TABLE: BLAST alignment of IAV neuraminidase sequences

Sequence ID	Strain	Residue (based on consensus sequence)																			
		14	**21**	**30**	**53**	**98**	**130**	**135**	**190**	**237**	**239**	**244**	**285**	**287**	**296**	**298**	**325**	**344**	**430**	**434**	**460**
C7FH46	H1N1	**C**	N	I	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**
P03468	H1N1	**C**	S	I	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**
P31349	H1N1	**C**	S	I	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**
P03469	H1N1	**C**	S	I	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**
P03482	H3N2	S	**C**	V	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**
Q1K9Q1	H2N2	S	**C**	A	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**
Q75VQ4	H3N2	S	**C**	V	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**
P03477	H8N4	S	G	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**
P03478	H6N5	S	N	T	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**
Q07574	H3N8	S	N	T	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**
P31510	H4N9	A	A	G	A	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**

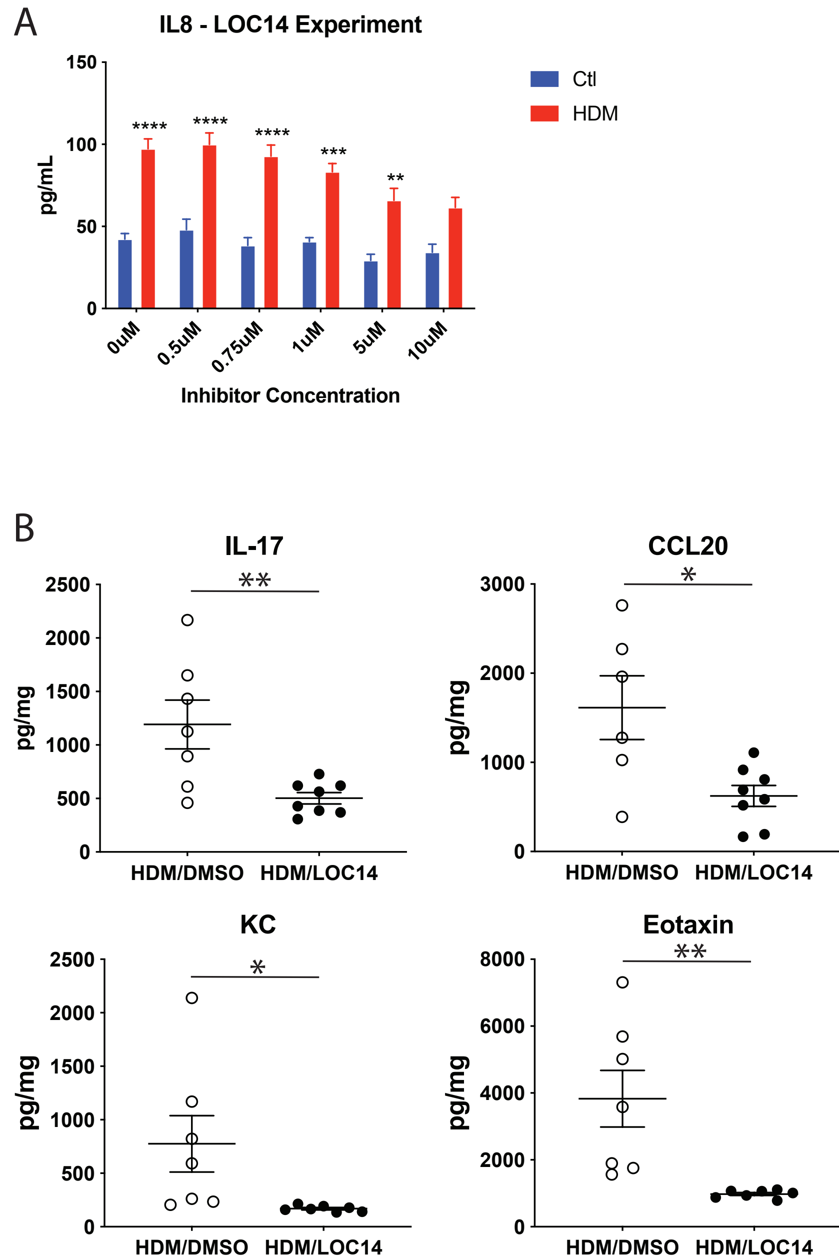
Sup Fig 2: LOC14 treatment attenuates viral induced inflammationin H3N2 infected mice



