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Inducible Epithelial Resistance Against Acute Viral Pneumonia And Chronic Asthma

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INDUCIBLE EPITHELIAL RESISTANCE AGAINST ACUTE VIRAL PNEUMONIA AND CHRONIC ASTHMA

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A

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Abstract

Inducible epithelial resistance against acute viral pneumonia and chronic asthma

By Shradha Wali, M.S.

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Viral pneumonia remains a global health threat despite worldwide vaccination and therapeutic programs. The influenza pandemic of 1918 and SARS-CoV2 pandemic of 2019-2020 are cautionary reminders demanding the need for novel treatment strategies. Moreover, in addition to causing acute disease, respiratory virus infections are often complicated by chronic lung pathologies, such as asthma induction, progression, and exacerbation. We have reported that mice treated with a combination of inhaled Toll-like receptor (TLR) 2/6 and TLR 9 agonists (Pam2-ODN) to stimulate innate immunity are broadly protected against respiratory pathogens, but the mechanisms underlying this protection remain incompletely elucidated. Here, we show in a lethal paramyxovirus model that Pam2-ODN-enhanced survival is associated with robust virus inactivation by reactive oxygen species (ROS), which occurs prior to internalization by lung epithelial cells. We also found that mortality in sham-treated mice temporally corresponded with $CD8^+$ T cell-enriched lung inflammation that peaks on days 11-12 after viral challenge, when the viral burden has waned to a scarcely detectable level. Pam2-ODN treatment blocked this injurious inflammation by reducing the viral burden. Alternatively, depleting $CD8^+$ T cells 8 days after viral challenge also decreased mortality. Notably, Pam2-ODN

treatment of mice lacking $CD8^+$ T cells at any point of SeV infection showed a similar degree of protection demonstrating Pam2-ODN mediated protection independent of $CD8^+$ T cell response. Further, Pam2-ODN treatment protected SeV challenged mice from progressing to asthma-like disease by initiating acute anti-viral effects. Interestingly, mice aerosolized with Pam2-ODN after viral clearance displayed reduced eosinophilia that was associated with reduced lung epithelial IL-33 production. Findings from this study reveal opportunities for targeted immunomodulation to protect susceptible individuals from mortality of respiratory virus infections and preventing progression to chronic asthma.

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Abbreviations

TLR	Toll-Like receptor
BAL	Bronchoalveolar lavage
ROS	Reactive oxygen species
IL	Interleukin
NK	Natural Killer
iNKT	Invariant natural killer T cells
TFH	T follicular helper
RSV	Respiratory syncytial virus
RV	Rhinovirus
SeV	Sendai
PRR	Pattern recognition receptor
TJ	Tight junction
AJ	Adherens junction
IFN	Interferon
IRF	Interferon regulatory factor
ISG	Interferon stimulated genes
NOX	NADPH Oxidase
Duox	Dual oxidase
mTOR	mammalian target of rapamycin
AMPK	activation of AMP-activated kinase

TNF	Tumor necrosis factor
GM-CSF	Granulocyte macrophage-colony stimulating factor
TSLP	Thymic stromal lymphopoietin
TGF	Transforming growth factor
CCL	chemokine (C-C motif) ligand
MCP	Monocyte chemoattractant protein
NET	neutrophil extracellular traps
Rag	recombination-activating genes
BCG	Bacillus Calmette-Guerin
MyD	Myeloid differentiation
ODN	Oligodeoxynucleotide
FBS	Fetal Bovine Serum
MLE	Mouse lung epithelial
mTEC	Mouse tracheal epithelial cells
LOQ	limit of quantification
COPD	Chronic obstructive pulmonary disease
NF-κB	Nuclear factor kB
NLR	NOD-like receptor
RIG-I	retinoic acid-inducible gene I
IRAK	interleukin-1 receptor-associated kinase
IRF	interferon (IFN)-regulatory factor
TRIF	TIR-domain-containing adapter-inducing interferon-β
TRAF	TNF receptor-associated factor

Chapter I: Introduction

1.1 Global burden of respiratory viral infections

Viral pneumonias remain a global health threat with seasonal influenza and emerging pandemic viruses such as SARS-CoV-2 inflicting morbidity and mortality in vulnerable subjects [1-3]. In addition to causing acute disease, respiratory virus infections are often complicated by chronic lung pathologies such as asthma initiation, progression, and exacerbation [4-8]. Therefore, novel therapeutic anti-viral strategies need to be developed to effectively prevent and treat respiratory virus infections and their associated chronic lung diseases.

Nearly 80% of asthma exacerbations are associated with respiratory viral infections [4-6, 8] but the mechanisms that link asthma exacerbations and virus infections remain incompletely understood. In this regard, Michael Holtzman's group found that in mice, infection with Sendai virus (SeV), a parainfluenza virus, causes long-term effects after viral clearance based on a "hit and run" strategy [6, 8-10]. Specifically, initial SeV infection (with viral replication peaking at 5 days and clearance from lungs by 14 days) is linked to changes in expression of epithelial immune response genes, inflammation, and subsequent acute hyperreactivity [8, 10]. Additionally, this transient SeV infection triggers potentially lifelong asthma-like disease characterized by goblet cell metaplasia, eosinophils, and airway hyperreactivity [6, 8, 9].

Therefore, controlling viral infections and associated disease severity could be an appealing approach to prevent virus-induced asthma-like disease and

exacerbations. However, none of the current strategies to treat asthma, such as corticosteroids and β2-adrenoceptor agonists, target virus-induced asthma exacerbations [11-13]. Additionally, despite multiple global vaccination programs to improve public health, these respiratory illnesses continue to significantly increase health care burden across the world [14-18]. Therefore, to inform future therapeutics for effective anti-viral programs, we must first note the challenges currently faced in designing anti-viral vaccination strategies.

1.2 Current challenges in anti-viral vaccine design

Currently licensed influenza vaccines focus on antibody generation specific to viral membrane proteins that mediate viral entry into host cells [17, 19]. However, due to antigenic drift resulting in mutated viral membrane proteins, strain-specific antibody generation is required, necessitating evaluations and reformulations 6-8 months a year to keep pace with antigenic drifts [17, 19]. Further, since systemic immunity is poor in the elderly population, current vaccines tend to be less efficacious in this group. This can be countered by using adjuvants or increasing the antigen dose, however, vaccine studies or systems biology approaches to improve immunogenicity in the elderly are lacking [16, 17]. Further, when animal-origin viruses acquire the ability to infect humans resulting in an antigenic shift, there ensues a lack of immunity against the novel virus [15, 17, 20]. The inability to predict the virus strains or subtypes that will result in future pandemics and the consequent delay in vaccine generation has generated great interest in a “universal influenza vaccine” that can produce broad, cross-reactive immunity, reducing the need for

annual vaccine evaluations [16, 20]. In this regard, recent advances have been made to understand how host innate immune cells can be induced to generate long-lasting and heterologous immunity, which will be discussed in the next section.

1.3 Trained innate immunity

For a long time, innate immunity was known to be non-specific and lacking memory, in contrast to adaptive immunity which is highly specific and long-lasting. However, invertebrate animals that lack adaptive immunity, display a prolonged functional state of their innate immunity after sufficient priming. Over the last few years, Mihai Netea's group has studied a similar phenomenon in detail, terming it 'trained innate immunity'. Studies on trained immunity using BCG, polio vaccine, and measles virus, among other immune stimuli, have shown generation of long-term protection against bacterial and viral challenges through epigenetic, metabolic, and functional changes in innate immune cells [21-23]. Perhaps an important functional change is the induction of memory in innate immune cells such as macrophages, monocytes, and NK cells that protect against secondary infections [21, 24, 25]. This protection is shown to persist even in the absence of adaptive immune compartments [21]. Training of macrophages and monocytes with BCG and β-Glucan causes metabolic shifts in these cells from oxidative phosphorylation to aerobic glycolysis that allow a faster supply of energy and metabolites after a secondary challenge. Further, trained macrophages undergo epigenetic changes that result in quick access to transcription factors, controlling the production of

proinflammatory genes, upon secondary infections [23, 26]. Therefore, epigenetic priming may be an important mechanism explaining the underlying characteristics of protective immune memory. One such epigenetic priming mechanism is the activation of gene transcription upon initial stimuli, which may be accompanied by specific chromatin marks acquisition such as H3K4me1, H3K4me2, H3K4me3 in macrophages [22, 27]. These chromatin marks appear to persist at least partially when the stimulus is removed [27]. This enhanced epigenetic status in the innate immune cells tagged by the chromatin marks such as H3K4me1 results in a robust response upon a secondary stimulus or challenge [27].

NK cells have shown both trained innate memory characteristics and classical immunological memory that is mediated by T and B cells [22, 28]. Studies in mice showed hapten-induced contact hypersensitivity was dependent on NK cells that were shown to be persisting for at least 4 weeks [29]. Additional studies using murine cytomegalovirus (MCMV) infection in mice showed evidence of immunological memory independent of T and B cells [29, 30]. This memory was mediated by NK cells that upon reinfection, produce IFN-γ and perforins, thus inducing a protective response [30, 31]. This NK cell memory is at least partially driven by epigenetic changes. One study with human CMV showed epigenetic priming of the IFNG gene locus in NK cells, which acquires specific chromatin marks [28, 29]. Upon reinfection, these NK cells then show greater IFN-γ generation thus leading to protective immune response [28, 29].

While modulation of trained immunity is generally shown to induce broad and long-term memory in animal studies, functional changes must be induced in the hematopoietic stem and precursor cells as these cells give rise to multiple generations of memory-consisting innate immune cells [21-24, 27]. Given the difficulties of effective vaccine design, the modulation of trained immunity can offer promising new treatments in the context of emerging new respiratory viruses such as SARS-CoV2.

This thesis project is focused on understanding how pharmacologic stimulation of the lung mucosal defenses can attenuate acute viral pneumonia and chronic asthma. Therefore, it is important to understand the lungs' mucosal immune response to viruses and anti-viral defense mechanisms, providing insights into the design of future therapeutics.

1.4 Pathogenesis of respiratory virus infections

This thesis project is mostly focusing on the mouse model of parainfluenza virus, Sendai (SeV) infection, but I also use influenza virus (H3N2 strain) in some experiments. Both influenza and parainfluenza infections are common infections found in children and adults leading to significant morbidity and mortality. To date, SeV infection in mice appears to resemble the response to human pathogens such as influenza, parainfluenza, and RSV and human type 2 immune response seen in asthma [8]. Since RSV replication in mice is difficult, the SeV mouse model offers an alternative model to study virus-induced pathogenesis that appears to resemble

human disease. I will briefly explain the pathogenesis of respiratory virus infection in this section. Influenza, SeV, or SARS-CoV-2 target the lung epithelium for infection by binding to the viral receptors on the lung epithelial cells [32-34]. The presence of viral RNA in the cells leads to the activation of intracellular signaling pathways, initiating an acute innate immune response via TLR signaling, RIG-I activation, or inflammasomes [35]. These pathways culminate in the activation of IRF3 or 7, which activates IFN-α/β transcription for viral clearance [35-37]. Activation of type I IFNs also leads to proinflammatory cytokine and chemokine production via NF-κB activation [35, 36]. Further, viral RNA in the cytoplasm can induce a conformational change in RIG-I that interacts with MAVS protein, which in turn activates IRF3 and NK-κB to produce type I IFNs and proinflammatory cytokines, respectively [38]. In addition, inflammasome proteins are induced via NF-κB activation [38]. In the presence of viral RNA, the NLRP3 inflammasome pathway gets activated which cleaves and activates caspase-1 producing IL-1β and IL-18, thereby augmenting the inflammation cascade [38]. Further, infection of lung epithelial cells leads to increase in damage-associated molecular patterns (DAMPs) that are recognized by dendritic cells, that migrating to the draining lymph nodes to activate cytotoxic $CD8^+$ T cells and $CD4^+$ T helper cells and memory $CD8^+$ T cells [38]. The chemokines and cytokines produced by NK-κB activation lead to the recruitment of neutrophils and differentiation of monocytes from peripheral blood to monocyte-derived alveolar macrophages [38]. Both neutrophils and macrophages amplify the inflammation during the on-going viral infection.

In the case of the novel SARS-CoV2 infection in humans, the virus uses the ACE-2 receptor on the lung epithelium for viral entry [32]. Upon transmission, symptoms appear to be minimal, with viral shedding restricted to the upper respiratory tract [32, 39]. However, the second week onward, viral replication in the lower respiratory tract and secondary viremia is seen with an attack on other organs and cells that express the ACE-2 receptor such as the kidney, heart, and gastrointestinal tract [32, 39]. This process of viral shedding correlates with clinical deterioration following disease onset in the second week. It appears that disease exacerbation in the late stage is not only due to direct viral damage but also a result of immune-mediated lung injury [39]. The progression to COVID-19 severe disease in humans is associated with reduced lymphocyte counts and increase neutrophils in the blood [32, 39]. This is also correlated with increased IL-6, MCP-1, MIP1A, and TNF-α in the blood plasma [32, 39]. Reduced lymphocyte counts and inflammatory cytokines appear to be associated with mortality of COVID-19 patients [32, 39]. However, the data regarding immune response and pathogenesis of SARS-CoV2 are evolving and several reports indicate different patterns of clinical manifestations depending on age, underlying health, and environmental conditions associated with severe disease induced by SARS-CoV2.

1.5 Host immune response to respiratory virus infections

Alveolar Macrophages: Residing within the alveoli airspaces are macrophages ideally located to respond to infectious agents in the respiratory tract. Phenotypically, alveolar macrophages are classified as classically activated (M1)

pro-inflammatory type producing high levels of TNF-α, IL-1β, and iNOS in response to virus infections, and alternately activated (M2) anti-inflammatory type, predominantly involved in tissue repair and maintaining homeostasis by TGF-β production [40]. Under homeostatic conditions, macrophage interactions with epithelial cells help to keep the alveolar macrophages in a suppressive, anti-inflammatory state [41, 42]. However, virus infection of epithelial cells and the subsequent death of infected epithelium leads to enhanced phagocytic activity in macrophages [41]. In addition to phagocytosis, macrophages release pro-inflammatory cytokines and chemokines such as CCL5, TNF-α, MCP-1, and IL-6, which in turn leads to additional immune cell recruitment to the lungs, ultimately resulting in viral clearance [41-43]. Macrophages can also act as a sink by absorbing the virus and preventing its spread to nearby cells [44]. The precise role of alveolar macrophages in viral infections is shown in depletion studies using clodronate-loaded liposomes that selectively induce macrophage death [42, 45]. Other studies have made use of GM-CSF$^{-/-}$ mice or administration of diphtheria toxin to CD169-diphtheria toxin receptor mice to study macrophage function in viral infections [41, 46, 47]. Mice depleted of macrophages by any of these methods have shown similar results with increased viral burden, enhanced inflammation, and disease severity, implicating their role in controlling viral infections [40, 43]. Overall, numerous studies have shown alveolar macrophages to be necessary and protective in function against viral infections.

Neutrophils: Another group of innate immune cells that respond quickly to an infection or injury are neutrophils. They arrive from the blood circulation to the lungs

by the action of proinflammatory cytokines such as IL-1β, IL-6, and TNF-α and chemokines such as CXCL1, CXCL2, and CXCL8 [48]. Neutrophils actively phagocytose virus particles and other dead cells containing virus particles [49]. Upon phagocytosis, neutrophils use a variety of enzymes, ROS, and anti-microbial peptides to kill pathogens [48, 49]. Another mechanism used by neutrophils is the release of neutrophil extracellular traps (NETs), after undergoing a form of programmed cell death. These NETs consist of proteases, chromatin, histones, and other anti-microbial peptides that inactivate virus particles, hence preventing the dissemination of viruses [48]. Several studies have shown that the recruitment of neutrophils and their activities are essential for controlling respiratory virus infections [48, 49]. However, there are also reports indicating excessive activity of neutrophils releasing ROS, enzymes, and myeloperoxidase that can injure lung epithelium during viral infections, leading to uncontrolled inflammation and host damage [50]. In addition to performing phagocytosis, neutrophils secrete chemokines and cytokines that can in turn not only recruit more neutrophils but a host of other immune cells to the injury site [48, 50]. It is therefore essential to carefully dissect the aspects of neutrophil functions to generate a well-balanced anti-viral therapy.

Natural Killer (NK) cells: NK cells are generally recruited between the time of neutrophil and T cell infiltration following a virus infection [51]. NK cells can differentiate between normal cells and virus-infected cells by up/downregulation of their unique receptors (inhibitory and activating) [52]. They are cytotoxic in function, killing virus-infected lung epithelial cells by secretion of IFN-γ and Granzyme B [51, 53]. Also, NK cells engage in a unique antibody-dependent cell-mediated

cytotoxicity (ADCC), in which antibodies bind to viral particles on infected cell surfaces, marking them for clearance via cell death [40, 41, 43, 51]. Most NK cell depletion studies are associated with an increased viral burden in mouse lungs and enhanced disease severity, implicating their role in controlling virus infection [51]. However, NK cells producing IFN-γ have also been shown to be injurious to lung epithelium during RSV infection [54]. Further, several influenza virus strains are shown to evade NK cell-mediated killing by activating the inhibitory receptor signaling that prevents the infected cells from being marked for death [55]. Hence, optimal NK cell activity seems to be dependent on virus strains for effective viral clearance from lungs or lung pathology [51].

Invariant Natural Killer T (iNKT) cells: Representing a diverse group of T cells that have the capacities of both NK and T cells is iNKT cells, which express a unique CD1d receptor on their cell surface that recognizes self-lipids during respiratory viral infections [36, 45, 53]. iNKT cells can perform effector responses by cytotoxic activities and secretion of cytokines. iNKT cells have been shown to boost early immune responses, reducing viral titers, and also controlling inflammatory monocytes in a highly pathogenic influenza mouse model [40, 53, 56]. CD1d deficient mice infected with influenza also showed that iNKT cell led IFN-γ production was necessary for optimal NK cell and $CD8^+$ T cell activity [56]. Overall, iNKT cells appear to be mostly beneficial in controlling acute respiratory virus infection.

Lymphocytes

B and CD4$^+$ T cells: The primary function of B cells in virus infections is to produce virus-specific antibodies that can neutralize, inactivate, and opsonize viral particles and also initiate other adaptive immune mechanisms that ultimately lead to viral clearance [35, 40]. The presence of neutralizing antibodies to rhinovirus and influenza in the serum has been correlated with protection from disease severity in patients [15, 16, 19, 20, 57]. Neutralizing antibodies prevent infection of respiratory epithelium by blocking viral surface proteins from attaching to the host cell receptors, and binding to infected cells marking them for death by ADCC [35, 58]. Another anti-viral mechanism of antibodies is to tag the virus for inactivation by the complement system and phagocytosis by macrophages and/or neutrophils (antibody-dependent cellular phagocytosis; ADCP) [35]. For an effective B cell response during viral infection, CD4$^+$ follicular T helper (Tfh) cells are required as they help in maturation and proliferation of neutralizing antibody-producing B cells in secondary lymphoid organs. The presence of Tfh cells after vaccination in young adults is correlated with the production of influenza-specific IgG and IgM antibodies [15, 17, 20, 57]. While B cells have proven to be largely useful for generating anti-viral immunity, the partial cytotoxicity required for viral clearance results in untoward consequences to the physiology of the epithelium.

CD8$^+$ T cells: Numerous studies on CD8$^+$ T cells' role in respiratory virus infections have shown them to be essential for viral clearance by cytotoxicity of infected cells. This is clearly shown in studies using athymic and/or *Rag1*$^{-/-}$ mice which display impaired virus clearance and, therefore, do not recover from the infections [59-61]. Similarly, adoptive transfer of influenza-specific activated CD8$^+$ T

cells into SCID mice led to reduced virus load and enhanced mouse survival [35, 57]. To induce cytotoxicity of infected cells, $CD8^+$ T cells use a variety of mechanisms such as engagement of death receptors expressed on virus-infected cells and release of cytotoxic factors like perforin and granzyme B to create holes in the cell membrane and initiate apoptosis of infected cells [40]. Despite the essential functions of $CD8^+$ T cell-mediated viral clearance, the respiratory epithelium is significantly compromised if $CD8^+$ T cell response is unregulated and can lead to catastrophic damage to the host. This is substantiated by multiple studies in *Rag1*$^{-/-}$ mice displaying delayed lung injury and mortality of influenza infections [59, 61-63]. Further, the adoptive transfer of RSV-specific $CD8^+$ T cells in *Rag1*$^{-/-}$ mice led to increased morbidity and mortality [63]. Additionally, $CD8^+$ T cell depletion studies showed impaired viral clearance but decreased lung injury [60, 61, 63, 64]. While the adaptive immune response to virus infections is essential for the resolution of infection and creates a long-term memory response against secondary infections, but if unregulated it can cause sufficient damage leading to host death.

1.6 Host survival of viral pneumonia

The primary goal for protection against viral pneumonia associated illness is host survival. One way of achieving host survival is to enhance virus elimination from the host, a concept termed '*host resistance'* [65, 66]. The other way is to mitigate damage to host tissues caused by a virus infection, a concept termed *'host tolerance'* [65, 66]. Most host leukocytes promote viral clearance by apoptosis of virus-infected cells causing excessive inflammation to the extent of destroying lung

physiology, resulting in acute respiratory distress and ultimately host death. Often, the factor that is required for viral clearance is also the cause for immunopathology, as was discussed earlier in this chapter about several leukocytes involved in controlling viral infections. Therefore, it is important to consider interventions that can eliminate the virus (host resistance) and control the damage done by the resistance mechanisms (disease tolerance), thereby enhancing host survival of viral infections [65-68]. Incidentally, lung epithelial cells, the target of most respiratory viruses, possess their own anti-viral defense mechanisms that have been largely overlooked as an alternative intervention against virus infections and related immunopathology [33]. Therefore, we will now discuss the lung epithelium defense mechanisms and how they can be harnessed to control viral infections and prevent chronic virus-induced asthma.