Sup Fig 3: LOC14 may have an effect on cytokine release



Sup Fig 4: LOC14 alters cytokine levels in non infectious inflammatory HDM models



Sup Table 5: Oligonucleotide primers used in this study

Primer Name	Primer Sequence (5'-3')
Polymerase acidic-FW	CGGTCCAAATTCCTGCTGA
Polymerase acidic-REV	CATTTGGGTTCCTTCCATCC
mPP1B-FW	TTTTCATCTGCACTGCCAAG
mPP1B-REV	TGCAGTTGTCCACAGTCAGC
mRP2-FW	TTGCCAGCAATTCGTGTGA
mRP2-REV	CCAGTTGACCTCTTCTGACA
mGAPDH-FW	AGGTCGGTGTGAACGGATTTG
mGAPDH-REV	TGTAGACCATGTAGTTGACCTCA
mIRF7-FW	GAAGACCCTGATCCTGGTGA
mIRF7-REV	CCAGGTCCATGAGGAAGTGT

Sup Table 6: Western blot antibodies used in this study

Antibody	Species	Company	Product Number
IAV H1 HA	Rb	Sino Biological	11684-RP01
IAV H3 HA	Rb	Sino Biological	11707-T38
IAV N1 NA	Sh	R&D	AF4858
IAV N2 NA	Rb	Sino Biological	40017-T60-50
PDIA3	Gt	LS Bio	LSB9768
GRP78	Rb	Abcam	ab21685
GRP94	Rat	Enzo Life Sciences	ADI-SPA-850
ATF6	Rb	Thermo Fisher	PA520216
B-actin	Mouse	Sigma	A5441
GAPDH	Rat	BioLegend	607902

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CHAPTER 4

DISCUSSION AND CONCLUSIONS

Overview

Viruses often have severe health and economic impacts, due to an overall ineffectiveness of conventional therapies owing to rapid viral mutation[1]. There is an urgent need to evaluate new targets for novel therapeutics that are less susceptible to these mutations. Current therapies are largely targeted towards viral proteins[2], though as obligate intracellular parasites viruses are almost entirely reliant on the host cell for replication[3]. An increased understanding of the interplay between the virus and subverted host biochemical pathways may provide valuable insight toward the development of more robust antiviral therapies.

Building off earlier studies illustrating the host protein disulfide isomerase isoform A3 (PDIA3) is directly involved in the folding of IAV proteins[4] and replication *in vitro*[5], herein we show airway epithelial PDIA3 is important in the establishment of IAV infection *in vivo*. We demonstrate overall PDI inhibition with the reversible PDI inhibitor LOC14 significantly alters disulfide bonds formation of the viral surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) resulting in diminished viral burden *in vitro*. Furthermore, we also show treatment with LOC14 in mice significantly decreases viral levels and IAV induced pathogenesis *in vivo*.

Current therapies

Despite the enormous impact of IAV there are currently only three classes of FDA approved drugs to specifically combat influenza infection; NA inhibitors, M2 inhibitors, and PA inhibitors, each one geared towards one specific influenza protein[2, 6, 7]. Both NA and M2 are found in the viral envelope on the surface of the viral envelope and are important in viral exit from the cell and deposition of the viral core during early infection respectively; while PA comprises one third of the viral RNA dependent RNA polymerase enabling viral transcription and replication in the nucleus[8, 9]. However, despite the differences between the three proteins, both in virion localization and overall function all three treatments suffer from similar limitations[2]. Owing to the comparatively short generational time of viruses and the lack of a proof-reading function on the viral polymerase the generation of drug resistant strains occurs rapidly[10]. Resistance to Amantadine, an early M2 inhibitor, has been linked to single amino acid substitutions at one of four positions in the transmembrane domain of the protein[11, 12]. M2 is a proton selective ion channel required for viral replication, acidifying the interior of the virion and facilitating conformational changes in membrane fusion proteins[9]. Strains resistant to amantadine are so prevalent M2 inhibitors are not recommended for use[2]. Resistance to NA inhibitors, while not as frequent nonetheless represents a significant concern[13]. Numerous countries stockpile oseltamivir, an oral NA inhibitor as part of response plans towards pandemic influenza and is considered a first line treatment option for patients with IAV infection[14]. Though in both seasonal influenza and during the most recent 2009 H1N1 pandemic oseltamivir resistance rapidly emerged[13]. This resistance was traced back to a single amino acid substitution close to the active site of NA[15], this H275Y