1.7 Anti-viral defense of respiratory epithelium

The lung epithelium for a long time was considered only essential for maintaining structural integrity and carrying out the function of gas exchange in the lungs. However, accumulating evidence suggests that lung epithelial cells are capable of mediating host defense against pathogens [69, 70]. Airway epithelial cells can enhance the host's barrier defense by the production of mucus, anti-microbial peptides, and reactive oxygen species (ROS) in response to several inflammatory insults, including viruses [71-73]. In addition to direct pathogen killing, alveolar epithelial cells communicate with the resident lung leukocytes by secretion of cytokines and chemokines that recruit leukocytes from lymphoid organs to the site of

infection [43, 45, 74]. While the epithelium signaling capacity to leukocytes has been explored, the innate anti-microbial capacity has received extraordinarily little attention. Our lab and several others have explored the inducibility of epithelial cells to kill pathogens as an important mechanism of innate resistance to infection. Therefore, further understanding of epithelial manipulation-based intervention is warranted for the prevention and treatment of various respiratory diseases.

Innate immune sensing of respiratory viruses: The lung epithelium, acting as an interface between the environment and the host, is constantly exposed to pathogens. Respiratory epithelial cells can recognize various pathogens through pathogen recognition receptors (PRRs) signaling on the plasma membrane or within endosomal vesicles [75, 76]. PRRs recognize specific conserved microbial patterns, collectively referred to as pattern-associated molecular patterns (PAMPs) [58, 75]. The lung epithelial cells express a variety of membrane and cytosolic PRRs such as Toll-like receptor (TLR), NOD-like receptor (NLR), C-type lectins, and retinoic acid inducible gene-I (RIG-I) among various other sensors. However, since this thesis project is focused on TLR stimulation mediated anti-viral immunity, we will only discuss TLR mediated immune sensing and anti-viral signaling. The lung epithelium expresses all known human TLRs including ones expressed on the plasma membrane and in the endosomes [75]. Viral proteins are recognized by cell surface TLRs 2 and 4 [76]. Intracellular TLRs 3, 7, 8, and 9 located in endosomes recognize viral nucleic acids [76]. TLR activation upon virus detection leads to recruitment of cytosolic Toll/IL-1R (TIR) adaptor proteins such as MyD88 (utilized by all TLRs except TLR 3), TRIF (utilized by TLR 3 and 4) or TRAM (utilized by TLR4) to the

cytosolic TLR domain [76]. These signaling cascades lead to the assembly of the Myddosome complex comprising of different IRAK and TRAF proteins, which eventually signal through NF-κB, activating the transcription of pro-inflammatory cytokines such as type I IFN [76]. TLR sensing of the virus eventually leads to the production of anti-viral proteins in epithelial cells and the production of cytokines and chemokines leads to the recruitment of innate and adaptive immune cells which ultimately clear the virus.

Physical Barrier: The lungs' mucosal surface is almost entirely covered with epithelium and consists of tight junctions (TJs), desmosomes, gap and adherens junctions (AJs) which regulate cell-cell activity [36, 69, 77]. TJs are important to maintain epithelial integrity and ensure communication between cells. AJs are located below TJs providing adhesive contacts for neighboring cells. Gap junctions are channels located between cells that allow ions and small metabolites to diffuse between cells. Desmosomes are intercellular junctions that form strong adhesive bonds between cells providing mechanical strength [36, 69, 77]. Therefore, by forming a strong physical barrier and the network of junctions between cells, the epithelium provides efficient first-line defense against viruses. Further, the mucociliary apparatus provides another layer of mucosal protection by trapping pathogens in the mucus and expelling them from the lungs with the aid of cilia [36, 70, 77]. To maintain tissue homeostasis, goblet cells of the epithelium continuously secrete mucus, mainly consisting of mucins made of glycoproteins to form a structural network and produce anti-viral substances [36, 69, 73, 78]. While increased mucin production during viral infections helps in viral elimination,

continuous and excessive airway mucus production can lead to airway obstruction, a prominent feature of asthma [79].

Type I and III interferons: Activation of type I interferons (IFN) is one of the main results of TLR recognition of virus infections [76]. For many years, TLR-mediated signaling leading to IFN production was mainly studied in phagocytic cells and the contribution of epithelial cells was less certain [36, 58, 75, 80]. However, it is now known that almost all cell types, including lung epithelial cells, can produce type I interferons. Most TLR signaling events have been linked to Interferon regulatory factor (IRF) 3 and IRF7 activating IFN stimulated genes (ISGs) that in turn code for anti-viral proteins [36, 73]. These ISGs encode for different anti-viral proteins such as Mx1, PKR, and Oas1, among several others, which function to control viral replication by inducing an anti-viral state [81]. For example, mouse studies showed Mx1 inhibits viral replication by blocking viral RNA transcription and a deficiency of this protein leads to susceptibility to influenza infection [82]. Similarly, Oas1 enzyme activates latent ribonuclease (RNAse L) resulting in viral RNA degradation and inhibition of protein synthesis [83].

Another group of IFNs called type III interferons are secreted by epithelial cells in different mucosal surfaces [37]. Both types of IFNs are known to induce ISGs that in turn trigger anti-viral peptide generation, apoptosis of infected cells, and activation of different immune cell pathways resulting in the recruitment of innate and adaptive immune cell types [81, 84, 85]. Increasing evidence suggests that type III IFNs are the dominant IFN response in the airways, especially under type I IFN deficiency [37, 86]. While type I IFNs are also known to induce immunopathology during viral

infections, type III IFNs are known to only induce an anti-viral state without much damage to the host [81, 84, 86, 87]. However, both type I and III IFNs appear to be important for viral clearance, and depletion or lack of either type of IFNs results in increased viral replication and impaired viral clearance leading to increased mortality from viral infections [37, 45, 76, 81, 84, 87].

Reactive Oxygen Species: The lung epithelium also produces ROS primarily by mitochondrial, NADPH oxidase (NOX), and Dual oxidases (Duox) sources in response to infectious stimulus [88-93]. Hydrogen peroxide and superoxide can promote virus elimination either directly by oxidative damage to virus particles or indirectly by acting as signaling molecules to promote innate and adaptive immune mechanisms [88, 92, 93]. Superoxide derived from NOX2 in epithelial cells can promote cell death by autophagy [92]. High ROS levels can reinforce the repression of mammalian target of rapamycin (mTOR) by activation of AMP-activated kinase (AMPK) [92]. Inhibition of the mTOR pathway is known to reduce viral burden by inducing autophagy, cell death of infected reservoirs, and regulating $CD8^+$ T cell memory [92]. However, most of these mechanisms of ROS-mediated anti-viral activity are derived from studies focusing on phagocytes such as neutrophils and macrophages. The ability of the lung epithelium in generating ROS as an anti-viral defense mechanism has not been explored significantly. We will cover this area of investigation in Chapter 6 on anti-viral mechanisms by lung epithelial cells.

Enhanced recruitment of host leukocytes: After virus sensing by TLR recognition, lung epithelial cells secrete a variety of proinflammatory cytokines and

leukocyte recruiting chemokines. These include cytokines like TNF-α, IL-1β, IL-6, GM-CSF, and chemokines like CXCL5, CXCL2, CCL20 that recruit neutrophils, macrophages, NK cells to the site of infection [89]. This process certainly is required for the host immune response to clear the virus. However, beyond the function of alerting the host immune system for viral clearance, epithelial cells produce cytokines like IL-33, IL-25, and TSLP that particularly promote T helper 2 adaptive immune response that in many cases can turn maladaptive [6, 9, 94]. Production of epithelial IL-33 represents a potential link to airway $CD4^+$ Th2 type inflammation associated with asthma. So far, mouse studies have indicated a correlation between IL-33 deficiency and failure in dendritic cell (DC) activation and migration to the draining lymph nodes [79, 95-98]. Several studies have shown IL-33 activated DCs when adoptively transferred exacerbate lung inflammation in allergen-induced asthma mouse models [99-101]. Therefore, epithelium targeted therapies seem to be a rational approach to controlling lung inflammation caused by epithelial IL-33 response upon virus or allergen assault.

1.8 Inducible epithelial resistance

Since respiratory epithelium is the target for many viruses, our lab hypothesized that the epithelium can be alternately harnessed to stimulate the local lung innate immune defense mechanisms. Although innate immune activation in the lungs is low at baseline, as reflected by the low level of anti-microbial activity without prior stimulation, there is generous evidence about the inducibility of local lung epithelial cells to perform anti-microbial activities traditionally carried out by leukocytes [36, 43,

69, 70, 77]. In this regard, our lab used this evidence and found that local stimulation of the lung microenvironment in mice with nontypeable *Haemophilus influenzae* (NTHi) lysate leads to broad resistance against multiple pathogens [71-73, 102, 103]. This broad resistance against various pathogens occurred rapidly within hours of aerosolization and did not rely on adaptive immune response [71]. This bacterial lysate-induced broad resistance was dependent on TLR signaling [103]. All TLR signaling requires adaptor proteins such as MyD88 or TRIF [76]. We found that NTHi-induced resistance was MyD88 dependent and not TRIF dependent [103]. This evidence of MyD88 dependency of innate resistance focused our future studies on TLRs among all PRRs. But, the lack of TRIF-dependent effect of the protection indicated that only a few TLR subsets were necessary for inducible resistance. This provided the basis for testing different synthetic TLR ligands in combination against multiple pathogens. However, individual TLR ligands failed to induce a strong resistance against pneumonia [103]. Therefore, to determine whether a combination of TLR ligands conferred protection, 21 non-redundant combinations of the several synthetic TLR ligands were tested in mice against pneumonia. Although not all tested TLR agonist combinations protected against pneumonia, a few combinations provided the protection that was statistically significant than PBS treatment [103]. However, no combination was as superior or effective as the combination of TLR2/6 (Pam2CSK4) and 9 (ODN2395) which resulted in 100% mouse survival against bacterial pneumonia [72, 102, 103]. Similarly, pathogen killing was associated with a synergistic combination of Pam2CSK4 and ODN2395 [103]. Further, the lab found that class C ODNs and not

class A or B ODNs provided synergistic interactions with Pam2CSK4 for maximum resistance against bacterial pneumonia [103]. An explanation for this phenomenon may come from the functions and structure of class C CpG ODNs. Class C ODNs have characteristics of both class A and B ODNs, and it may be hypothesized that stimulation with Class C ODN results in both IRF-7 and NF-κB pathways required for the protective Pam2-ODN effect. Interestingly, by fluorescent microscopy, we found that Pam2CSK4 and ODN2395 co-localize intracellularly in endosomes in lung epithelial cells [103]. To this effect, several studies have shown that TLR2 and TLR9 cooperate in controlling several pathogens including *Mycobacterium tuberculosis*, *Helicobacter pylori*, and herpes simplex virus [104-106]. However, further studies are required to uncover the synergistic interactions of TLR 2 and 9 signaling components.

To date, our lab has shown that mouse inhalation of an aerosolized combination of Pam2CSK4 and a class C ODN-M362 (together this combination is called Pam2-ODN) led to resistance against respiratory pathogens including viruses [71, 72, 102, 107, 108]. Further, we have shown that stimulated innate resistance with Pam2-ODN in the lungs is dominantly carried out by the epithelium as anti-microbial resistance is still intact in mice deficient of individual leukocytes (neutrophils, macrophages, dendritic cells, NK cells, and lymphocytes) [102]. This is further supported by mice showing no resistance against other treatment routes (intraperitoneal or intravenous) suggesting that resistance mechanisms are local [72, 73]. In addition, mice conditionally deleted of MyD88 (required for TLR signaling) only in lung epithelial cells showed no benefit from Pam2-ODN stimulation, strongly

indicating the role of lung epithelial cells in driving the protective Pam2-ODN response [102, 103]. Further, *in vitro* stimulation of isolated lung epithelial cells with bacterial lysates or Pam2-ODN showed efficient pathogen killing ability [107, 109]. Therefore, our lab described this broad innate resistance as '*Inducible epithelial resistance'*.

This evidence of the inducibility of lung epithelial cells to efficiently kill pathogens seems a rational approach to treating respiratory virus-related illness. In this thesis project, we will focus on uncovering mechanisms of enhanced survival of respiratory virus infections by therapeutic stimulation of lung epithelial cells to initiate anti-viral effects. Further, we also focus on the prevention of virus-induced chronic asthma-like disease by viral killing and viral-killing independent mechanisms.

Chapter 2: Specific Aims

2.1 Working model and hypothesis

Using the knowledge on cooperative abilities of TLR signaling to induce protective responses, our lab found that combinatorial treatment with TLR2/6 (Pam2-CSK4) and TLR 9 (ODN-M362) agonists showed the maximal protection against a broad set of pathogens [71, 72]. Further, we have shown prevention of chronic virus-induced asthma in mice treated with Pam2-ODN [110]. However, the mechanisms underlying protection against viral pneumonia-related mortality, chronic asthma-like disease, and anti-viral killing mechanisms by Pam2-ODN stimulation of lung epithelial cells has not been fully elucidated by our group. Therefore, this thesis project will focus on understanding three aspects of respiratory virus infections – 1) enhancing the host survival during viral infections, 2) preventing virus-induced asthma-like disease, and 2) uncovering virus-killing mechanisms by Pam2-ODN stimulation of lung epithelial cells. The central hypothesis of this project is that ***"Inducible epithelial resistance enhances mouse survival of respiratory viral infections by reducing viral burden by epithelial ROS production and prevents progression to virus-induced asthma-like disease by controlling IL-33 production".*** To address this hypothesis, studies under the following aims will be performed.

2.2 Specific Aims

<u>Aim 1:</u> Determine Pam2-ODN mediated enhanced survival of viral pneumonia in mice. *Rationale and Hypothesis:* Several studies have indicated that the host immune response during viral infections is often also the cause of fatal immunopathology [21, 65, 66]. And, our preliminary studies using the SeV mouse model have shown that peak mortality of SeV pneumonia occurs at the time of lymphocytic lung inflammation. I hypothesize that *$CD8^+$ T cell inflammation contributes to mouse mortality of SeV pneumonia which can be prevented by Pam2-ODN pretreatment* and we will address this hypothesis in three sub aims **1a)** Determine the kinetics of $CD8^+$ T cells in Pam2-ODN treated and untreated SeV-infected mice. **1b)** Identify the antiviral and immunopathology causing role of $CD8^+$ T cells by depletion studies. **1c)** Determine the role of Pam2-ODN induced ROS in reducing the lymphocytic inflammation during SeV infection.

<u>Aim 2:</u> Determine mechanisms of Pam2-ODN mediated prevention of virus-induced asthma. *Rationale and Hypothesis:* Our lab has previously shown that Pam2-ODN aerosolization in mice protects against SeV-induced asthma-like disease with reduced airway hyperreactivity, mucus metaplasia, and eosinophils. Interestingly, asthma is also reduced in mice aerosolized with Pam2-ODN after viral clearance by viral-killing independent mechanisms, which are not elucidated. Therefore, I hypothesize that *Pam2-ODN treatment after SeV clearance can reduce asthma by reducing lung IL-33*. To address this hypothesis, studies under the following sub aims will be performed. **2a)** Determine the kinetics of IL-33 reduction in mice treated with Pam2-ODN and SeV challenged. **2b)** Determine the cellular

source of IL-33 in lung cells during SeV infection. **2c)** Determine whether IL-33 is reduced in SeV challenged mice treated with Pam2-ODN after viral clearance.

<u>Aim 3:</u> Determine the viral-killing mechanisms of Pam2-ODN treatment in lung epithelial cells. *Rationale and Hypothesis*: Our lab has reported that Pam2-ODN induced ROS is essential for protection against viral infections *in vivo* and in isolated lung epithelial cells. *I hypothesize that Pam2-ODN induced ROS directly inhibits SeV particles thereby reducing SeV replication in the lung epithelial cells.* To address this hypothesis, studies under the following sub aims will be performed. **3a)** Determine whether Pam2-ODN reduces SeV burden before or after SeV entry in lung epithelial cells. **3b)** Determine the role of Pam2-ODN-induced ROS in SeV inactivation before or after SeV entry in lung epithelial cells.

Chapter 3: Materials and Methods

The following chapter is based on my publications, used with permission granted by mBIO and British Journal of Pharmacology and American Thoracic Society.

Kirkpatrick, C.T., Y. Wang, M.M. Leiva Juarez, P. Shivshankar, J. Pantaleon Garcia, A.K. Plumer, V.V. Kulkarni, H.H. Ware, F. Gulraiz, M.A. Chavez Cavasos, G. Martinez Zayes, S. Wali, A.P. Rice, H. Liu, J.M. Tour, W.K.A. Sikkema, A.S. Cruz Solbes, K.A. Youker, M.J. Tuvim, B.F. Dickey, and S.E. Evans, *Inducible Lung Epithelial Resistance Requires Multisource Reactive Oxygen Species Generation To Protect against Viral Infections.* MBio, 2018. **9**(3).

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Wali, S., J.R. Flores, A.M. Jaramillo, D.L. Goldblatt, J. Pantaleon Garcia, M.J. Tuvim, B.F. Dickey, and S.E. Evans, *Immune Modulation to Improve Survival of Viral Pneumonia in Mice.* Am J Respir Cell Mol Biol, 2020.

Goldblatt, D.L., J.R. Flores, G. Valverde Ha, A.M. Jaramillo, S. Tkachman, C.T. Kirkpatrick, S. Wali, B. Hernandez, D.E. Ost, B.L. Scott, J. Chen, S.E. Evans, M.J. Tuvim, and B.F. Dickey, *Inducible epithelial resistance against acute Sendai virus infection prevents chronic asthma-like lung disease in mice.* Br J Pharmacol, 2020. **177**(10): p. 2256-2273.

3.1 Mice and cell culture

All *in vivo* experiments were performed using 6- to 10-week-old C57BL/6J mice of a single-sex (male or female) purchased from (Jackson laboratory) or bred in-house according to the Institutional Animal Care and Use Committee of MD Anderson Cancer Center, protocol 00000907-RN01. Mouse lung epithelial (MLE-15) cells were kindly provided by Jeffrey Whitsett, Cincinnati Children's Hospital Medical Center, and cultured in DMEM with 2% Fetal Bovine Serum (FBS), 1% insulin, and transferrin. MLE-15 cells were authenticated by the MD Anderson Characterized Cell Line Core Facility. To harvest tracheal epithelial cells, mice were anesthetized to expose and excise tracheas. These tracheas were then digested in pronase (1.5 mg/ml, Sigma Aldrich) overnight at 4º C. Tracheal epithelial cells were then isolated and cultured on collagen-coated transwells in Ham's F12 media supplemented with differentiation growth factors and hormones as previously described [111].

3.2 TLR treatments and viral challenge

For *in vitro* treatments, cells were treated with Pam2CSK$_4$ (2.2 µM) and ODN M362 (0.55 µM), 4 h before SeV inoculation as previously described (ref). For *in vivo*

treatments, 10 ml solution of $Pam2CSK_4$ (4 µM) and ODN M362 (1 µM) in endotoxin-free water was delivered by Aerotech II nebulizer (Biodex Medical Systems) driven by 10 l/min along with CO2 (5%) in air for 30 minutes as previously described [112]. SeV was purchased from ATCC (Manassas, VA) and grown in Rhesus monkey kidney cells obtained from Cell Pro labs (Golden Valley, MN). For *in vitro* challenges, multiplicity of infection (MOI) = 1 was used. Unless otherwise stated, mice were challenged with 1×10^8 plaque-forming units (pfu) in PBS inserted into the oropharynx of mice, under isoflurane anesthesia as described [110]. Mice were weighed before and daily after challenge as a measure of morbidity and criteria for euthanasia.

3.3 Bronchoalveolar lavage and differential Giemsa staining

After deep anesthesia, mouse tracheas were exposed, cannulated with a 20-gauge syringe, and instilled with 1.5 ml of PBS. Approximately 1 ml of BAL fluid was collected per sample. The BAL fluid was then spun down at 4º C at 300 *g* to collect the cells in the pellet. The cell pellet was resuspended in 1 ml of ice-cold PBS and 200 µl of this cell suspension was then subjected to cytocentrifugation at 300 *g* for 5 min. Cells were stained with Giemsa stain for differential count determination and total cells were counted by hemocytometer.

3.4 Flow cytometry

For *in vivo* experiments, mouse lungs were perfused with 5 to 10 ml ice-cold PBS, dissected, cut into 1 mm^3 piece, and digested with collagenase/DNAse I (5 mg/ml, Worthington biochemical) for 30 min at 37° C. After digestion, single cells were collected by passing through a 70 µm filter. These single cells were washed with FACS staining buffer (PBS supplemented with 1% FBS) and stained for specific cell types, as indicated in the antibody table (Table 1). For *in vitro* experiments, MLE-15 cells were seeded on 24 well plates for treatment with Pam2-ODN and SeV inoculation. Cells were trypsinized and washed with FACS staining buffer 2X. Cells were blocked in 5% donkey serum for 30 min before proceeding to stain with Rabbit SeV antibody (MBL International) overnight at 4° C, followed by staining with secondary Alexa488 anti-rabbit antibody (Jackson Immunologicals) for 1 h. Cells were fixed and acquired on BD LSRII (BD Biosciences) for Alexa488 positive cells.

3.5 SeV Immunofluorescence

MLE-15 cells were grown on chamber slides (Labtek), treated with Pam2-ODN for 4 h before inoculation with SeV (MOI 1). Cells were then fixed with 2% paraformaldehyde before staining with a rabbit anti-SeV antibody (MBL International) and detected using a secondary anti-rabbit antibody. For each experimental condition, specimens were imaged using an Olympus BX60 microscope using identical parameters for time of exposure, color intensity, contrast, and magnification. Images were then loaded on ImageJ software to calculate the mean fluorescence intensity for each group.

3.6 Hematoxylin and eosin staining

Mouse lungs were fixed by intratracheal inflation with 10% formalin for 24 h, and then transferred to 70% ethanol embedded in paraffin. Tissue blocks were then cut into 5 µm sections, mounted onto frosted glass slides, deparaffinized with xylene, washed with ethanol, then rehydrated and stained with hematoxylin and eosin for morphological changes.

3.7 Epithelial proliferation assays

Mice were injected intraperitoneally with 0.1 ml EdU (1 mg/mouse). After 24 h, mouse lungs were inflated and fixed with 10% formalin for 24 h at 4° C, and then lungs were embedded in paraffin. Paraffin sections were cut into 5 µm transverse sections of the axial airway, between lateral branches 1 and 2. Lung sections were then stained following the Click-iT EdU Imaging Kit protocol for EdU (Abcam,) followed by staining with DAPI for 30 min at room temperature. Images were collected using an Olympus BX60 microscope using identical parameters for all conditions. Some lung sections were subjected to antigen retrieval and then stained for Ki67 (1:1000; Invitrogen) or cCasp3 (1:500; Cell Signaling). EdU, Ki67, or cCasp3 positive cells were quantified using a cell counter plugin in ImageJ and normalized to DAPI positive cells in every field of view (number of fields surveyed per mouse sample = 3).

3.8 $CD8^+$ T cell depletion

Anti-CD8-β antibody (200 μg/mouse, Bioxell) was delivered to mice intraperitoneally at indicated time points. $CD8^+$ T cell depletion was confirmed by flow cytometry analysis 24 to 48 h after depletion.

3.9 Viral burden quantification

For *in vivo* experiments, mouse lungs were collected 5 days after SeV challenge. RNA from mouse lungs was extracted using the Qiagen RNeasy kit. 500 ng of total RNA was converted to cDNA using Biorad iScript cDNA conversion kit. Viral burden was determined by reverse transcription quantitative PCR (RT-qPCR) of the Sendai Matrix (M) protein normalized to house-keeping gene 18SRNA. 18S forward primer – GTAACCCGTTGAACCCCATT; reverse primer – CCATCCAATCGGTAGTAGCG. SeV M gene forward primer – ACTGGGACCCTATCTAAGACAT; reverse primer – TAGTAGCGGAAATCACGAGG. The Limit of quantification (LOQ) was established for the SeV qPCR assay as the highest dilution of the template still maintaining the linearity of the assay. The threshold cycle (C_T) value of the LOQ was set as the lower limit for the assay.

3.10 ROS inhibition in vitro and in vivo

NADPH oxidase activity was inhibited by exposing the cells to GKT137831 (10 µM; Selleckchem) 12 h prior to treatment with Pam2-ODN or PBS. Mitochondrial ROS production was inhibited using the combination of FCCP (400 nM, Cayman Chemicals) and TTFA (200 µM, Cayman Chemicals) for 1 h before Pam2-ODN or PBS treatment. For *in vivo* experiments, mice were aerosolized with 10 ml TTFA (200 mM) and FCCP (800 µM) 2 h before Pam2-ODN aerosolization and 2 h before SeV challenge and then again 4 days after SeV challenge.

3.11 Viral attachment assays

For most enveloped viruses, internalization into epithelial cells is inhibited at 4º C without affecting viral binding to epithelial cells [34]. MLE-15 cells were cultured in 24 well plates or chamber slides for treatment with Pam2-ODN for 4 h. Cells were placed on ice 30 min before inoculation with SeV to prevent viral entry into the cells. After 4 h inoculation on ice, cells were vigorously washed 5X with media to remove unattached virus. Cells were then harvested to measure SeV burden using immunofluorescence or flow cytometry. For RT-qPCR assays, epithelial cells were treated with Pam2-ODN or PBS, followed by SeV infection on ice (to prevent viral entry into cells). Virus particles were allowed to attach to the epithelial targets for 4 h on ice. These cells were then extensively washed to remove unattached virus particles, and then the cells were lysed by passing through a syringe 10X. The liberated virus particles were then transferred to naïve epithelial cells that had no prior exposure to Pam2-ODN. SeV M gene expression was assessed by qPCR after 24 h of SeV replication in the new cells. In some experiments, mitoROS inhibitors

(FFCP-TTFA) were used before Pam2-ODN treatment to determine the role of Pam2-ODN induced ROS in SeV inactivation prior to internalization.

3.12 IL-33 Immunohistochemistry

Mouse lungs were fixed, embedded, sectioned, and deparaffinized, then exposed for 10 min to 3% H_2O_2 in 90% methanol, and then heated for 10 min in 10 mM sodium citrate, pH 6.0, for antigen retrieval. Slides were rinsed in water, blocked in horse serum (Jackson ImmunoResearch, West Grove, PA) for 1h, then rinsed again and incubated with goat anti-mouse IL-33 (R&D Systems, Minneapolis, MN; 1:1000) diluted in blocking solution at 4° C overnight. After incubation, secondary antibody - biotinylated horse anti-goat IgG (Vector) was added for 2 h at room temperature. Tissue sections were then washed with PBS, counterstained with H&E, and mounted with VectaMount (Vector).

3.13 IL-33 Immunofluorescence

Lungs were frozen in OCT and cut into 10-µm sections. Sections mounted on glass slides were thawed, washed with water, blocked with donkey serum (Jackson ImmunoResearch), then incubated with primary antibodies overnight at 4°C. Primary antibodies used were: rabbit anti-prosurfactant protein C (EMD Millipore, Burlington, MA; 1:1000), rabbit anti-keratin 14 (KRT14, RB-9020-P0, Thermo Fisher, Waltham, MA; 1:500), rat anti-mouse CD68 (BioLegend, San Diego; 1:200). This was followed by the addition of secondary antibodies conjugated to Alexa 555 (Cy3), Alexa 488

(FITC), and DAPI (Jackson ImmunoResearch) for 2 h. A confocal microscope (A1plus, Nikon) was used to acquire all images. For quantitative studies, random images were acquired by investigators blinded to subject identity. Percentages of IL-33 positive cells were counted using ImageJ. To measure IL-33 fluorescence intensity, the left lung was sectioned at the axial bronchus between lateral branches 1 and 2 using a precision cutting tool then imaged using an upright microscope (Olympus BX 60) with a 40X lens objective and identical parameters of exposure time, color intensity, contrast, and magnification. Images were uploaded to ImageJ, and a red (IL-33) background intensity threshold was established by first measuring fluorescence intensity for background regions (those without blue nuclear staining) and regions of interest (double-positive red and blue staining). The threshold was then set as the mean between maximal background regions and mean regions of interest and subtracted from all fields.

3.14 Type I Interferon ELISA

Mouse tracheal epithelial cells were grown at an air-liquid interface. Cells were treated with Pam2 and ODN as described earlier and infected with either influenza A or SeV. Cell supernatants were collected at various times post-infection and used for IFN-β ELISA (R&D Systems) according to the ELISA manufacturer's instructions.

3.15 IL-33 ELISA

Mouse lungs were snap-frozen in liquid nitrogen and stored in -80 ºC. Mouse lung samples were homogenized in protein lysis buffer with protease inhibitors and EDTA (Thermo Fisher). Homogenized samples were kept on ice for 30 mins to separate the protein components from cell debris. Samples were then spun at high speed at 4 ºC to collect the supernatant containing the total protein. Total protein from the samples was measured using a BCA kit (Thermo Fisher) and 3 µg of total protein was used for IL-33 ELISA (R&D Systems). The ELISA manufacturer's instructions were followed for the rest of the assay. Data collected was represented as total protein per µg of tissue.

3.16 Statistics

All statistical analysis was performed using GraphPad Prism software (Version 8 for Windows, GraphPad Software, La Jolla, CA). Data are representative of one experiment from at least three independent experiments and presented as mean +/- standard error of biological replicates. To determine pairwise differences in viral burden or cell numbers, Student's *t*-test was used. Mouse survival analysis of viral challenges was analyzed using Log-rank, Mantel-Cox test. One-way analysis of variance (ANOVA) with multiple comparisons was used to determined differences between multiple experimental conditions.

Table 1: List of antibodies used

Antibodies	**Vendor**	**Catalog numbers**
CD3	Tonbo	65-0031-U100
CD4	Tonbo	60-0042-U100
CD8	Tonbo	25-0081-U100
Live dead	Tonbo	13-0870-T500
CD25	Biolegend	102038
Foxp3 Treg kit	eBiosciences	72-5775
CD8-Depleting Ab	Bioxell	BE0223
CD19	Biolegend	115507
B220	BD Biosciences	562922
Anti-SeV virus Ab	MBL International	PD029
Ki67	Invitrogen	MA5-14520
cCasp3	Cell signaling	9662S

Chapter 4: Determine Pam2-ODN mediated enhanced survival of viral pneumonia in mice.

The following chapter is based on my publication, used with permission granted by American Thoracic Society. The figures and data shown in this chapter are entirely my work.

The American Journal of Respiratory Cell and Molecular Biology is an official journal of the American Thoracic Society.

(https://www.atsjournals.org/doi/10.1165/rcmb.2020-0241OC)

Wali, S., J.R. Flores, A.M. Jaramillo, D.L. Goldblatt, J. Pantaleon Garcia, M.J. Tuvim, B.F. Dickey, and S.E. Evans, *Immune Modulation to Improve Survival of Viral Pneumonia in Mice.* Am J Respir Cell Mol Biol, 2020.

Our lab has previously found the survival benefit of Pam2-ODN aerosolization in the influenza mouse model, but the mechanisms of host survival remain incompletely elucidated [107]. Using the SeV mouse model, we found host mortality

of infections occurring after viral clearance, as discussed in this chapter. Therefore, to elucidate the mechanisms of host survival of viral pneumonia, we characterized the cellular changes in the host occurring after infection in mice with or without Pam2-ODN treatment and the results are presented below.

4.1 Pam2-ODN treatment enhanced mouse survival of SeV infections is associated with reduced lung viral burden

Aerosolized Pam2-ODN treatment one day prior to SeV challenge increased mouse survival of SeV challenge (Figure 1A) like the protection afforded against lethal influenza pneumonia [107]. The survival benefit was associated with reduced lung SeV burden, as measured by SeV M gene expression (Figure 1B).

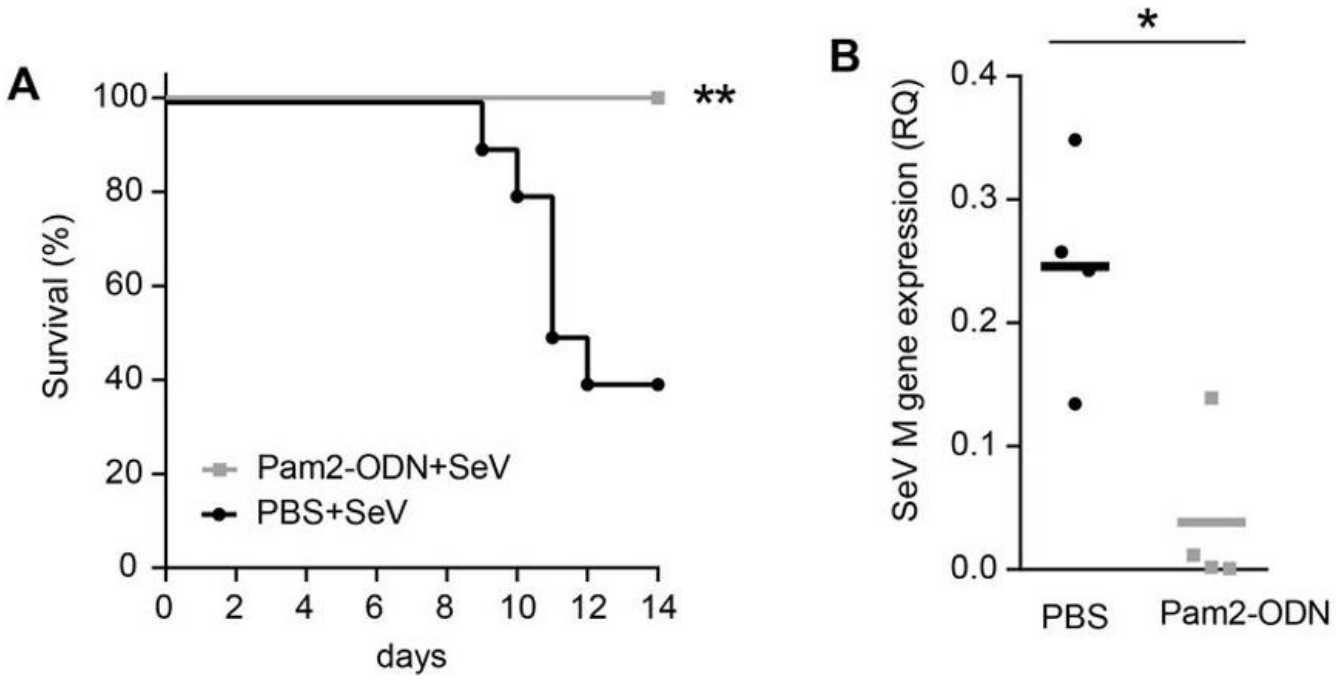

Figure 1: Pam2-ODN treatment enhanced mouse survival of SeV infections is associated with reduced lung viral burden (A) Survival of mice treated with PBS or Pam2-ODN one day prior to SeV virus challenge. **(B)** Mouse lung SeV burden 5 days after infection assessed by qPCR for Sendai Matrix (M) gene (Relative quantification, RQ to 18S) relative to 18S. n=10 mice per group in survival plot **(A)**, n=4 mice/group in virus burden experiments. *p<0.05, **p<0.005.

4.2 Mouse mortality of SeV infections occurs after viral clearance from the lungs

Investigating the natural progression of infection revealed that SeV lung burden was maximal on day 5 and gradually decreased until falling below the limit of quantification (LOQ) by day 11 (Figure 2A). Pam2-ODN pretreatment reduced SeV burden on all assessed days (Figure 2A). Although the lethality of SeV infection was exquisitely dependent on the inoculum size, we strikingly found that peak mortality paradoxically occurred around days 10 to 12 after infection irrespective of inoculum size, even though SeV is essentially undetectable that long after challenge (Figure 2A). This temporal dissociation between peak virus burden and peak mortality led to the hypothesis that SeV-induced mortality may not be exclusively driven by excessive virus burden but may also result from untoward SeV-induced host immune response.

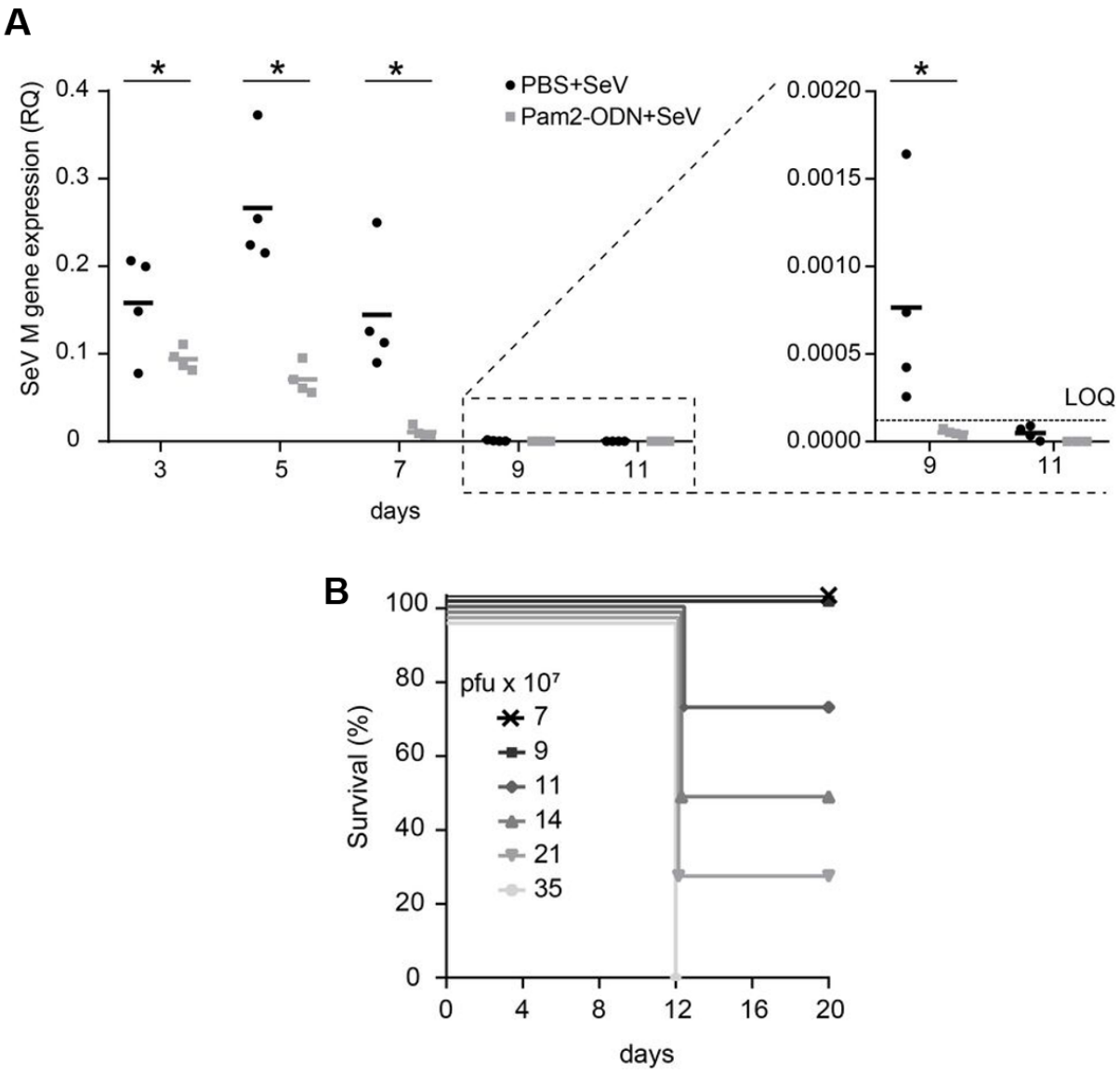

Figure 2: Mouse mortality of SeV infections occurs after viral clearance from lungs (A) Time course of lung SeV burden in mice treated with PBS or Pam2-ODN. **(B)** SeV inoculum dependent mouse survival. Data are representative of three independent experiments. n=10 mice per group in survival plot **(B)**, n=4 mice/group in virus burden experiments. LOQ, limit of quantification. *p<0.05.

4.3 Pam2-ODN treatment reduces epithelial cell death and proliferation during acute SeV infection

We found increases in lung epithelial cleaved caspase 3 (cCasp3), a marker for programmed cell death, on days 7 to 11 after SeV infection (Figure 3A, upper panel). Virus infection-related epithelial cell injury and death are typically associated with proliferative repair mechanisms [113]. Staining the infected mouse lung tissue for Ki67 and EdU revealed maximum signals for both markers in the second week after infection (Figure 3B-E, upper panel). These events of lung epithelial cell death and proliferation coincided with the peak of mortality (day 12, Figure 1E). Further, hematoxylin and eosin staining of lung tissues infected with SeV showed profound increases in inflammatory cells from days 7 to 10 with evidence of damaged airway and parenchymal tissue (Figure 4). However, Pam2-ODN pretreatment of mice reduced epithelial cell injury and proliferation (Figure 3A-E, lower panel). This temporal association of epithelial injury and death after viral clearance supported the hypothesis that mouse mortality caused by SeV infection is due in part to the host immune response to SeV infections. Therefore, to explore this hypothesis, the host leukocyte response to SeV infection was characterized.

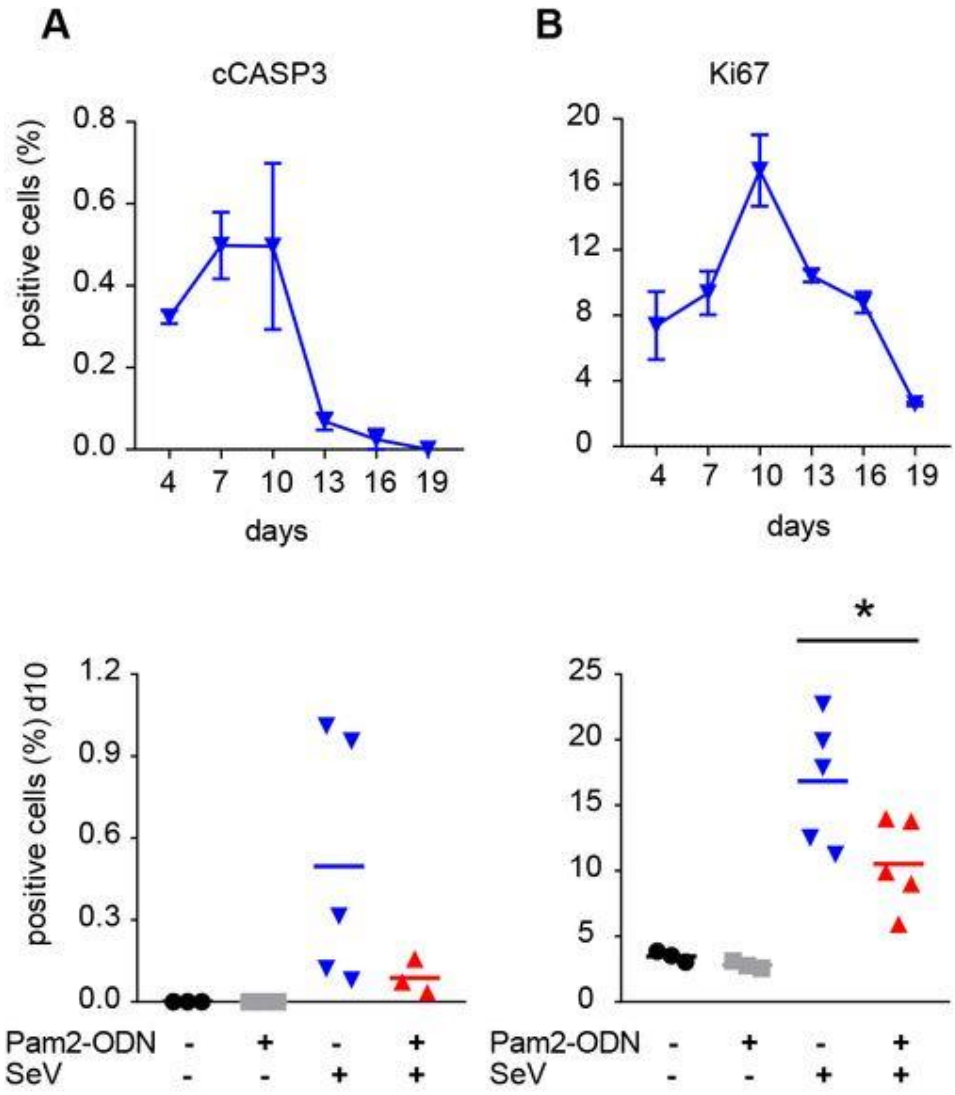

Figure 3: Pam2-ODN pretreatment reduces epithelial cell death and proliferation during acute SeV infection. Cleaved caspase 3 (cCasp3) **(A)** or Ki67 **(B)** positive cells in mouse lung epithelium after SeV infection with or without Pam2-ODN treatment (lower panel). n=5 mice per condition. Data are representative of two independent experiments. Scale bar = 100 µm. *p<0.05.