mutation alone is capable of decreasing the effectiveness of oseltamivir by over 400-fold[16]. In addition to conferring resistance to oseltamivir, this mutation has been shown to increase the IC50 of peramivir, a related intravenous NA inhibitor, *in vitro*[17]. The PA inhibitor baloxavir represents the most recent class of anti influenza drug approved by the FDA, the first in roughly 20 years[18, 19]. However, despite the novelty of the drug and its mechanism of action[20], protein variants conferring resistance to baloxavir were detected in in both its phase 2 and 3 clinical trials[21]. As in NA inhibitors, the resistance to baloxavir stems from a single amino acid substitution[21, 22]. Thus, all the currently approved classes of drugs suffer from low barriers to resistance.

One potential avenue around this limitation is the use of combination therapy, utilizing antivirals with different modes of action. A combination of the M2 inhibitor rimantadine and the broad-spectrum antiviral ribavirin has been shown to decrease the incidence of amantadine resistance *in vitro*[23]. While combination therapy involving multiple NA inhibitors found combinations of NA inhibitors dd not provide any additional benefit over monotherapies with the same agents *in vitro*[24], suggesting no greater antiviral effect than the more potent drug would be expected when combining compounds that utilize the same mechanism.

All three classes of compounds are also limited by factors independent of viral mutation[2]. As they target various points of the viral replicative cycle their effectiveness is by their very nature limited to points during infection where the virus is actively replicating. While there is some level of viral replication throughout the course of

infection, the majority of viral replication is largely limited to the first 24 to 72h following infection, typically peaking at approximately 48h and tapering off during the remainder of illness[25]. The effectiveness of antivirals is significantly diminished when administered over 48h after the onset of symptoms[26, 27], and rapid symptom onset combined with pleomorphic presentation can make acute timely diagnosis difficult[27]. Meaning later infection treatment is largely limited to symptomatic care rather than treatment of the underlying etiology, especially in the case of severe influenza infection[28, 29].

Host targeted therapies

Viruses are obligate intracellular parasites and as such are almost entirely reliant on host factors for nearly every step of their lifecycle[3]. Thus, each one of these host factors is a potential drug target. While viral proteins can mutate rapidly, in an effort to ensure core functionality is retained, mutations typically are limited to certain domains in an attempt to limit the fitness cost to the virus. Host targeted therapies are by their nature less prone to resistance than therapies targeting viral proteins, as resistance would require swift extensive mutations to the pathogen to utilize an alternative host factor to successfully replicate. Disulfide bonds are important for stability and functionality of both host and viral proteins[30-32], and as such are often conserved[33]. The HA trimer is stabilized by 18 disulfide bonds, 6 per monomer[32], each one has been experimentally shown to be required for HA functionality[34, 35]. Similarly, the mature NA tetramer contains 32 disulfides[36], again each one has been experimentally shown to be required for proper NA function[37].

PDIs are the major disulfide bond forming mechanism within the cell[38], and the relationship between IAV and PDIs has been demonstrated in the literature[4, 39, 40]. PDIs represent an intriguing target for a host-based therapy due in part to the high conservation of cysteine residues involved in disulfide bonds within influenza proteins, but also to the large size of the PDI family. The average human protein family contains 2.5 members[41], the PDI family currently contains 21, making it almost 8.5 times larger[42, 43]. While the size of the family speaks to the importance of oxidative folding in protein maturation and functionality, it also allows for an incredible amount of redundancy. While not all PDIs are catalytically active there is high conservation of the CxxC active site and extensive structural homology between members[40, 43]. Solda et al. demonstrated using PDIA3 knockout lines that other PDIs could assist in folding PDIA3 client proteins, most notably PDIA4, possibly owing the fact that it has three CxxC sites rather than two[4]. Additionally, they showed that certain proteins specifically require PDIA3 and no other PDIs examined were able to assist in folding, IAV HA is one on these PDIA3 specific proteins[4].

One major consideration in host targeted therapies is toxicity towards the host, with therapies geared towards viral targets, generally, there is not a host equivalent process meaning high doses of inhibitors can be given with minimal impact on the host. Conversely, direct modulation of host processes must be more subtle as these pathways and factors are evolutionarily conserved and required for normal host functionality. PDIA3 has been shown to be required for certain tissue development and PDIA3 knockouts are embryonic lethal[44, 45]. However, despite the importance of PDIA3 in development,

knockouts are generally very well tolerated on a cellular level[4, 46], illustrating the redundancy provided by the numerous members of this family. Additionally, the utilization of interferon therapy, especially for the treatment of hepatitis infection demonstrates the efficacy of utilizing host processes as antiviral therapies[47].