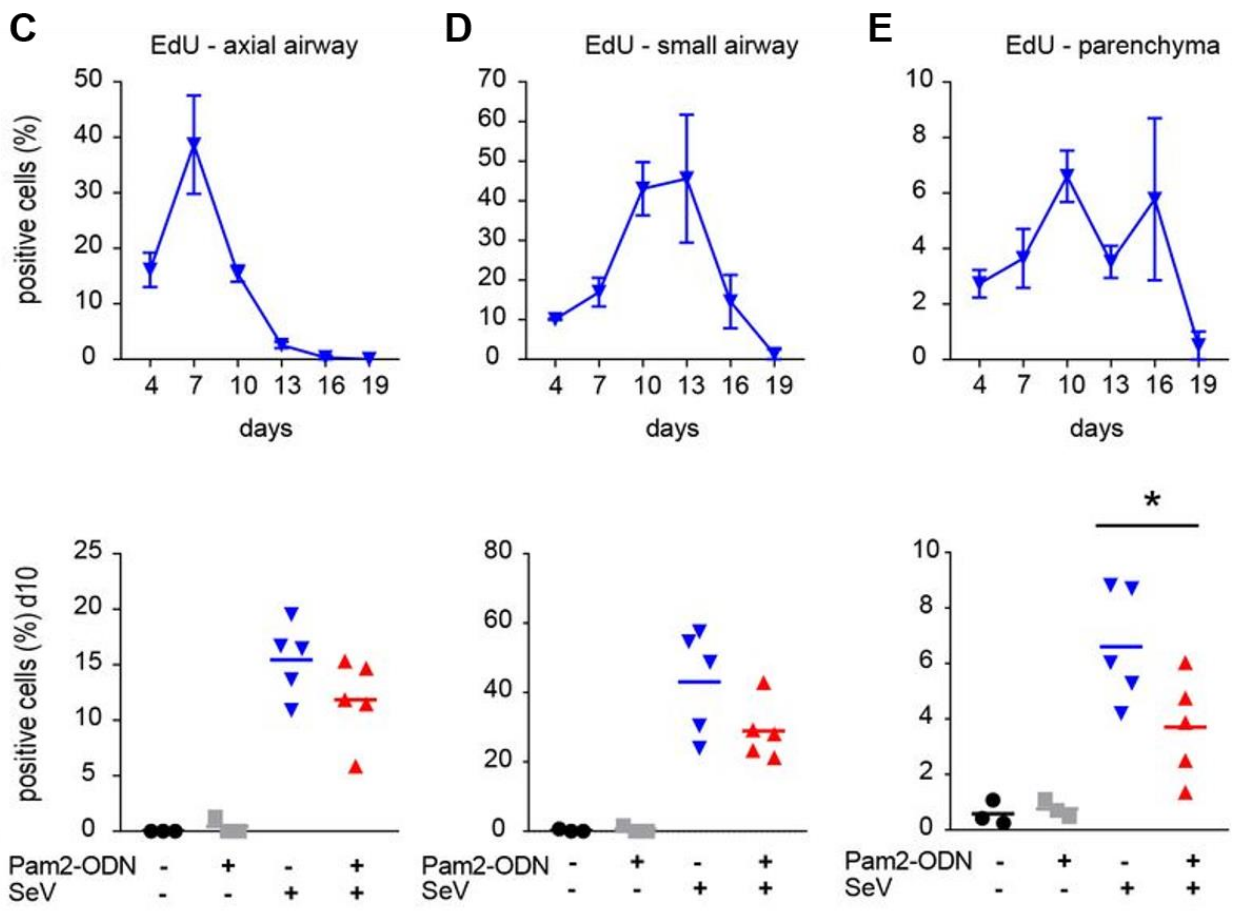

Figure 3 continued: Pam2-ODN pretreatment reduces epithelial cell death and proliferation during acute SeV infection. EdU positive cells in axial **(C),** small airways **(D),** and parenchyma **(E)** after SeV infection with or without Pam2-ODN (lower panel). Data are representative of two independent experiments. Scale bar = 100 µm. *p<0.05.

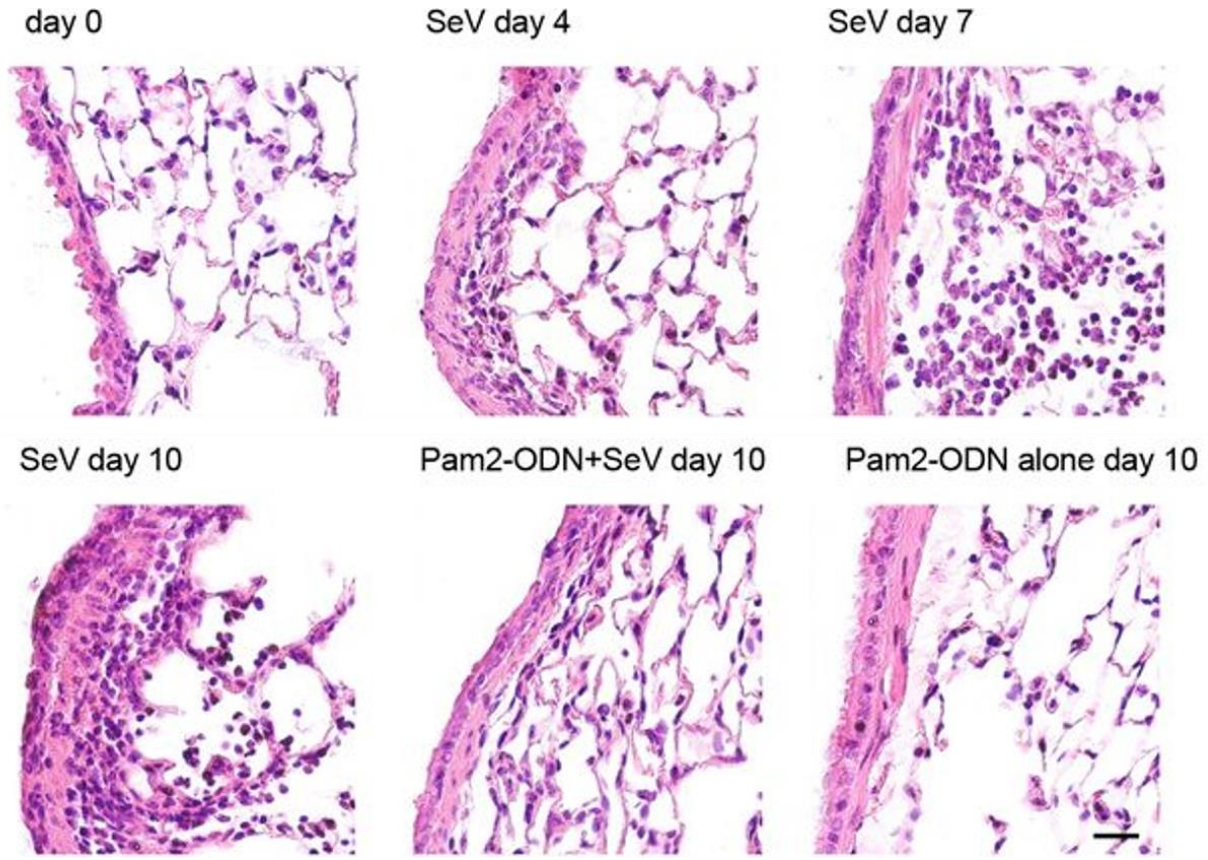

Figure 4: Mouse lung histology following SeV challenge with or without Pam2-ODN.

4.4 Pam2-ODN treatment reduces SeV induced lung CD8$^+$ T cells.

Differential Giemsa staining of bronchoalveolar lavage (BAL) cells revealed increased neutrophils on days 2 to 5 and increased macrophages on days 5 to 8 (Figure 5A, B, solid grey line) after SeV challenge. Congruent with our prior studies, inhaled treatment with Pam2-ODN in the absence of infection led to a rapid rise in neutrophils that was resolved within 5 days (Figure 5A, dashed line). The neutrophil response to SeV challenge was modestly increased among mice pretreated with Pam2-ODN (Figure 5A, solid dark line). Pam2-ODN-treated, SeV-challenged mice showed almost no difference in macrophage number compared to PBS-treated, SeV-challenged mice (Figure 5A, solid dark line). A rise in lymphocytes was observed on days 8 to 11 in PBS-treated, SeV-challenged mice (Figure 5C, solid grey line), temporally corresponding with peak mortality. However, Pam2-ODN treated, SeV-challenged mice displayed significantly reduced lymphocyte numbers at every time point assessed (Figure 5C, solid dark line). Specific lymphocytes (B cells, CD4$^+$, or CD8$^+$ T cells) in the lungs of SeV infected with or without Pam2-ODN were determined by flow cytometry showing only modest differences between the groups [114]. The biggest difference between groups was in CD8$^+$ T cells, with Pam2-ODN-treated, SeV-challenged mice displaying a significantly lower number and percentage of CD8$^+$ T cells than PBS treated, SeV-challenged mice (Figure 5D, E). Since the greatest difference after Pam2-ODN treatment was in CD8$^+$ T cell levels and there was a tight correlation between peak mortality and the increase in lung CD8$^+$ T cells on days 8 to 11, we investigated the role of CD8$^+$ T cells in SeV-induced mortality.

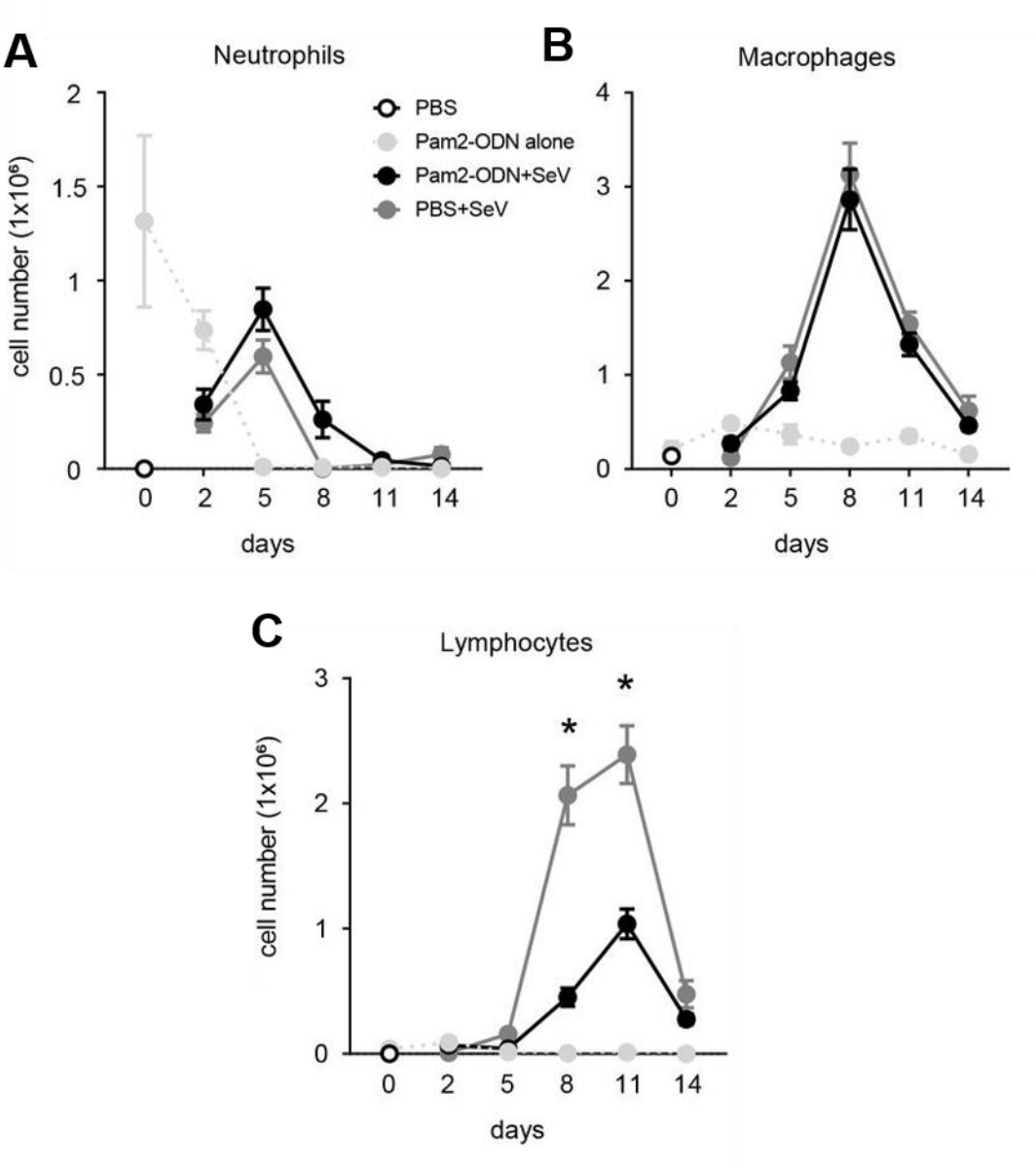

Figure 5: Pam2-ODN pretreatment reduces SeV induced lung CD8$^+$ T cells. Differential Giemsa staining of BAL cells showing **(A)** neutrophils, **(B)** macrophages, and **(C)** lymphocytes from mice challenged with SeV with or without Pam2-ODN pretreatment. Data are representative of three independent experiments. *p<0.05 compared to PBS+SeV.

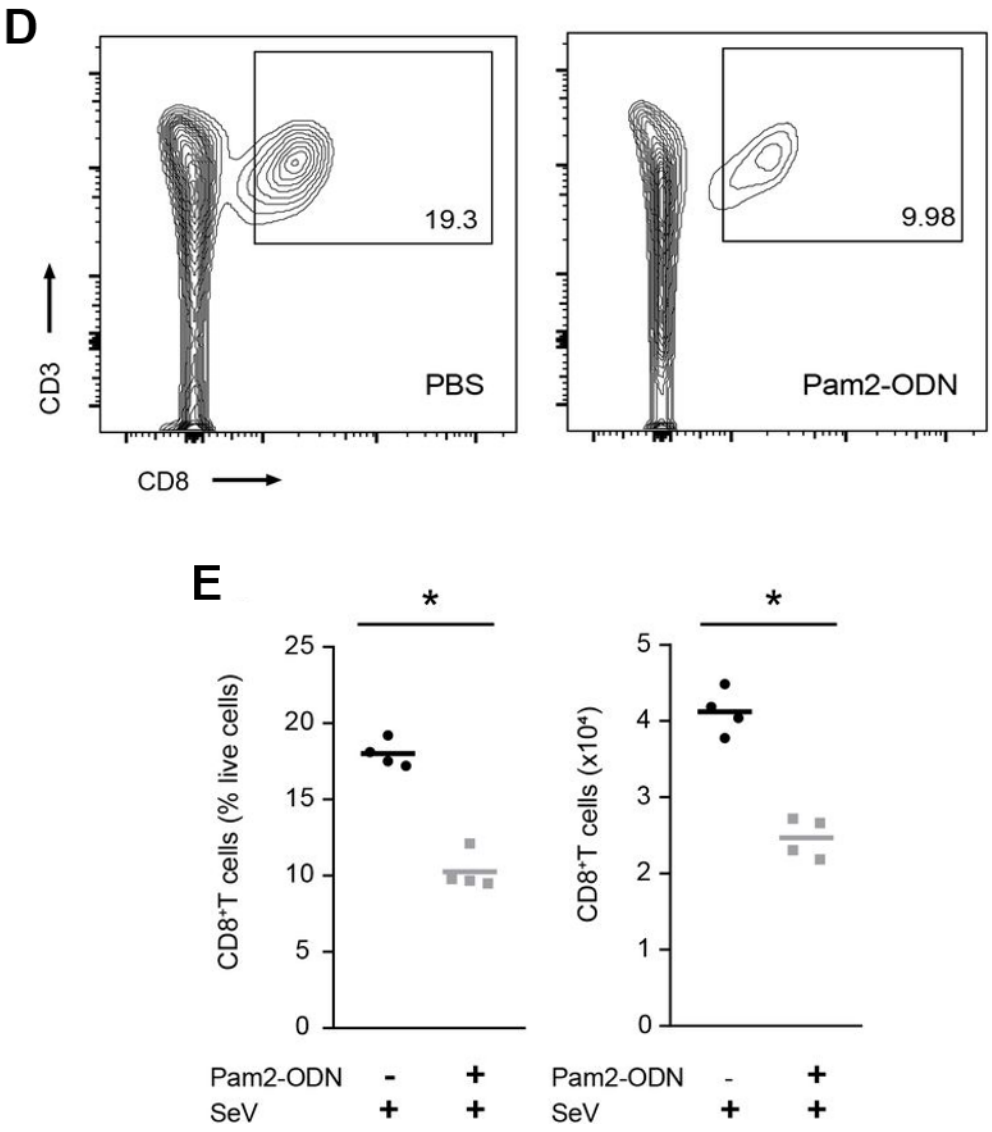

Figure 5 continued: Pam2-ODN pretreatment reduces SeV induced lung CD8$^+$ T cells. (D) Flow cytometry for CD8$^+$ T cells from disaggregated mouse lungs 11 days after SeV infection with or without Pam2-ODN. **(E)** Lung CD8$^+$ T cells 11 days after SeV challenge in mice pretreated with PBS or Pam2-ODN. Data are representative of five independent experiments for **(D)** and **(E)**. *p<0.05 compared to PBS+SeV.

4.5 Pam2-ODN treatment reduces CD8$^+$ T cell-associated SeV induced immunopathology

To understand the apparent contributions of host immunopathology to mouse outcomes, we depleted CD8$^+$ T cells on day 8 -- after virus burden was substantially reduced but before peak mouse mortality (Figures 2A, 6A). Mice depleted of CD8$^+$ T cells displayed significantly enhanced survival of SeV challenge compared to mice with intact CD8$^+$ T cells (Figure 6B). Depletion of CD8$^+$ T cells was confirmed by flow cytometry in disaggregated lung cells 10 days after SeV challenge (Figure 6C). This supported our hypothesis that CD8$^+$ T cells contribute to fatal SeV-induced immunopathology.

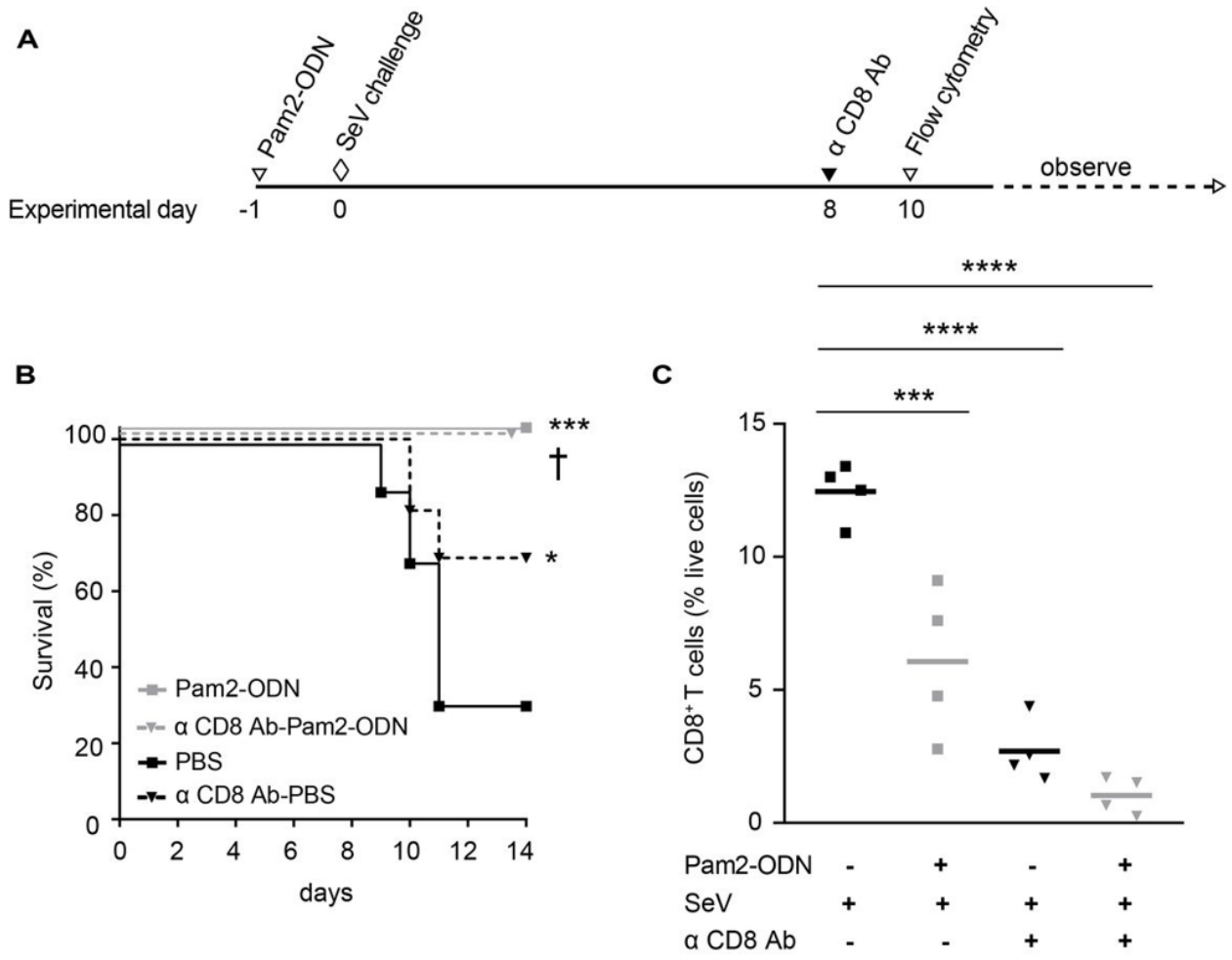

Figure 6: Pam2-ODN treatment reduces CD8⁺ T cell-associated SeV induced immunopathology. Experimental outline **(A)**, survival **(B),** and percentage of $CD8^+$ T cells **(C)** from disaggregated mouse lungs 10 days after SeV challenge following pretreatment with PBS or Pam2-ODN and with or without $CD8^+$ T cells depleted on day 8 of SeV challenge. n=16 mice/group for survival in experiment A and n=4 mice/group in experiment **(B)**. ****p<0.0001 compared to PBS in **(C)**, ***p<0.0005 compared to PBS in **(B)** and **(C)**, †p<0.05 compared to PBS, *p<0.05 compared to PBS.