A particular advantage of host targeted therapies is the development of potential broad-spectrum antivirals. Most anti-virals are specific for certain viruses, nucleoside analogs notwithstanding. The recent Ebola and Zika outbreaks as well as the current Coronavirus pandemic clearly demonstrate that new anti-viral compounds are few and far between, despite an urgent need. A recent study determined time spent in clinical development by new anti-virals has increased dramatically despite numerous initiatives designed to speed up access[48]. The closest SARS CoV-2 vaccine is not expected until the end of 2020 or early 2021 despite unprecedented effort and collaboration[49], and even then, adequate production may prove difficult. And, as previously mentioned, baloxivir is the first new class of approved anti influenza compounds approved in nearly 20 years[19]. We have demonstrated PDI inhibition significantly impacts influenza pathogenesis and alters viral protein maturation[39]. Influenza is not the only virus that utilizes disulfide bonds to stabilize its proteins. Hepatitis C (HCV) virus also uses disulfides in its envelope protein, and numerous PDIs, including PDIA3 are involved in the HCV life cycle[35, 50]. A group has shown the abscisic acid-based compound origamicin can modulate PDI activity and blocks HCV protein folding[50]. While LOC14 has not been tested against HCV, given its effects on PDIA1 and A3 it is not unreasonable to assume that it would also alter HCV protein folding, though experimentation is needed to determine with any

certainty. Nonetheless, two very different viruses infecting two very different tissues are impacted by the same mechanism[39, 50-52]. The spike protein of SARS CoV-2 as well as SARS-CoV and MERS-CoV, the two other coronaviruses with significant pandemic potential, are also stabilized by disulfide bonds[40, 53]. Large amounts of unpaired cysteines in the spike protein of SARS-CoV are believed to assist in binding with the ACE2 receptor, the authors speculate the unpaired cysteines may in fact play a role in SARS-CoV crossing host barriers[54]. In addition to adding disulfides to protein PDIs are also capable of removing unwanted extraneous disulfides from proteins as well through reductive unfolding of the nascent polypeptide[55]. While the exact nature of PDIs in the maturation of this protein are not currently known, PDI inhibition warrants further investigation as an anti-coronavirus strategy, especially in the context of the current pandemic.

While targeting host processes subverted by viruses are less prone to resistance, it is still possible for the virus to mutate around these restrictions. HIV envelope glycoprotein contains 10 conserved disulfide bonds, only five of which are responsible for folding, and eight for proper functionality[34]. Demonstrating the possibility that certain viral proteins may have remarkable tolerance to the manipulation of its tertiary structure. Further work by the same group demonstrated one particular disulfide bond required for folding, C385/C418, could be replaced by the acquisition of local features within the beta barrel, primarily the condensation of amino acid sidechains to increase local interactions[56]. It should be noted, however, that HIV is the prototypical fast evolving virus, and is known for its high genetic variability. HIV produces every possible single base substitution within a patient multiple times every day[57]. While host targeted therapies have a higher barrier

to resistance than viral targeted therapies, given short generation times and error prone replication mechanisms[10], these barriers can be overcome.

Upon infecting the cell influenza is sensed through intracellular pattern recognition receptors RIG-I, MDA5, or various Toll-like receptors (TLRs)[51], upon stimulation these sensors trigger the activation of the NF-kB and interferon pathways activating cellular antiviral responses[58]. These pathways lead to the expression of pro-inflammatory cytokines like IL-1, IL-6, and TNF-a, and chemokines to cause rapid influx of inflammatory cells to the lung[59]. While a robust inflammatory response in necessary for control of the virus it can cause unwanted damage to underlying tissues, leading to decreases in lung function[60, 61]. Normally these responses are self-limiting over the course of infection, though rarely during the infection with seasonal influenza strains and more commonly with pandemic and highly pathogenic strains these responses can lead to a positive feedback loop resulting in aberrant immune activation and systemic cytokine signaling resulting in severe tissue damage and inflammation, termed cytokine storm[62]. The exact causes of cytokine storm are not currently well understood[62]. Current therapies are not considered directly immunomodulatory, though by diminishing viral levels they can result in decreased immune responses, they are not given to directly lower inflammation[2]. Even in the absence of cytokine storm lung damage from the host inflammatory response is a significant cause of mortality[51]. For this reason, compounds directed toward excessive proinflammatory host immune responses have been proposed for use in conjunction with antiviral therapies.

Statins are low cost, potent anti-inflammatory compounds though combination therapies involving them has yielded mixed results[63]. One study showed a combination simvastatin and oseltamivir did not improve efficacy over oseltamivir treatment alone in mice infected with lethal doses of avian H5N1[64]. Whereas a separate study investigating a triple cocktail of oseltamivir, fenofibrate, and simvastatin showed improvement over oseltamivir monotherapy[65]. Interferon therapy has been classically used to treat viral infections, especially HCV[47], as well as other conditions such as cancer and autoimmune disorders, and results in potent immune suppression[66]. However, interferon therapy is commonly associated with severe and varied side effects[66, 67]. Glucocorticoids are commonly used concurrently with conventional antiviral therapies to treat acute respiratory distress syndrome (ARDS) and influenza associated pneumonia[68]. They act through the glucocorticoid receptor and ultimately lead to the down regulation of proinflammatory genes while simultaneously upregulating anti-inflammatory genes. Though their use has been associated with increased duration of viral replication in certain patients. And observational studies during the 2009 H1N1 pandemic found systemic glucocorticoid treatment was associated with increased secondary infections and higher mortality in intensive care unit patients[69]. While the control of aberrant or excessive immune responses represents a valuable tool in curbing morbidity and mortality associated with influenza infection care must be taken to avoid compromising the body’s ability to adequately control viral replication. There are other potential complications to consider with combination therapies such as the route of administration, availability of the drug, complexity of the dosing regimen, and potential drug interactions.

We show treatment with the reversible PDI inhibitor LOC14 has direct anti-viral effects, altering the maturation of viral proteins by impacting oxidative folding[39]. We also demonstrate LOC14 treatment has a separate anti-inflammatory effect, though the exact mechanism needs to be more thoroughly investigated. Previous work in the lab has shown siRNA mediated knockdown of PDIA3 in C10 lung epithelial cells altered oxidative folding of eotaxin[5], a chemokine important in the selective recruitment of eosinophils to inflammatory sites[70]. Interestingly, we show discrepancies between cytokine levels of certain proinflammatory markers in the BAL fluid and the lung homogenates of IAV infected mice following PDI inhibition. Levels of KC, CCL20, and IL-6 that are significantly decreased in the BAL remain elevated in the lung tissue, suggesting a possible decrease in the release of these cytokines and chemokines. Generally, proteins that do not properly fold aggregate together and are retained within the cell and do not proceed normally through the secretary pathway[38]. While further experimentation is needed to determine the nature of this retention within the cell, the previously demonstrated role of PDIA3 in eotaxin maturation[5] and the effect of LOC14 suggest defects in oxidative folding are likely a contributing factor. That we do not observe this effect in other cytokines may also demonstrate a role in other PDIs in their maturation, though again further experimentation with siRNA knockdowns of additional PDIs or additional PDI inhibitors more specifically targeted towards other members of the PDI family.

Coagulation cascade

Coagulation is highly ordered intricate process involving endothelial cells, blood platelets, and various clotting factors[71]. While there are numerous pathways, the

mechanism generally involves the activation and aggregation of platelets and the accumulation and maturation of fibrin, resulting in a clot[71, 72]. Anticoagulants control the coagulation process and help to limit thrombus propagation[73]. Viral infections are commonly associated with coagulation disorders and they can affect the coagulation pathways in a variety of ways[74]. Experiments in C57BL/6 mice have demonstrated IAV infection activates coagulation through the increased generation of thrombin and fibrin deposition[75]. The H1N1 2009 pdm09 strain has been reported to cause both hemorrhagic and thrombotic complications[76], while both H7N9 and H5N1 strains have been found to cause coagulopathy[77, 78]. Influenza induced overactivation of coagulation is linked to an increased risk of various cardiac and pulmonary diseases like pulmonary embolism and myocardial infarction[79, 80]. And oseltamivir treatment has demonstrated decreases in viral loads are associated with decreases in the incidence of cardiac complications[81]. Influenza infection can lead to the depletion of anticoagulant factors by the induction of a pro-thrombotic state[82]. This exhaustion of anticoagulant elements can in turn lead to abnormal coagulation and hemorrhage which is commonly seen in patients with severe IAV infection and cytokine storm[83]. PDIs, primarily PDIA1[84] and A3[85], play critical roles in the early activation of the coagulation cascade. Given their roles in early activation much attention is being given to PDI inhibitors in an attempt to determine if they can help abate aberrant thrombosis. One study has shown PACMA31 treatment inhibits both platelet deposition and fibrin formation in a mouse model of stenosis in the inferior vena cava[86]. Others have shown 16F16 blocks tissue factor activation, though these results are somewhat inconsistent[87]. The effect of LOC14 on