4.6 Pam2-ODN treatment protects against SeV infections despite lack of CD8$^+$ T cells

To assess the role of CD8$^+$ T cells throughout infection, mouse CD8$^+$ T cells were depleted prior to and during SeV challenge (Figure 7A). This depletion resulted in significantly reduced survival of SeV infection (Figure 7B), compatible with the known antiviral functions of CD8$^+$ T cells. However, it is notable that Pam2-ODN treatment still significantly enhanced survival of SeV challenge even in the absence of CD8$^+$ T cells (Figure 7B). This finding was congruent with our previous studies showing Pam2-ODN inducible resistance against bacterial pneumonia despite the lack of mature lymphocytes (*Rag1*$^{-/-}$).

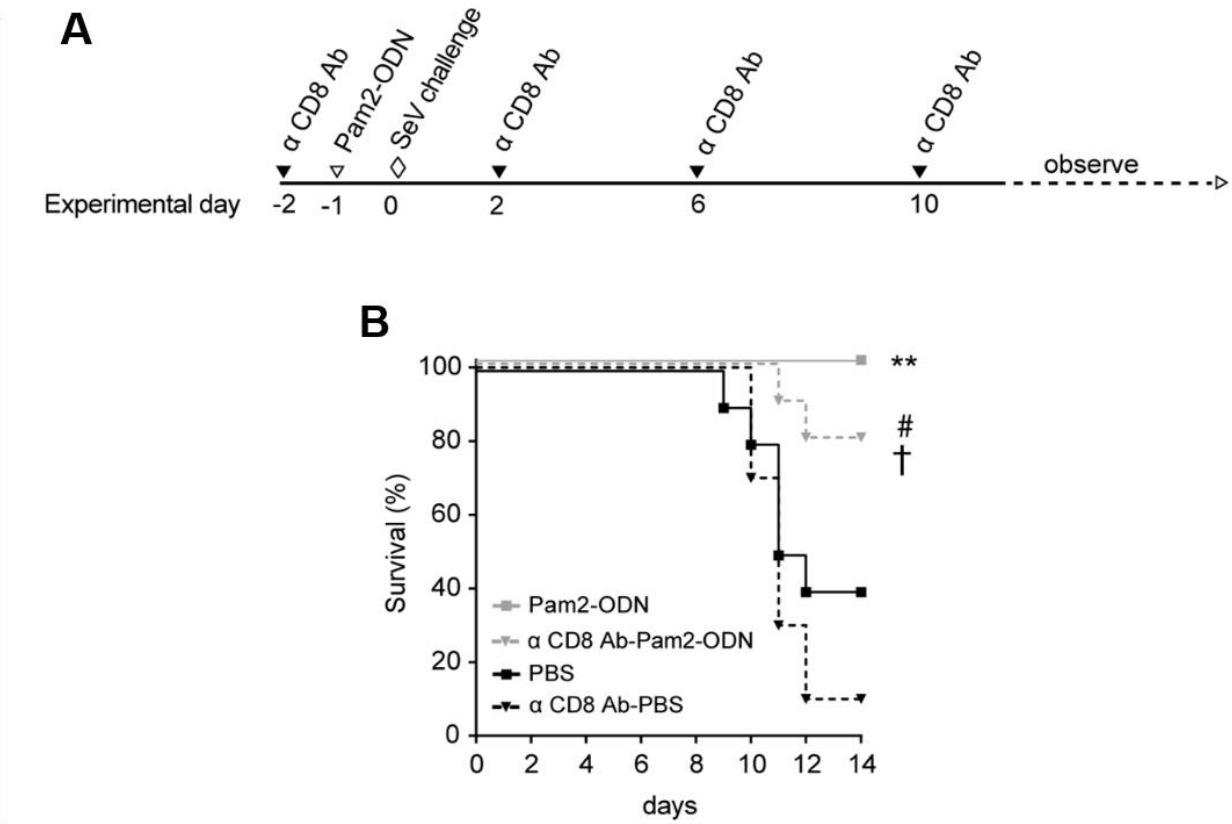

Figure 7: Pam2-ODN treatment protects against SeV infections despite the lack of CD8⁺ T cells. Experimental outline **(A)**, survival **(B)** of mice SeV challenge following PBS or Pam2-ODN treatment, and with or without preinfection $CD8^+$ T cell depletion. ***p*<0.005 compared to PBS, #*p*<0.005 compared to α CD8 Ab-PBS, †*p*<0.05 compared to PBS.

4.7 Summary and discussion

While Pam2-ODN treatment provided a significant host survival benefit in SeV infection, we observed this survival benefit occurring after the time when PBS-treated mice had cleared the virus. This observation prompted the hypothesis that host mortality is not the exclusive result of direct viral injury to the lungs, but due at least in part to the host response to the virus infections. We observed enhanced survival of SeV infections in mice depleted of $CD8^+$ T cells 8 days after infection (Figure 6), revealing the importance of balancing the dual functions of $CD8^+$ T cells in anti-viral immunity and in causing fatal immunopathology. Our findings suggested that the surge in $CD8^+$ T cells within the lungs after most virus has been cleared causes physiologic impairment via lung injury and cell death [59, 61-63].

Previous reports support the concept of counter-balanced immune protection and immunopathology by $CD8^+$ T cells during virus infections [59, 62, 63]. Some reports have shown that antigen-experienced memory $CD8^+$ T cells enhance RSV clearance, but also mediate severe immunopathology [59, 62-64]. However, our study is the first to demonstrate the survival advantage in paramyxovirus respiratory infection of either stimulating the lungs' mucosal defenses early in the infection or of suppressing the $CD8^+$ T cells later in the infection. Our findings are also congruent with reports on the role of $CD8^+$ T cells in non-respiratory viral infection models, such as in West Nile virus infection, where $CD8^+$ T cell-deficient mice display decreased mortality [61].

While host survival of viral infections certainly improved by suppression of $CD8^+$ T cell-associated immunopathology late in infection, some $CD8^+$ T cell-depleted mice were still susceptible to SeV infection i.e. we didn't achieve 100% survival upon depletion of $CD8^+$ T cells on day 8 of SeV infection. This indicates there are other mechanisms of virus-induced host mortality that play a complementary role to $CD8^+$ T cell-associated immunopathology. These might include direct viral cytopathic effects that result in epithelial apoptosis or secondary bacterial infections. Several other virus infection studies in mice have suggested excessive activity of NK cells and neutrophils to be associated with enhanced host mortality [48, 50]. Investigations to resolve the contributions of various factors in inducing host mortality of virus infections will be the scope of future studies.

A limitation of our study is also in addressing the pathology of virus infections that led to mouse mortality. Epithelial cell death and proliferation remain largely associated with the timing of $CD8^+$ T cell infiltration and mouse mortality peak. Therefore, the next step in this area of research will be to address the effect of $CD8^+$ T cell depletion on SeV-induced lung epithelial cell death and proliferation. I hypothesize that the increase in SeV-induced epithelial cell death may reach a threshold level where essential functions performed by lung epithelial cells are impaired. To this effect, some groups have studied the failure of respiratory function in mice infected with lethal influenza strains that succumb to the infection. Mice infected with the PR/8 strain of influenza succumbed between days 9-12 (similar to SeV infected mice in our model) with mortality corresponding to alveolar damage reaching a threshold level beyond which the mice cannot survive [115]. Although

there was no evidence of replicating virus on day 9 onward in infected mouse lungs, the mouse mortality coincided with the loss of type I pneumocytes and reduced ability to take oxygen and expel carbon dioxide from the infected lungs [115]. Thus, poor gas exchange and reduced arterial blood oxygenation could eventually result in compromised lung function leading to host death. These data indicating poor respiration between days 9 to 12 also coincided with poor gross pathology in mice characterized by necrotizing bronchiolitis, diffuse alveolar damage (DAD), fibrin deposition, and hyaline membrane formation along the alveolar lining. Further, the post-mortem report of patients that succumbed to influenza infection revealed DAD with viral RNA present only in a few patients [116]. Another possible contributor to poor respiration is the indirect effect of edema resulting from poor barrier function [115, 117]. Sepsis is found to be associated with severe disease during viral infections, which might result from secondary bacterial infections or a dysregulated pro-inflammatory host response to virus infections. One study found that progression to bacterial sepsis was 6 fold higher in patients with influenza pneumonia [118]. Data collected from influenza-infected mouse models and infected humans support our findings that mortality from respiratory virus infection results from a dysfunctional immune response or impaired viral clearance. In this regard, a clear advantage of Pam2-ODN treatment in mice is the rapid and robust reduction in lung viral burden that leads to reduced host immune response ($CD8^+$ T cells) and reduced epithelial cell death. This is a clear advantage of early viral clearance enhancing mouse survival by reducing the gross pathology of virus infections.

While findings from our study and others reveal that the harmful effects of $CD8^+$ T cell-mediated immunopathology may supersede the benefits of T cell-mediated viral clearance, the question arises of what might be the adaptive value of the vigorous late $CD8^+$ T cell response. One possibility is that it might ensure that the infection does not flare again, but that seems implausible since the host has successfully defended itself against the initial infection, and innate immune mechanisms presumably remain intact and are possibly primed [21, 24, 27], in addition to the multiple adaptive immune mechanisms that increasingly come into play. The possibility that the immunopathology simply results from an error on the part of the immune system also seems implausible given the substantial rate of host mortality, suggesting there is likely an adaptive value to the response. A third possibility, that the persistence of pockets of low level infection might lead to chronic lung pathology, is supported by a recent study showing that sites of viral RNA remnants following influenza infection are linked to chronic lung disease [7]. Thus, a trade-off may exist between the adaptive value of a vigorous $CD8^+$ T cell response to prevent chronic lung disease and the acute mortality it can cause. Manipulating this balance therapeutically will need to account for both the benefits and costs of the response. It is particularly appealing to develop inducible anti-microbial strategies that do not rely on conventional T cell-mediated microbial clearance and are also effective in vulnerable immune-deficient populations.

Chapter 5: Determine mechanisms of Pam2-ODN mediated prevention of virus-induced asthma

The following chapter is based on my publication, used with permission granted by the British Journal of Pharmacology. The figures and data shown in this chapter are entirely my work.

Goldblatt, D.L., J.R. Flores, G. Valverde Ha, A.M. Jaramillo, S. Tkachman, C.T. Kirkpatrick, S. Wali, B. Hernandez, D.E. Ost, B.L. Scott, J. Chen, S.E. Evans, M.J. Tuvim, and B.F. Dickey, *Inducible epithelial resistance against acute Sendai virus infection prevents chronic asthma-like lung disease in mice.* Br J Pharmacol, 2020. **177**(10): p. 2256-2273.

Our lab has found that SeV-induced asthma-like disease can be mitigated by Pam2-ODN treatment one day prior to SeV challenge. While it is clear that the treatment of mice with Pam2-ODN one day prior to SeV challenge reduced viral burden (Figure 1, 2) and that probably directly had effects on reducing virus-induced asthma-like symptoms (mucus metaplasia, airway hyperreactivity, and eosinophilia). However, Holtzman and group showed that SeV infection in mice is translated into a chronic asthma-like disease requiring a persistent IL-33 production in the lungs [6, 8, 9]. Therefore, our data showing reduced SeV induced asthma-like disease

suggested that IL-33 may be reduced by Pam2-ODN treatment and it was important to elucidate this in multiple ways and to understand the cellular source of IL-33 during SeV infection.

5.1 Pam2-ODN treatment reduces IL-33 production from lung cells during SeV infection

We measured IL-33 protein levels in homogenized lung samples at different times during SeV infection with or without Pam2-ODN to understand the time course of IL-33 production in the lungs. We found that SeV infection leads to an increase in IL-33 in mouse lungs from day 21 onward up to day 49 (Figure 8A-C) in comparison to naïve mice or mice treated with Pam2-ODN alone. Of the time points assessed, the highest IL-33 production by SeV infected mice was on day 49, with Pam2-ODN treated SeV infected mice consistently reduced IL-33 production at all time points (Figure 8A-C). By immunohistochemistry, we found naïve or Pam2-ODN alone treated mice showed faint staining for IL-33 in lung cells. Mice challenged with SeV without Pam2-ODN treatment showed a dramatic increase in the frequency and intensity of IL-33 in the lung cells (Figure 8D). However, Pam2-ODN treated, SeV challenged mice displayed drastically reduced IL-33 staining consistent with the ELISA results from day 49 (Figure 8D). Further, to quantify the fluorescence intensity of IL-33, we used immunofluorescence staining of mouse lung sections and found Pam2-ODN treated SeV challenged mice displayed drastically reduced IL-33 intensity (Figure 8E).

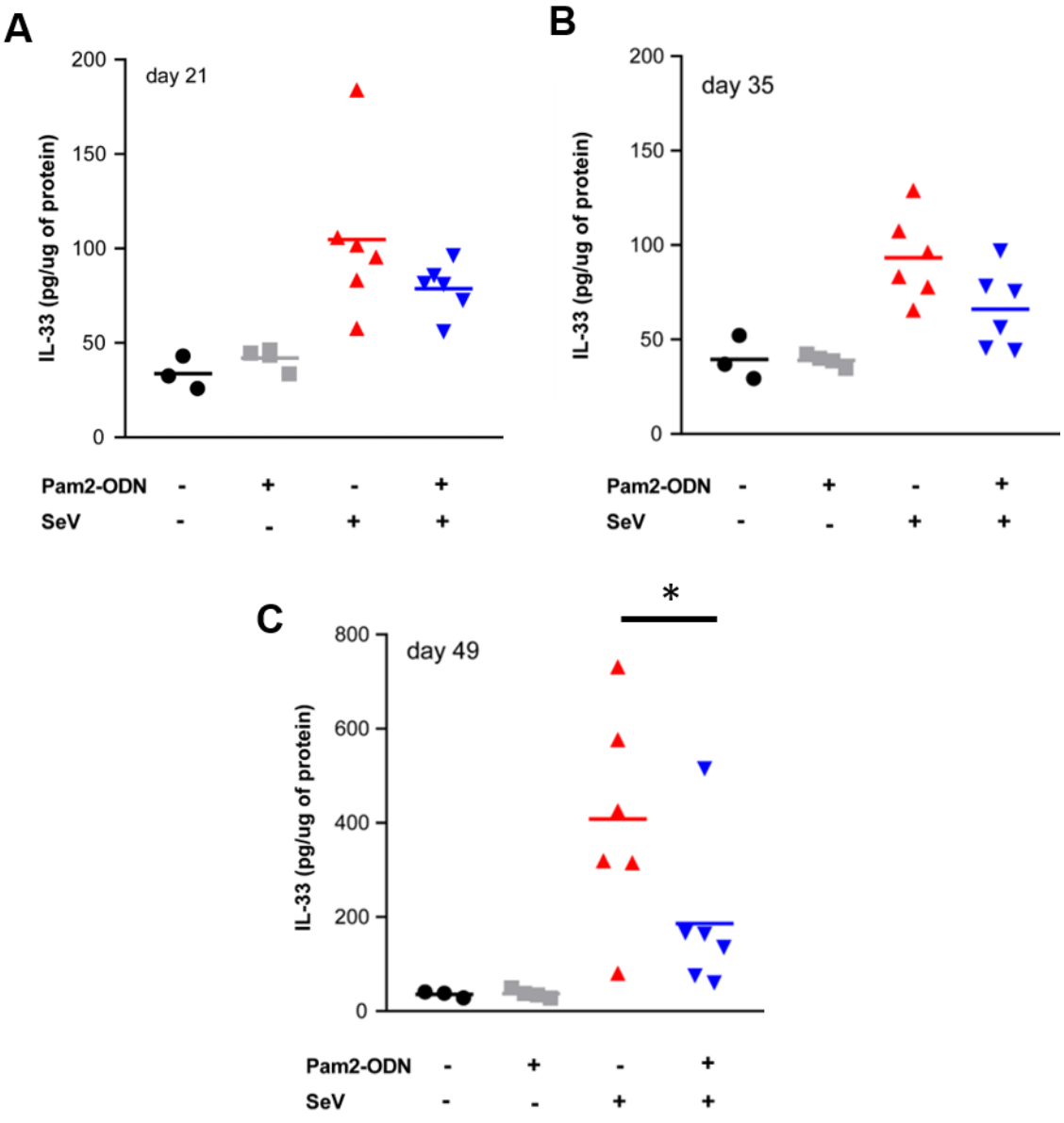

Figure 8: Pam2-ODN treatment reduces IL-33 production from lung cells during SeV infection. IL-33 protein levels from homogenized mouse lungs at day 21 **(A)**, 35 **(B)**, and 49 **(C)** after SeV challenge with or without Pam2-ODN treatment, determined by ELISA. *p<0.05 by unpaired Student's t -test.

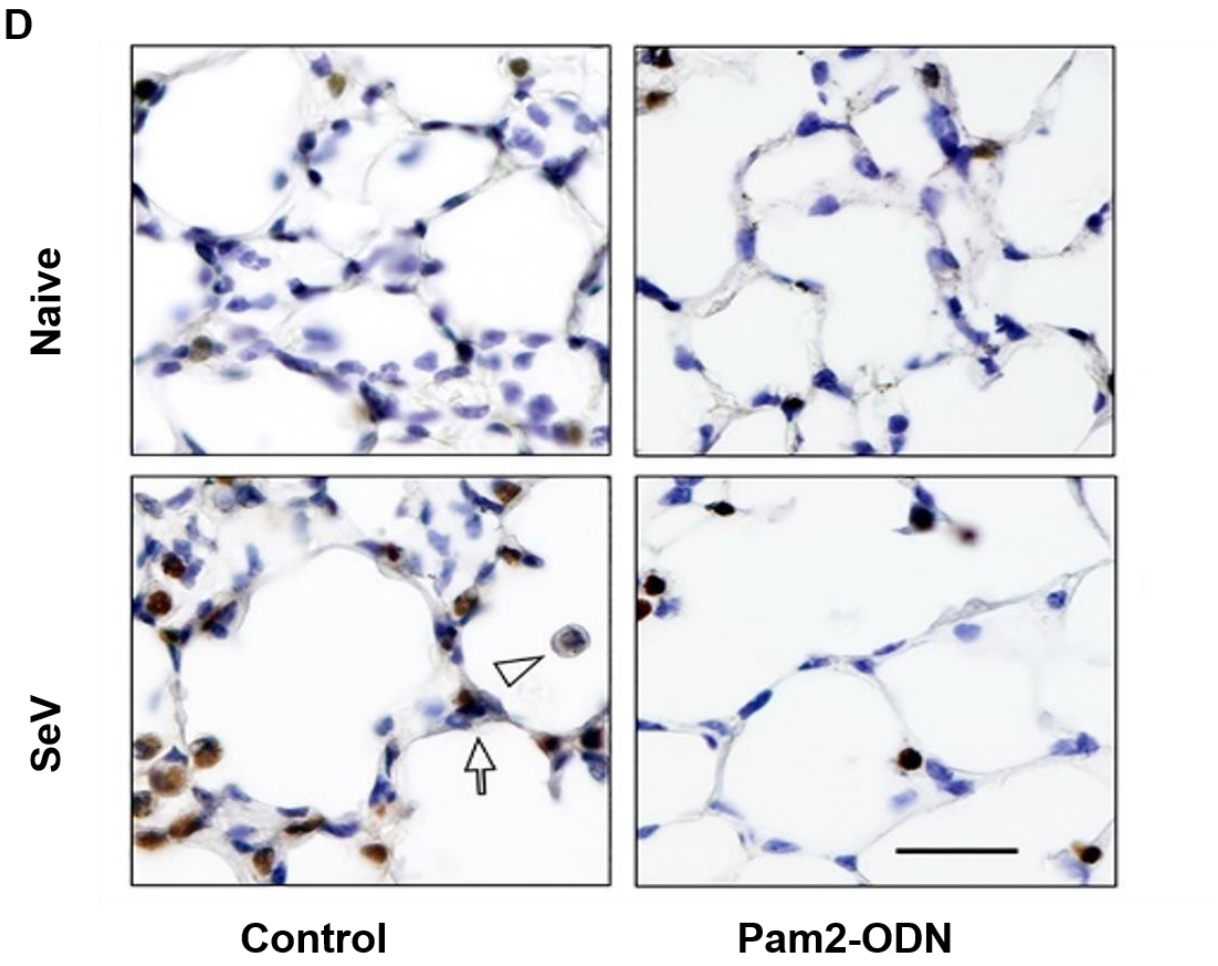

Figure 8 continued: Pam2-ODN treatment reduces IL-33 production from lung cells during SeV infection. (D) Images of mouse lungs from day 49 with or without SeV challenge and with or without Pam2-ODN treatment. The brown color is immunohistochemical staining for IL-33. Arrow points to an intensely stained Type 2 pneumocyte, and the arrowhead points to a faintly stained macrophage. Scale bar = 100 μm.