coagulation has not been explored, but given its demonstrated effects on viral pathogenesis, it would be interesting to explore.

Secondary bacterial infections

Secondary bacterial infections commonly associated with influenza infections, most notably with pandemic influenza strains[88]. *Haemophilus influenzae* was so frequently found in patient samples during the 1918 H1N1 pandemic it was originally believed to be the causative agent until the early 1930s[89]. While the exact causes of secondary bacterial infection are not completely understood it is currently thought a combination of viral damage to the lung epithelium providing the bacteria with an enhanced niche and a shift of the immune system towards antiviral activity rather than antibacterial activity may play a role[88]. Up to 75% of those that acquire influenza associated pneumonia are confirmed to have bacterial coinfection[90]. During the recent 2009 H1N1 pandemic pneumonia as a result of secondary infection was found in almost 50% of mortalities[91]. Not surprisingly immunosuppression is associated with increased susceptibility to secondary bacterial infection[92]. Secondary infection is typically treated with antibiotics in conjunction with conventional antivirals[93]. However, with antibiotic resistance becoming increasingly prevalent and limitations in the current dosing of anti-influenza drugs novel treatments may be required. Disulfide bonds are important in both prokaryotic and eukaryotic proteins, and bacteria, like eukaryotes have evolved a system to aid in their formation[94]. Bacteria utilize the Dsb family of proteins to catalyze disulfide bond formation, this family has four members, DsbA, B, C and D which behave much like the PDI family of proteins[94]. In fact, like PDIs, these proteins contain

thioredoxin like domains and utilize the same CxxC active site architecture[43, 94]. The conservation of the active site suggests that PDI inhibitors like LOC14 may also inhibit these proteins, though further experimentation is needed. There are currently small molecule inhibitors of Dsb proteins available, most notably for DsbB, which plays a role akin to Ero1 in eukaryotes and acts a primarily as a recycling enzyme for DsbA, though DsbB has no direct homologs[95]. These compounds decrease virulence, and some are being explored as potential antibiotics[95, 96], though none have been tested in the context of bacterial infection secondary to influenza.

Potential pitfalls and considerations

While we have established PDIA3 plays an important role in IAV infection further experimentation is required to elucidate its exact role in pathogenesis. Though PDIA3 is known to assist in the oxidative folding of viral proteins, mass spectrometry experiments would help determine exactly which disulfides are catalyzed specifically by PDIA3. This could provide valuable insight on HA/NA maturation and glycoprotein folding in general. Moreover, further characterization of the virus itself by examination of viral titers combined with detailed structural analysis by electron microscopy under PDIA3 deficient conditions could shine additional light on the role of PDIA3 in the viral lifecycle. Along the same line histopathologic analysis of infected tissues and pulmonary function analysis following PDI inhibition would be useful in evaluating PDIA3 as a pharmacological target. At present, no specific PDIA3 inhibitors are available, though there are several plant flavonoid compounds have been identified as potential inhibitor candidates that are more targeted towards PDIA3 as opposed to other members of the PDI family[97]. The use of

these compounds as well as other newly identified PDI inhibitors would prove useful in determining PDIA3's role, as well as the role of other PDIs in IAV infection.

We deliver LOC14 systemically via intraperitoneal injection, though the evaluation of a more targeted delivery method, such as intratracheal injection or oropharyngeal delivery may allow the same antiviral effects at a lower dosage. Similarly, pharmacokinetic studies would provide insight on the distribution, absorption, and metabolism of LOC14, which could lead to the refinement of dosing regimens and concentrations. Further studies on the interaction of LOC14 with PDIA3 specifically, along with other PDIs may also help to improve these regimens.