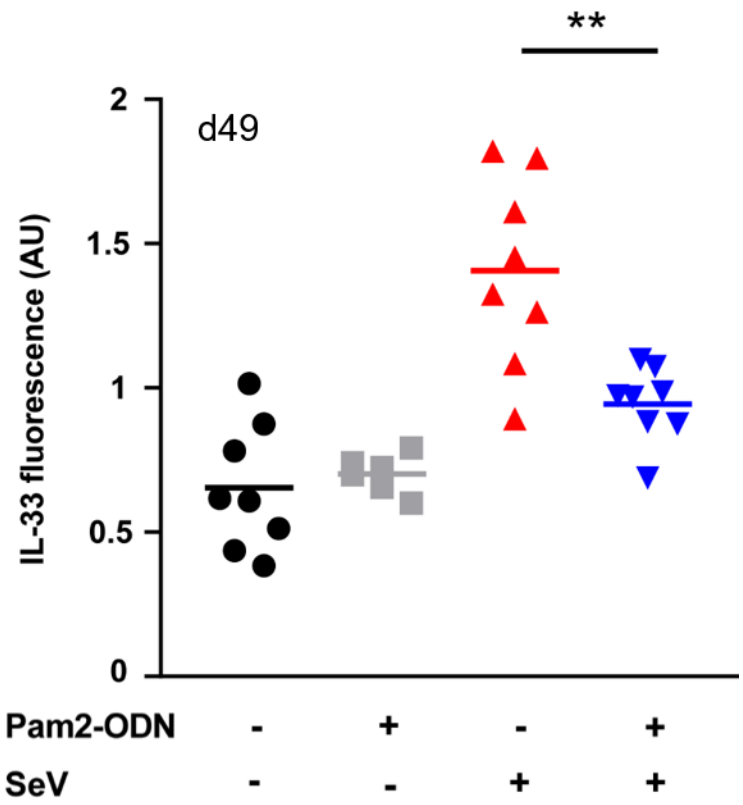

Figure 8 continued: Pam2-ODN treatment reduces IL-33 production from lung cells during SeV infection. (E) Same groups as in **(D)** showing quantification of total IL-33 immunofluorescence intensity. ***p*< 0.01 unpaired Student's *t* -test; n = 6–8 mice, with three fields examined per mouse.

5.2 SeV infection in mice leads to the production of IL-33 from mainly type II alveolar epithelial cells.

Immunohistochemical staining for IL-33 in lung cells indicated localization (corners of polygonal alveoli) and morphology (cuboidal and protruding into the alveolar lumen) of type II alveolar cells (Figure 8B). This finding was congruent with reports about substantial IL-33 expression in type II alveolar epithelial cells in naive mice [119, 120]. Therefore, we performed immunofluorescence co-localization of IL-33 staining with lineage markers and found high IL-33 expression in SeV-challenged mice occurring exclusively in type II alveolar cells, with 97% of IL-33-positive cells also positive for pro-SP-C (pro-surfactant protein C). We found no measurable IL-33 staining in Krt14-positive basal cells of the conducting airways (Figure 9B) or in CD68-positive alveolar macrophages (Figure 9C) (N = 100 cells in 3 mice for each lineage marker). This definitively proved that the predominant source of IL-33 during SeV infection is the alveolar lung epithelium.

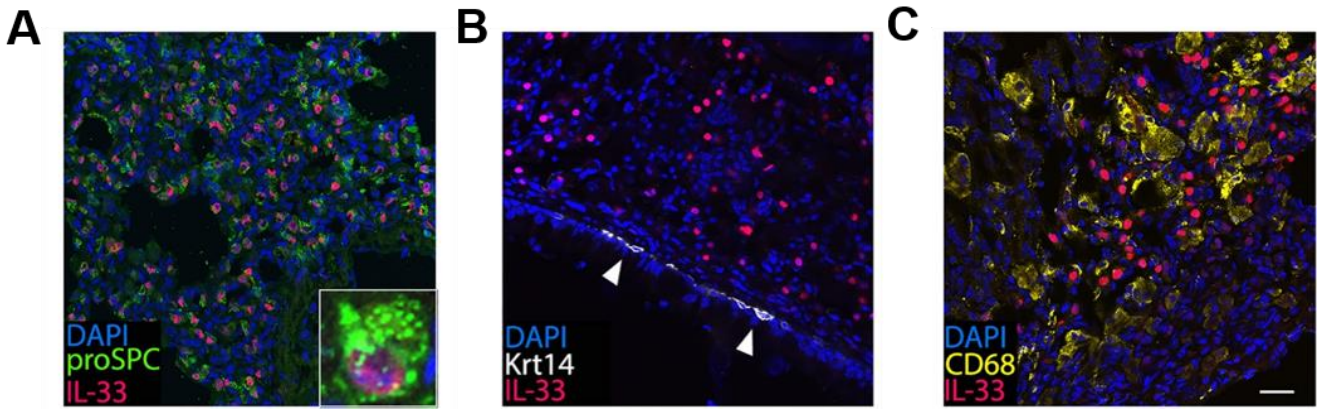

Figure 9: SeV infection in mice leads to the production of IL-33 from mainly type II alveolar epithelial cells. (A-C) Images of the lungs of mice challenged with SeV and then killed 49 days later. **(A)** Fluorescence staining for pro-surfactant protein C (proSPC) to identify type II alveolar cells (green), IL-33 (red), and DAPI to identify nuclei (blue). Inset shows IL-33 expression in a type II alveolar cell. **(B)** Fluorescence staining for cytokeratin 14 (Krt14) to identify airway basal cells (white arrowhead), IL-33, and DAPI as in **(A)**, shows no apparent expression of IL-33 in basal cells. **(C)** Fluorescence staining for CD68 to identify macrophages (yellow), and IL-33 and DAPI as in **(A)**, shows no apparent expression of IL-33 in macrophages (scale bar for (A-C) = 200 μm, and for inset in (A) = 30 μm; n = 3 mice/antibody pair)

5.3 Pam2-ODN treatment after viral clearance reduced eosinophils by reducing IL-33 production in lung cells.

While it became clear that Pam2-ODN pretreatment reduced asthma features by way of reducing the viral burden and IL-33 production in the lungs. This proved the capacity of Pam2-ODN treatment in preventing the progression of SeV infections to asthma-like disease. We now wanted to know whether Pam2-ODN treatment after virus clearance from the lungs can reduce asthma-like disease on day 49, thus acting as an immunomodulator. Therefore, we aerosolized mice with Pam2-ODN 20 days after SeV challenge (when virus is no longer detected in the mouse lungs – Figures 1 and 2) and measured eosinophils as a measure for asthma features. We found reduced eosinophilia on day 49 in mice treated with Pam2-ODN on day 20 after SeV challenge in comparison to untreated SeV challenged mice (Figure 10A). There was no significant difference in BAL eosinophils between mice treated with Pam2-ODN on day -1 or day +20 (Figure 10A). Therefore, Pam2-ODN treatment certainly has an immunomodulatory capacity to reduce eosinophilia. We next hypothesized if the reduction in eosinophilia is due to a reduction in IL-33 by Pam2-ODN treatment on day +20. Therefore, we measured IL-33 protein levels from homogenized lungs by ELISA and found that IL-33 is reduced on day 49 by Pam2-ODN treatment on day +20 of SeV challenge. Like in the case of eosinophilia, there was no significant difference in IL-33 levels from mice treated with Pam2-ODN on day -1 or day +20 (Figure 10B). Therefore, these datasets provide evidence that Pam2-ODN treatment after viral clearance can reduce asthma features such as eosinophils and IL-33 production.

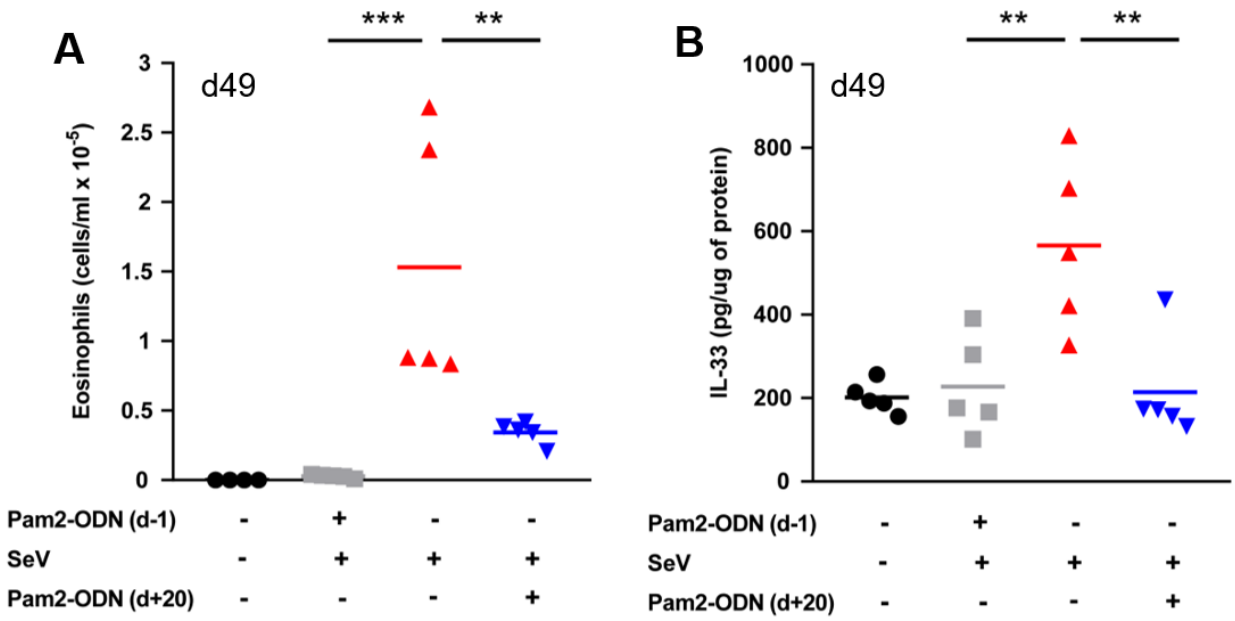

Figure 10: Pam2-ODN treatment after viral clearance reduced eosinophils by reducing IL-33 production in lung cells. (A) Eosinophils after differential Giemsa staining of BAL cells on day 49 of SeV challenge with or without Pam2-ODN treatment at day -1 or day +20. **(B)** IL-33 protein levels determined by ELISA on day 49 of SeV challenge with or without Pam2-ODN treatment on day -1 or day +20. ***p*<0.01; ****p*<0.005; One-way ANOVA with multiple comparisons.

5.4 Summary and discussion

In the previous chapter, we found the major mechanism of Pam2-ODN inducible resistance via reduction of pathogen burden, thereby enhancing the host survival of respiratory infections. However, a major consequence of acute respiratory viral infections is the development of the chronic asthma-like disease. Holtzman and colleagues have demonstrated that an acute SeV infection in mice leads to the production of IL-33 from the airway and type II epithelial cells [6, 9]. And, this IL-33 production is necessary for the development of asthma characterized by eosinophilia, airway hyperreactivity, and mucus metaplasia [9]. Using the SeV model, our lab has found that Pam2-ODN treatment can prevent the progression to asthma-like disease by primarily reducing the viral burden. In this study, we found further evidence that Pam2-ODN can prevent progression to asthma by additionally reducing IL-33 production from lung epithelial cells at all times assessed (Figures 8, 9). The finding that IL-33 was predominantly produced by type II alveolar epithelial cells because of SeV infection was important given that IL-33 was essential in the development of SeV-induced chronic asthma. Holtzman's group showed IL-33 to be expressed in both airways and type II epithelial cells in mice upon SeV infection [9]. Interestingly, we found IL-33 expression only in type II alveolar epithelial cells and none in airway basal cells [110]. Further, Holtzman's group also showed IL-33 to be expressed by airway basal cells in human COPD patients, which is not reproducible in the mouse model of SeV induced asthma disease [9]. Our findings prompted us to investigate whether there are additional benefits to Pam2-ODN treatment other than the reduction in pathogen burden. Interestingly, we found reduced eosinophils on

day 49 of SeV infection when mice were challenged with SeV and then treated with Pam2-ODN after viral clearance (day +20) (Figure 10A).

Given our prior observations that Pam2-ODN inducible resistance is local, it made sense to investigate the extent of epithelial IL-33 reduction in this approach of late Pam2-ODN treatment after viral clearance. Indeed, we found reduced IL-33 on day 49 of SeV infection, suggesting that Pam2-ODN treatment may possess immunomodulatory functions that work by lowering epithelial IL-33. Further, this is an important proof-of-principle finding that suggests therapeutic stimulation of lungs' mucosal responses may redirect the lungs microenvironment into non-inflammatory and protective response and/or induce tolerance against the impact of acute SeV-led disease. One way to understand the mechanisms of epithelial IL-33 reduction by Pam2-ODN treatment is by analyzing transcriptomic changes in the lung at the single-cell level. Future investigations in the lab combining cellular, transcriptomic, and epigenetic changes upon Pam2-ODN treatment are required to uncover the immune gene activity in lung epithelial cells and may explain IL-33 regulation in lung epithelial cells.

These studies may prove helpful in informing future therapeutics on unique epithelial-driven mechanisms on reduction in organ injury and may help in defining host resistance vs host tolerance functions of Pam2-ODN treatment.

Chapter 6: Determine the viral-killing mechanisms of Pam2-ODN treatment in lung epithelial cells

The following chapter is based on my publications, used with permission granted by mBIO and American Thoracic Society. The data and figures presented in this chapter are entirely my work.

The American Journal of Respiratory Cell and Molecular Biology is an official journal of the American Thoracic Society.

(https://www.atsjournals.org/doi/10.1165/rcmb.2020-0241OC)

Wali, S., J.R. Flores, A.M. Jaramillo, D.L. Goldblatt, J. Pantaleon Garcia, M.J. Tuvim, B.F. Dickey, and S.E. Evans, *Immune Modulation to Improve Survival of Viral Pneumonia in Mice.* Am J Respir Cell Mol Biol, 2020.

Kirkpatrick, C.T., Y. Wang, M.M. Leiva Juarez, P. Shivshankar, J. Pantaleon Garcia, A.K. Plumer, V.V. Kulkarni, H.H. Ware, F. Gulraiz, M.A. Chavez Cavasos, G. Martinez Zayes, S. Wali, A.P. Rice, H. Liu, J.M. Tour, W.K.A. Sikkema, A.S. Cruz Solbes, K.A. Youker, M.J. Tuvim, B.F. Dickey, and S.E. Evans, *Inducible Lung Epithelial Resistance Requires Multisource Reactive Oxygen Species Generation To Protect against Viral Infections.* MBio, 2018. **9**(3).

Our findings from chapters 4 and 5 indicate that pretreatment of mice with Pam2-ODN initiates anti-viral effects to enhance host survival, and therefore prevents infected mice from developing asthma-like symptoms. As the antiviral protection consistently correlated with reduced viral burden *in vivo*, (Figure 1 and 2) and as the reduced virus burden likely contributes to the reduced $CD8^+$ T cell levels (Figure 4), we next sought to determine how Pam2-ODN-induced responses cause antiviral effects. We investigated at what stage of viral entry into lung epithelial cells, does Pam2-ODN mediate anti-viral effects. Viral entry into epithelial cells is via attachment to receptors on lung epithelial cells, which mediates the virus entry into the cells. Therefore, we investigated whether the anti-viral effect of Pam2-ODN occurs before or after virus internalization to epithelial cells.

Further, our lab had previously investigated host survival of virus-infected cells with or without Pam2-ODN pretreatment and found that Pam2-ODN treated virus-infected cells demonstrated enhanced survival. More interestingly, Pam2-ODN treatment of cells alone displayed greater enhanced XTT conversion and MitoTracker Red signals without any virus infection [107]. This led to the finding that Pam2-ODN treatment-induced ROS that proved to be necessary for Pam2-ODN mediated anti-viral protection [107]. Therefore, using the SeV model of viral pneumonia, we also investigated whether Pam2-ODN induced ROS is also necessary for anti-SeV protection in isolated lung epithelial cells and in mice.

6.1 Pam2-ODN treatment leads to extracellular inactivation of virus particles

Assessing the effect of Pam2-ODN on SeV burden in immortalized mouse epithelial cells (MLE-15) and primary mouse tracheal epithelial cells (mTEC), we found that Pam2-ODN treatment reduced SeV burden at every time point measured, reflecting the inducible antiviral capacity of isolated epithelial cells (Figure 11). Further, we investigated whether the principal Pam2-ODN effect occurred before (extracellular) or after (intracellular) virus internalization into their epithelial targets. SeV inoculation was carried out at 4º C preventing SeV internalization while allowing SeV attachment to epithelial cells. Using multiple methods to determine the effect of Pam2-ODN on SeV attachment, we found no differences in the attachment of SeV to lung epithelial targets (Figure 12A-D).

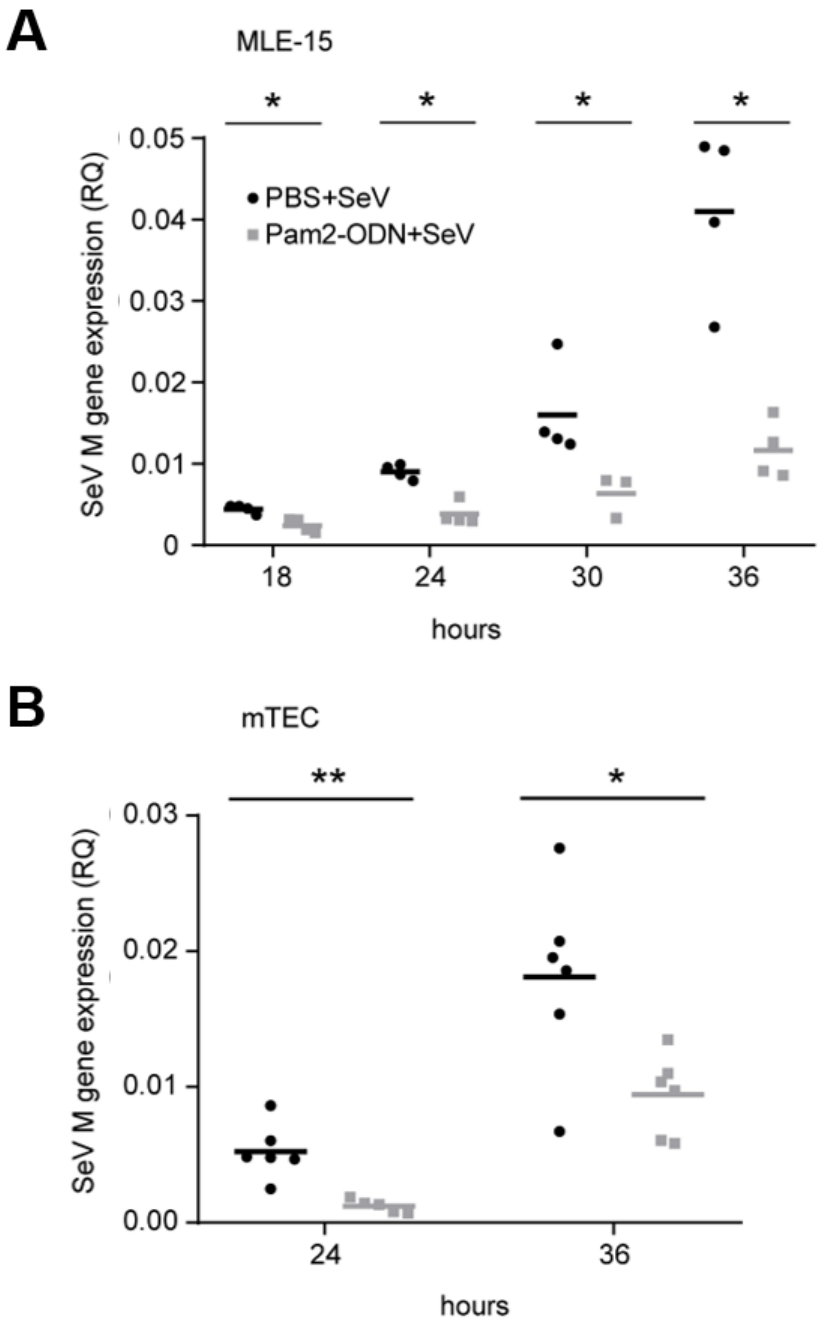

Figure 11: Pam2-ODN pretreatment reduced virus burden in isolated lung epithelial cells. SeV M gene expression assessed by qPCR at various time points upon SeV inoculation with or without Pam2-ODN pretreatment in MLE-15 **(A)** or mTCEs **(B).** *p<0.05, **p<0.01.

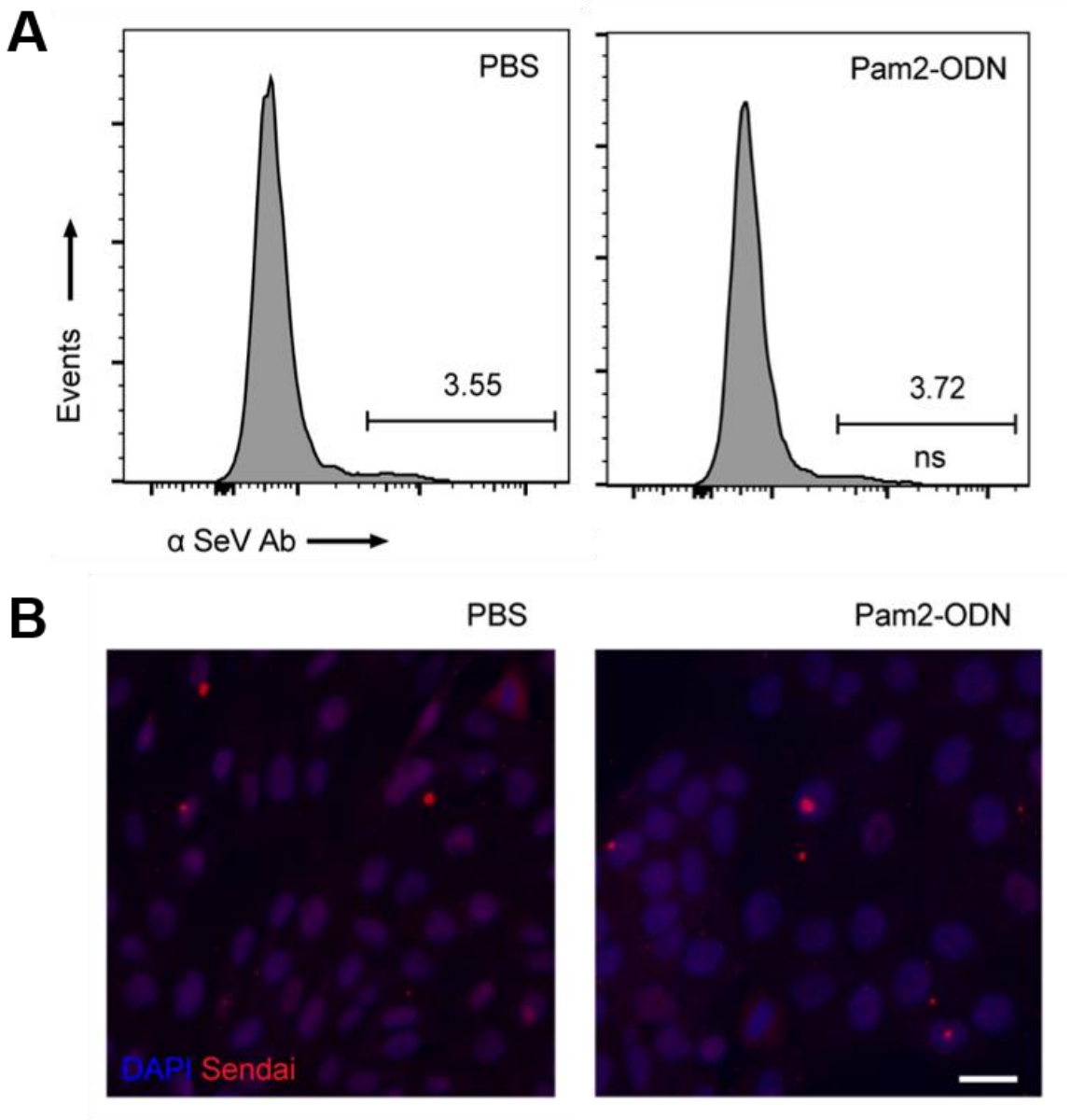

Figure 12. Pam2-ODN inhibits SeV without altering attachment. **(A)** Flow cytometry to measure virus attachment to epithelial cells 4 h after SeV challenge. **(B)** Representative examples of immunofluorescence for virus attachment.