Additionally, we have focused solely on the oxidoreductase activity of PDIA3; however, it is a multifaceted protein that plays numerous roles within the cell[40, 98]. It has been demonstrated to be a critical part of the peptide loading complex (PLC) of MHC class I, which is an important part of the anti-viral immune response[99]. While it has been shown that the redox activity of PDIA3 is dispensable for its role in the PLC it is not known what effect PDI inhibition will have on MHC class I signaling[98]. However stable knockout PDIA3 cells do not show defects in MHC class I activity[46]. Similarly, PDIA3 can act as a receptor for vitamin D3, acts in cell migration, as well as apoptosis[98]; any one of which may play an important role in active viral infection beyond oxidative protein folding.

Disulfide bonds are utilized by many viruses to impart stability in their proteins, particularly glycoproteins. Various respiratory syncytial virus (RSV)[100], coronaviruses (CoV)[40], HIV[34], and HCV[35] proteins contain disulfide bonds, though their reliance on host PDIs in their formation is not currently known in all cases. HIV is known to require PDIA1 activity to enter cells[101]shuffling disulfide bonds of envelope proteins to facilitate viral entry. As mentioned above, PDI inhibition with origamicin can block HCV protein folding[50]. And PDIA3 inhibition through nitazoxanide, a thiazolide, has been shown to block F-protein folding[102]. Intriguingly, nitazoxanide has also demonstrated antiviral activity against HCV, rotavirus, and IAV[102]. Additional experimentation could determine if the utilization of host PDIs is a common strategy employed by other viruses and thus a promising target for future broad spectrum anti-virals. However even if host PDI utilization is limited to a few select viruses, PDI inhibition still warrants further investigation for its anti-inflammatory and potential immunomodulatory effects which may have a beneficial role in viral treatment.

Another important consideration is our model usage, most of the work presented is in a mouse model of influenza infection or in isolated primary mouse epithelial cells. While mice are common models of human diseases and conditions, they can often respond to stimuli drastically differently than do humans[103]. Mouse hemoglobin has a lower affinity for oxygen than does human hemoglobin, supporting the higher rodent metabolism[104]. Action potentials in neurons are known to be shorter in mice than humans[104]. There are also differences in the immune system of the two organisms[105]. The PDIA3 gene is located on chromosome 2 in the mouse while it is present on chromosome 15 in humans, this can lead to differences in regulatory sequences and overall

gene expression which can have profound effects on the protein itself. While utilizing isolated human primary cells is a potential solution, they too are not without issues as typical cell culture techniques can also lead to differences in gene expression from that of the original organism. This may be avoided by the use of human organoids, self-organizing tissues derived from isolated stem cells[106]. These organoids develop more or less as normal and recapitulate typical organ architecture[106]. Focusing specifically on the lung, numerous techniques exist to allow for the differentiation of airway-like structures containing different cell types[107]. And these cultures have been already utilized to monitor influenza infection *in vitro*[108].

A final consideration is the nature of drug discovery and development itself, which fails far more often than it succeeds. Briefly, the development process proceeds from basic research, to preclinical development, to actual clinical trials, and finally FDA approval; though the actual process is much longer, difficult, and complex[109]. The average time for the development of a novel antiviral is roughly 6 years[109], though as we are utilizing a host process as a target rather than a unique viral protein this time frame would undoubtably be increased due to the associated complexities Our work is firmly in the basic research phase of the process, as there is still much we do not know about how LOC14 behaves in mice, much less humans.

Conclusion

PDIs play important roles in influenza infection, from direct utilization by the virus to the host response to infection. And their inhibition represents a novel target for the potential development of future antivirals at a time when we are woefully underprepared

to manage the emergence of new zoonotic viruses or novel pandemic strains of influenza. As a host targeted therapy PDI inhibition with small molecule inhibitors set up a high genetic barrier to resistance potentially circumventing rapid viral mutation. Moreover, the use of various PDI inhibitors in numerous animal models of disease illustrates that they are generally well tolerated[110-112], possibly due to the redundancy built into the PDI family. Likewise, by targeting a system utilized by both the host and virus we have the potential to generate a broad-spectrum antiviral compound that mediates its effect on different fronts. However, further investigation and analysis is needed to determine proper targeting and dosing to avoid potential cytotoxicity or off target effects, potentially worsening the infection. Despite these limitations and concerns PDI inhibition with LOC14 represents an intriguing platform for the development of potential future anti-virals.

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