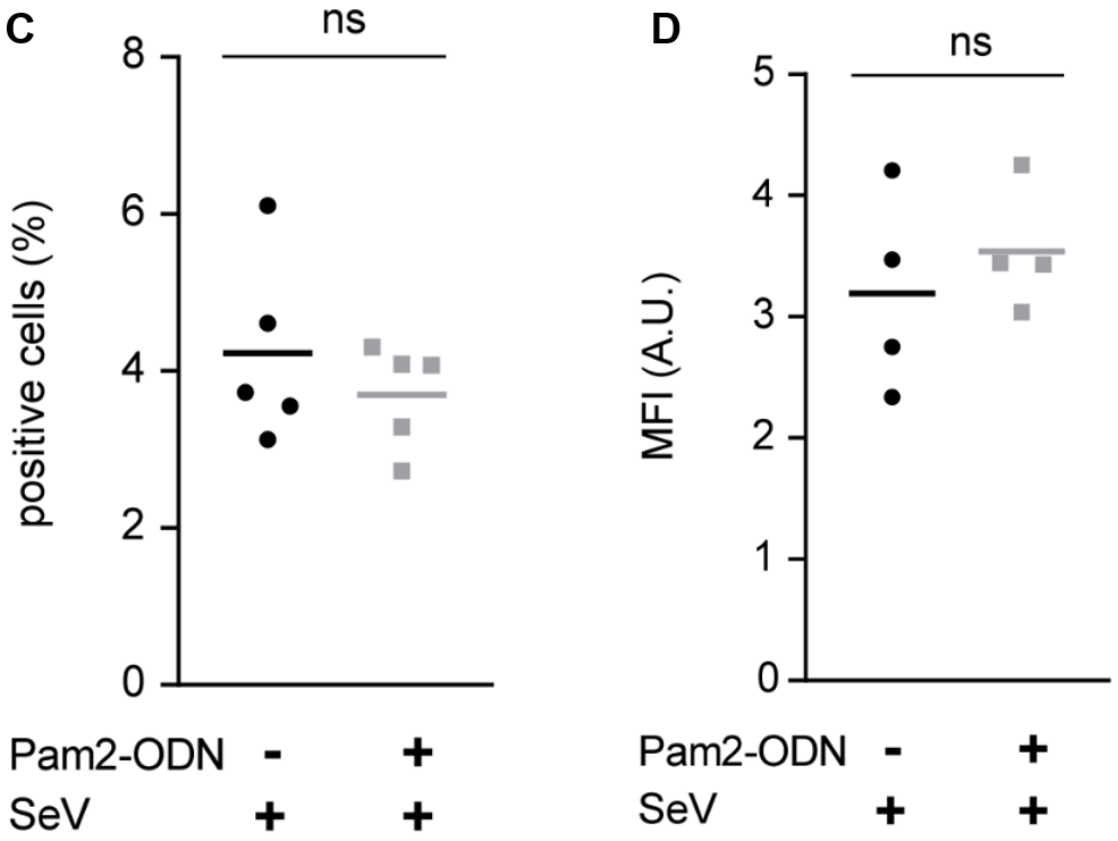

Figure 12 continued. Pam2-ODN inhibits SeV without altering attachment. **(C)** Percentage of SeV positive epithelial cells from **(A)**. **(D)** Mean fluorescence intensity of SeV-exposed epithelial cells 4 h after SeV challenge.

Even though similar numbers of virus particles were attached to epithelial cells, when these attached virus particles were liberated from the epithelial cell targets, virus particles from Pam2-ODN-treated epithelial cells were less able to subsequently infect other naive epithelial cells (Figure 13A-C). As the number of attached virus particles was the same, this difference in SeV burden in cells that

received liberated virus particles from PBS vs Pam2-ODN treated cells indicated that SeV is inactivated prior to epithelial internalization (Figure 13A-C).

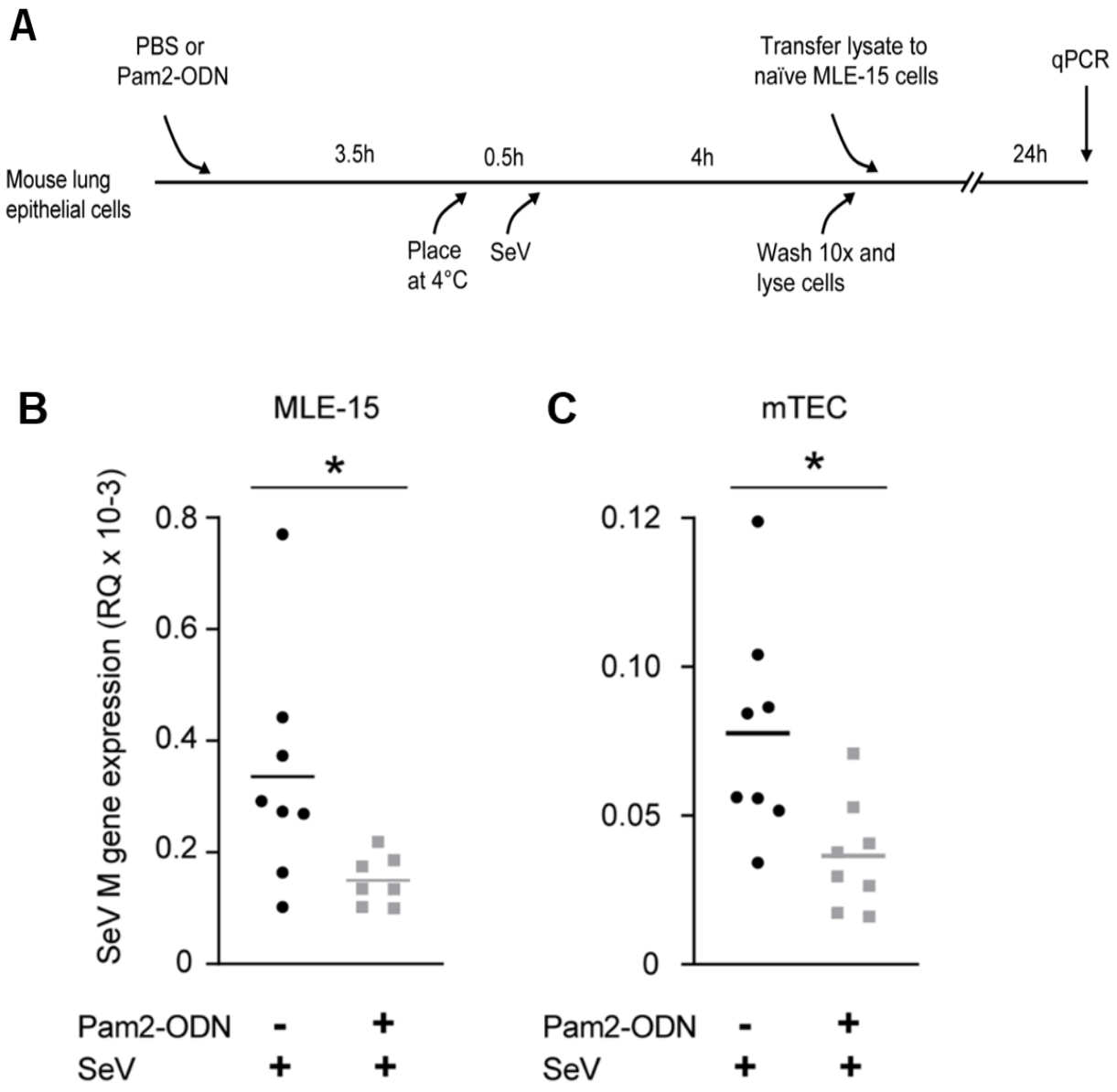

Figure 13. Pam2-ODN inhibits SeV prior to epithelial internalization. **(A)** Experimental outline showing viral attachment and prevention of virus internalization by epithelial cells. SeV M gene expression in untreated MLE-15 cells **(B)** or primary tracheal epithelial cells **(C)** challenged with the liberated virus (uninternalized virus

particles) from cultures that had been pretreated with PBS or Pam2-ODN prior to SeV infection 24 h after transfer of liberated virus to new cells. Data are representative of five independent experiments. *p<0.05

6.2 Pam2-ODN-induced epithelial ROS protect against SeV infection and CD8$^+$ T cell immunopathology

The anti-influenza response initiated by Pam2-ODN requires epithelial generation of ROS from both NADPH-dependent dual oxidase and mitochondrial sources [107]. Extending these findings to the SeV model in lung epithelial cells, an NADPH oxidase inhibitor (GKT 137831) fully abrogated the Pam2-ODN-induced anti-SeV response (Figure 14A). Similarly, treatment with a combination of FCCP (an uncoupler of oxidative phosphorylation) and TTFA (a complex II inhibitor) obviated the Pam2-ODN-induced anti-SeV response (Figure 14B) [107, 109] in isolated lung epithelial cells.

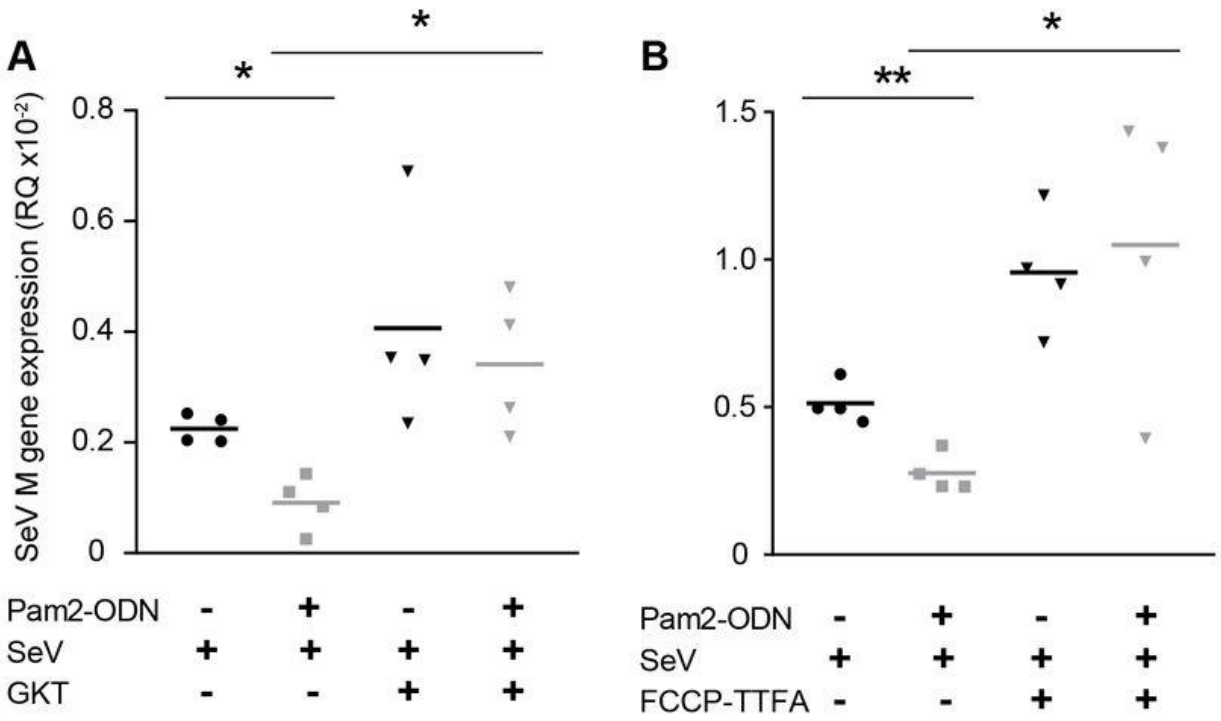

Figure 14: Pam2-ODN induced reactive oxygen species protects against SeV virus infection in isolated lung epithelial cells. SeV burden in MLE-15 cells with or without treatment with Pam2-ODN and/or NADPH inhibitors **(A)** or mitoROS inhibitors **(B)**. Data are representative of three independent experiments. ***p*<0.01, **p*<0.05

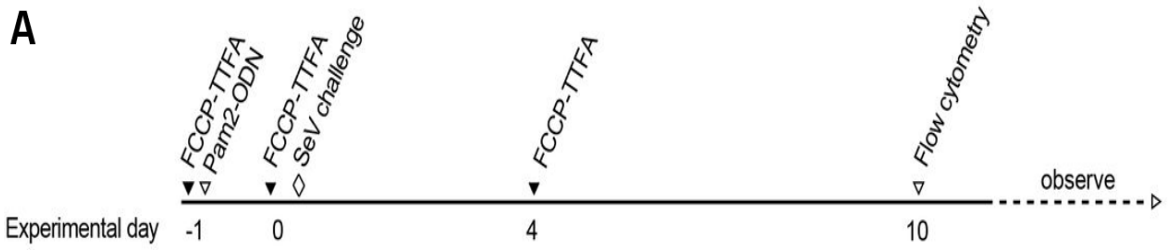

Figure 15: Pam2-ODN induced reactive oxygen species protects against acute SeV virus infections in mice. (A) Experimental outline.

Further, congruent with these *in vitro* studies, mice treated with FCCP and TTFA before Pam2-ODN treatment and SeV challenge (Figure 15A) demonstrated reduced survival (Figure 15B), increased SeV burden (Figure 15C). Since we had earlier found that SeV induced immunopathology and mouse mortality was associated with lung $CD8^+$ T cells on day 10, we found that mtROS blockade in Pam2-ODN treated, SeV challenged mice displayed enhanced lung $CD8^+$ T cells on day 10 (Figure 16). Further, it was found that Pam2-ODN induced epithelial generation of mitochondrial ROS were required for inactivation of SeV before epithelial entry (Figure 17).

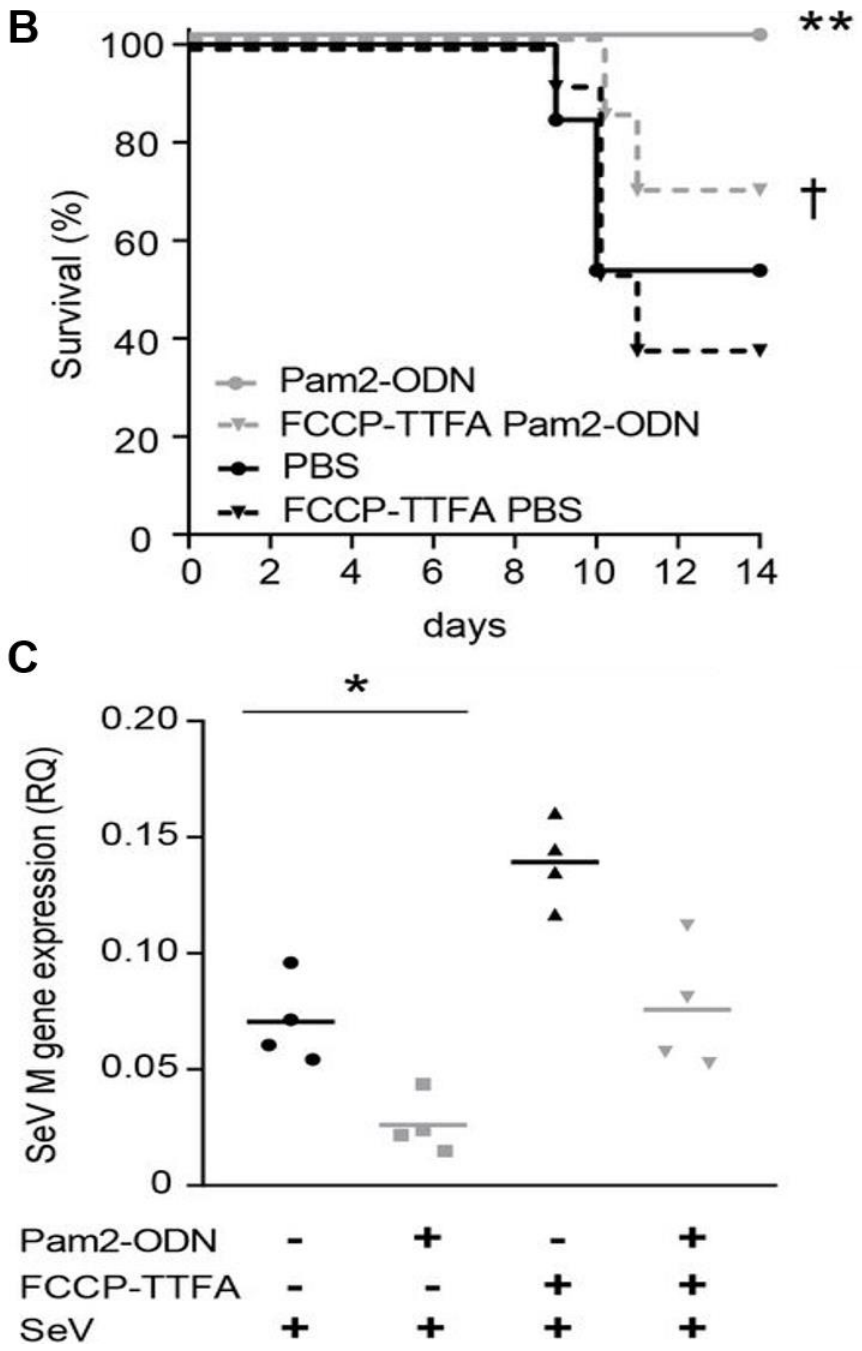

Figure 15 continued: Pam2-ODN induced reactive oxygen species protects against acute SeV virus infections in mice. (B) Survival of SeV challenge in mice treated with PBS or Pam2-ODN and/or mtROS inhibitors. **(C)** Lung SeV burden

measured on day 5. n=13 mice/group in experiment **(B)**. Data are representative of three independent experiments. **p<0.01 compared to PBS, †p<0.05 compared to Pam2-ODN-treated mice without ROS inhibition, *p<0.05 compared to PBS.

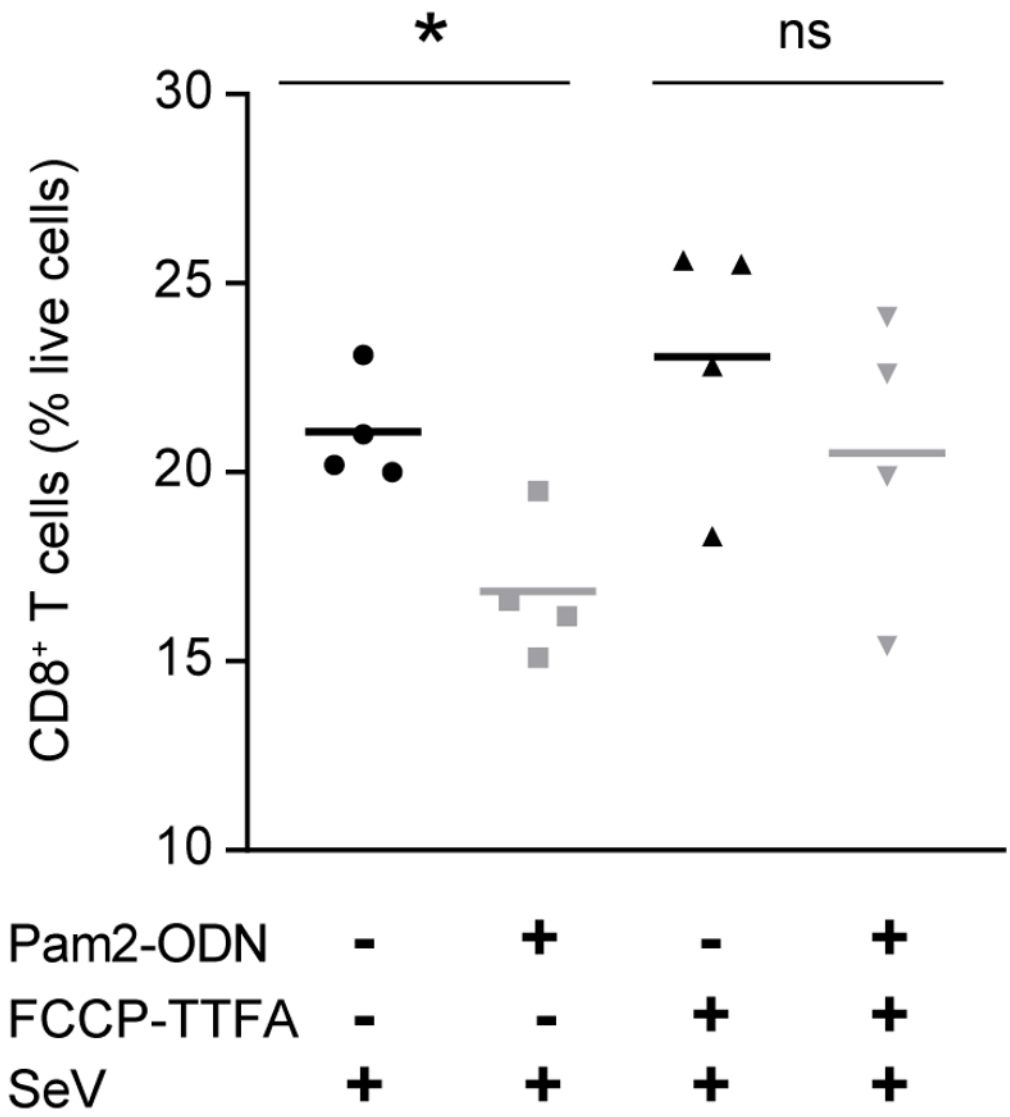

Figure 16. **Pam2-ODN induced reactive oxygen species reduce SeV induced CD8$^+$ T cells**. Lung CD8$^+$ T cells assessed on day 10. Data are representative of three independent experiments. *p<0.05, ns – not significant.

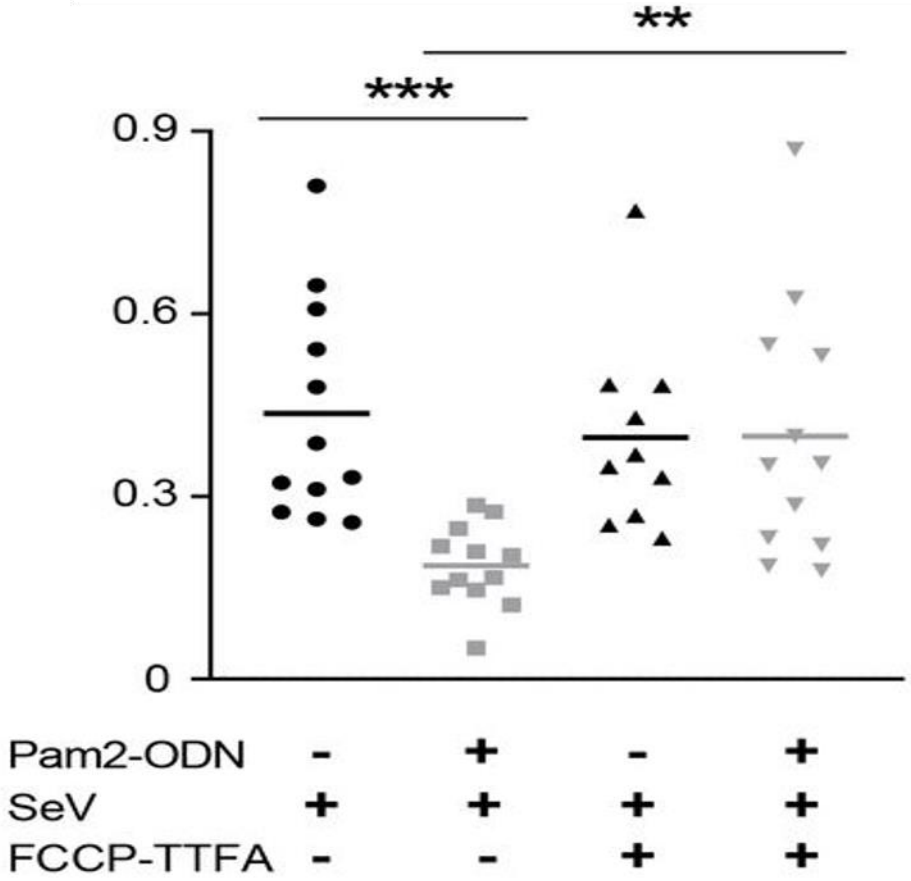

Figure 17: Pam2-ODN induced ROS is essential for SeV inactivation prior to epithelial internalization. SeV M gene expression in untreated MLE-15 cells challenged with liberated virus from cells that had been pretreated with PBS or Pam2-ODN burden and mitoROS inhibitors. ****p*<0.0001; *p*<0.005

6.3 Role of Type I IFN in Pam2-ODN mediated anti-viral effects

In addition to ROS mediated anti-viral protection by Pam2-ODN treatment, we also investigated whether Pam2-ODN treatment-induced type I IFN as an anti-viral mechanism. Prior studies from our lab have demonstrated that there was no association of inducible resistance with type I IFN sensitive genes by microarray analysis [103, 108]. This was evident in isolated lung epithelial cells and mouse lung homogenates after Pam2-ODN treatment [71, 72, 103]. Hence, it was likely that type I interferons were not involved in Pam2-ODN induced resistance against viral pneumonia. However, these studies only demonstrated that Pam2-ODN treatment alone did not lead to induction of type I IFN responsive genes, not whether Pam2-ODN treatment alone and/or together with a viral challenge led to the induction of type I IFNs. Therefore, it was hypothesized that type I IFNs are induced in lung epithelial cells after Pam2-ODN pretreatment and subsequent viral challenge.

6.4 Pam2-ODN mediated anti-viral immunity is mediated in a Type I IFN independent manner

When mouse tracheal epithelial cells (mTECs) were treated with Pam2-ODN and PBS alone, at various times post-treatment, we found no induction of IFN-β (Figure 18). As expected, PBS treated, virus challenged (Flu or SeV) mTECs produced copious amounts of IFN-β congruent with prior reports on IFN induction upon viral infection (Figure 18). These data suggest that while virus infection of epithelial cells led to the production of IFN-β, Pam2-ODN pretreatment of virus

challenged cells significantly decreased IFN-β probably due to reduced viral burden associated with Pam2-ODN treatment. As congruent with prior microarray gene expression data from our lab, Pam2-ODN treatment alone did not produce any IFN at any time points assessed in lung epithelial cells [107].

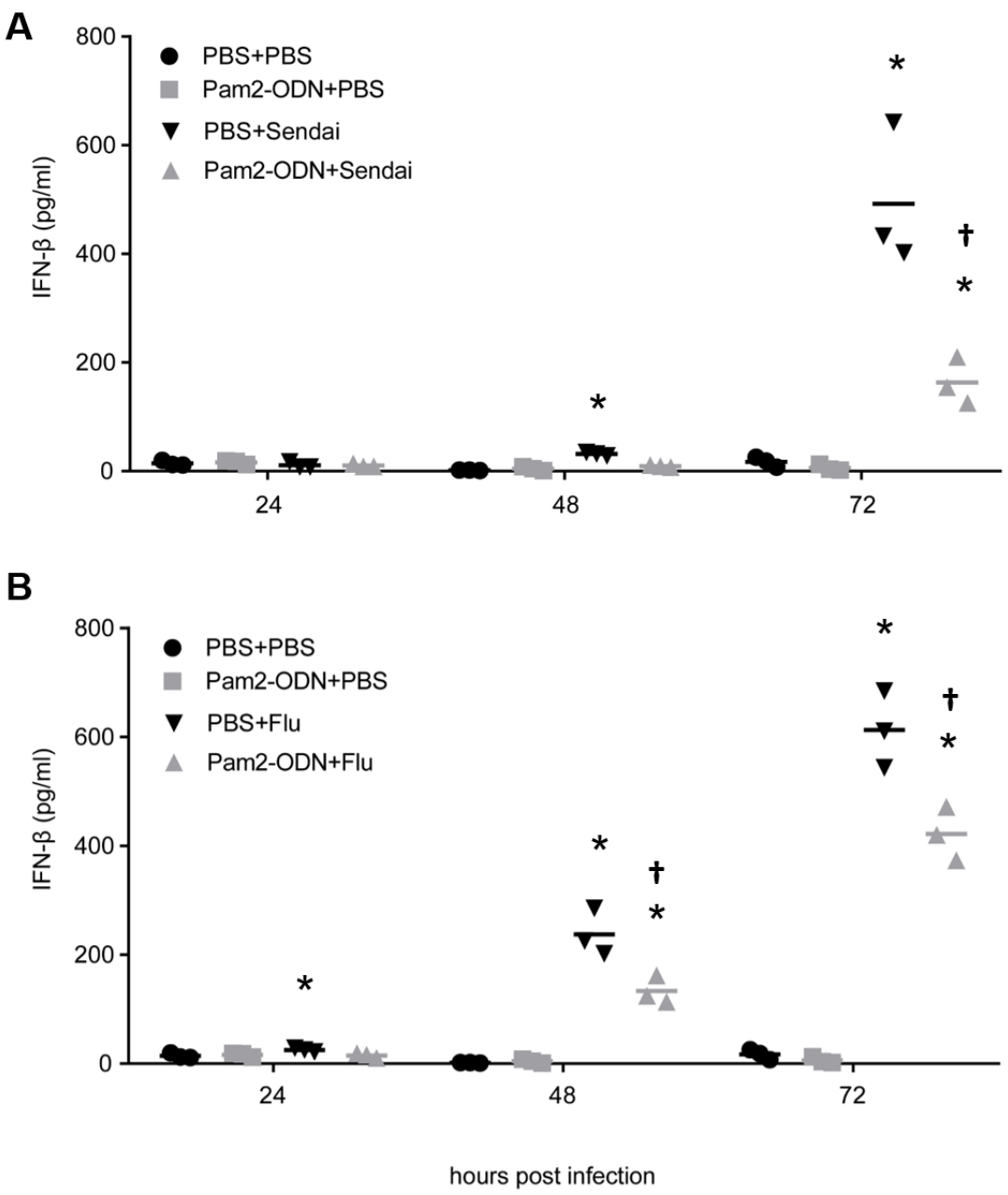

Figure 18: **Pam2-ODN treatment alone or with viral challenge does not induce type I IFN production**. IFN-β levels from mouse tracheal epithelial cells treated with Pam2-ODN or PBS 4 h prior to SeV **(A)** or Flu challenge **(B).** *$p < 0.05$ versus PBS-treated samples; †$p < 0.02$ versus PBS-treated and virus-infected cells.

Since Pam2-ODN treatment alone or with virus challenge did not produce significant IFN-β in lung epithelial cells, we wanted to confirm that Pam2-ODN mediates anti-viral immunity via type I IFN independent pathway. To assess the role of IFN signaling in Pam2-ODN mediated anti-viral immunity, we used $IFNAR^{-/-}$ mice that lack type I IFN signaling. While PBS treated, Flu challenged $IFNAR^{-/-}$ mice displayed enhanced susceptibility to Flu challenge, Pam2-ODN pretreatment of $IFNAR^{-/-}$ mice rescued these mice from mortality of Flu challenge (Figure 19A). Further, the increased virus burden in $IFNAR^{-/-}$ mice was associated with increased mortality of Flu infection (Figure 19A, B). However, Pam2-ODN treatment of $IFNAR^{-/-}$ mice displayed significantly reduced virus burden and did not differ from the WT mice further confirming that type I IFN signaling is not required for controlling virus burden by Pam2-ODN (Figure 19B). These data were remarkably interesting because not only were type I IFN not required for Pam2-ODN mediated protection, but Pam2-ODN treatment rescues these mice from mortality of virus infections despite the lack of IFN signaling.

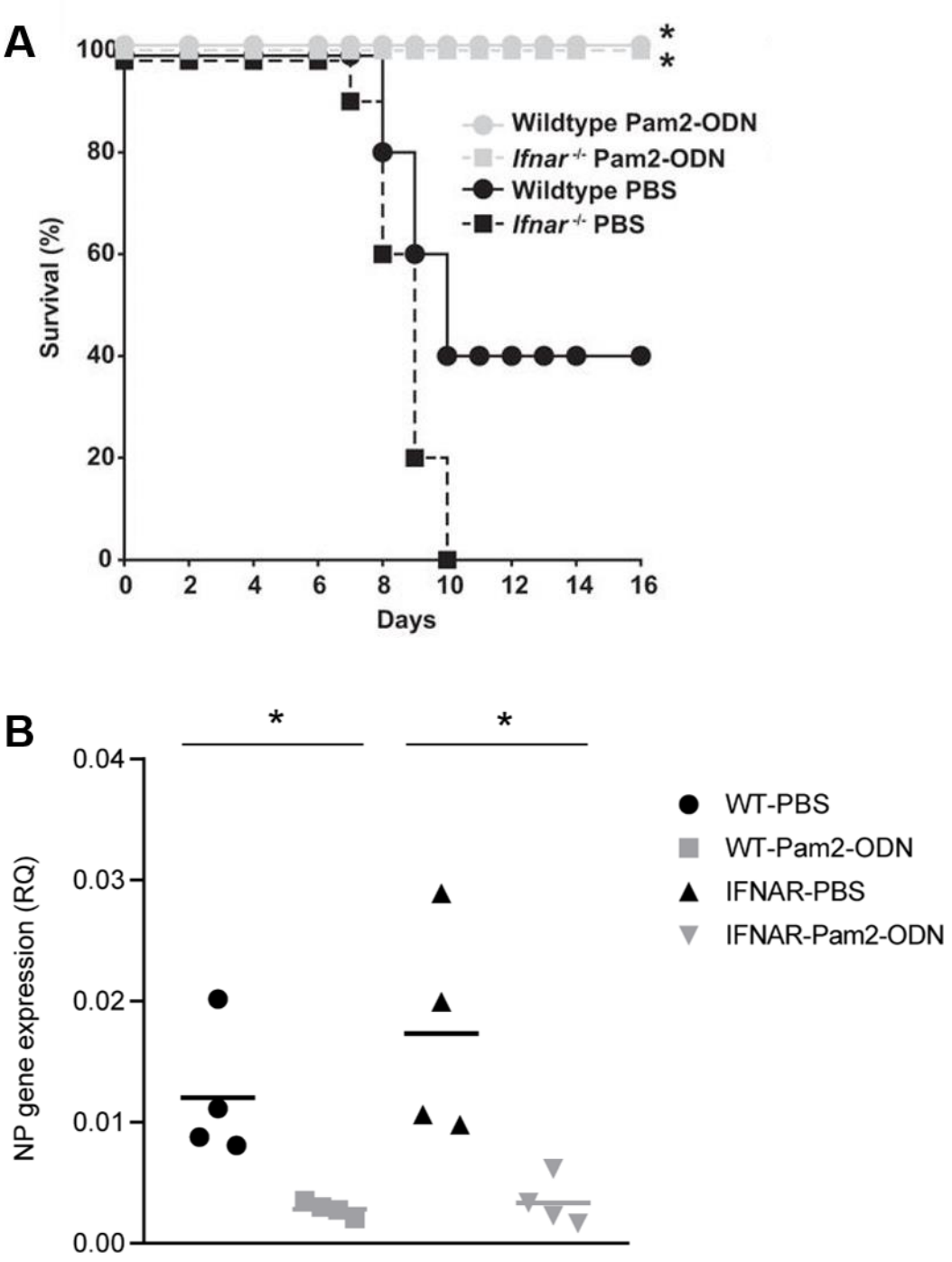

Figure 19: Pam2-ODN inducible anti-viral resistance is independent of type I IFN signaling. (A) Viral burden assessed by Influenza NP gene expression from mice treated with Pam2-ODN or PBS before influenza A challenge in IFNAR$^{-/-}$ or

C57BL/6J mice. **(B)** Mouse survival of influenza A challenge in IFNAR$^{-/-}$ or C57BL/6J mice pretreated with Pam-ODN or PBS.

6.5 Summary and discussion

These data revealed that mouse lung epithelial cells can be therapeutically stimulated to induce robust antiviral responses reducing viral burden and this can, in turn, enhance mouse survival during viral infections. Knowing that Pam2-ODN-inducible resistance required ROS production to protect against influenza [107], we studied the role of ROS in Pam2-ODN-mediated reduction in SeV burden and found that ROS inhibition led to the loss of Pam2-ODN mediated anti-viral effects in isolated lung epithelial cells (Figure 14). *In vivo* ROS inhibition not only led to attenuation of Pam2-ODN's anti-viral effect but allowed increased lung CD8^{+} T cell numbers, implicating Pam2-ODN-induced ROS in preventing both identified mechanisms of mouse mortality in SeV pneumonia (Figures 15, 16). ROS inhibition led to the loss of Pam2-ODN-inducible *in vitro* inactivation of SeV prior to epithelial internalization (Figure 17), demonstrating for the first time that epithelial ROS directly contribute to virus inactivation. While our group has demonstrated inducible resistance against multiple respiratory pathogens including viruses [73, 102, 103] these studies demonstrate for the first time when in the virus lifecycle the anti-viral effects begin (viz., prior to internalization), and substantiate the role of ROS in protection against SeV (Figures 12, 13).

The antiviral protection afforded by Pam2-ODN pretreatment did not require type I IFN signaling, which was congruent with our prior microarray data that did not show any IFN inducible genes (Figure 18, 19). Further, Pam2-ODN treatment enhanced mouse survival of viral infections despite the lack of type I IFN signaling, suggesting its applicability in IFN deficient populations (Figure 19). Importantly, this finding also placed importance on novel type I IFN independent mechanisms such as induction of epithelial ROS from mitochondrial as well as NADPH oxidase sources. Production of ROS as a microbicidal mechanism has been widely reported in phagocytic cells [89-93]. However, this mechanism has not been extensively studied in non-phagocytic cells where it apparently acts predominantly extracellularly rather than intracellularly as in phagocytes. These findings of viral inactivation by epithelial ROS production reveal an essential component of inducible epithelial resistance. Future investigations in the lab will include understanding the role of type III IFNs and inflammasome activation as possible additional anti-viral mechanisms utilized by Pam2-ODN treatment.

Chapter 7: Discussion and Future Directions

In this thesis project, we have demonstrated that therapeutic stimulation of the lung epithelium enhances mouse survival of acute SeV infections by reducing virus burden and attenuating host immunopathology. Further, Pam2-ODN treatment before or after viral clearance prevented the progression to SeV-induced chronic asthma by reducing the viral burden and reducing IL-33 produced by lung epithelial cells.

Our findings are potentially informative in the context of treating viral pneumonia in human subjects, including those suffering from lung injury associated with SARS-CoV-2 (Figure 20). Of note, the median period from SARS-CoV-2 exposure to respiratory distress is 9 to 13 days, identical to the period from viral inoculation to death from pneumonitis in this study of SeV pneumonia in mice [2]. Further, it was recently reported that dexamethasone treatment confers a survival advantage to COVID-19 requiring respiratory support and in those recruited after the first week of their illness [121]. This suggests a stage of disease dominated by immunopathology, rather than active viral replication, similar to our observation in the SeV model nine days after viral challenge. It will be interesting to test the effects of dexamethasone on $CD8^+$ T cell activity and host mortality in the SeV model.

Host survival of acute infections is the goal when defining immunity against infectious diseases. However, there are additional life-long host changes that can be life-threatening long after the host survives the infection. One such example is where respiratory viruses can induce molecular and structural changes in the lungs'

microenvironment leading to chronic asthma [4-6]. Therefore, the outcome of any interventional strategies against respiratory viral infections must not only be host survival but also inducing host tolerance by limiting the health impact caused by virus infections. In that regard, Pam2-ODN treatment of mice before viral challenge seems to be a reasonable approach to prevent the progression of virus-induced asthma. This approach relied heavily on host resistance of infection by limiting pathogen burden. However, we also tested the efficacy of Pam2-ODN in mitigating the SeV-induced asthma-like disease long after viral clearance from the lungs (thus relying on non-viral killing mechanisms). Very interestingly, we found reduced asthma-like features when mice were treated with Pam2-ODN 20 days after SeV infection, revealing the immunomodulatory capacity of Pam2-ODN treatment. Since lung epithelial IL-33 is essential for SeV-induced asthma, it was important to find the extent of IL-33 reduction in late Pam2-ODN treatment. The reduction in lung epithelial IL-33 in mice treated with Pam2-ODN on day +20 of SeV challenge (Figure 10), indicates that Pam2-ODN treatment perhaps directly limits the damage caused by viral infections at the epithelial level. However, other possible immunomodulatory mechanisms may play a role as well since we don't fully understand the mechanism of lung IL-33 production after an acute respiratory virus infection.

Several possible explanations for IL-33 reduction in our model may be inferred from prior literature on IL-33. IL-33 has been traditionally identified as a nuclear alarmin released because of cells underdoing necrosis during infections or other assaults [6, 9, 79, 94, 101]. Virus-led host inflammation and injury may lead to necrosis and as a result, IL-33 is released as a damage signal to initiate repair and

proliferative mechanisms in the lungs. The mice that survive the SeV infection begin to produce IL-33 from day 21 to 49 (Figure 8) (long after acute cell death events - Figure 3), possibly for an indefinite time. Indeed, when mouse lungs undergo repair, SeV-induced proliferation of lung epithelial cells and appearance of progenitor basal stem cells was observed on day 14 (after viral clearance) [122]. These basal stem cells continue to persist on day 49 of SeV infection and possibly long after that. Further, these basal stem cells are normally present in young mice, not abundantly found in adult mice, and function to restore homeostasis and give rise to type II alveolar epithelial cells [6, 9, 113, 122]. And, our finding that type II epithelial cells are the dominant source of IL-33 production during SeV infection might indicate that Pam2-ODN treatment can reprogram the transcriptional machinery in these epithelial basal stem cells to initiate a restorative epithelial cell fate that does not lead to injurious IL-33 production. The reprograming may occur at a transcriptional and/or epigenetic level where changes are persistent, long-lasting, and durable. It remains to be seen however, how the lung epithelial compartment is changed at the cellular and transcriptional level by Pam2-ODN treatment. Therefore, it can be broadly hypothesized that Pam2-ODN treatment in addition to inducing broad anti-microbial responses in the lungs, may also induce a favorable transcriptional program in the lung epithelium initiating reprogrammed cell repair and proliferation pathway to restore homeostasis in the lungs (indicative of host tolerance).

Indeed, prior investigations on transcriptomic changes in mouse lungs and isolated lung epithelial cells after Pam2-ODN revealed a flux of differentially expressed genes (DEGs) that may play a role in inducing a broad anti-microbial

resistance and/or disease tolerance upon a secondary assault [108]. If we combine the lung transcriptomic changes with chromatin mapping analysis, we could potentially find epigenetically poised genes that may have unrealized potential for disease tolerance or protective immune activity. The concept of realized and unrealized epigenetic potential was recently reported in detail in structural cells where low expression of immune active genes coupled with higher open chromatin marks suggested unrealized epigenetic potential which turned on after a systemic viral infection [123]. Similarly, in our model of Pam2-ODN pretreatment, we could hypothesize that the lung epithelial genes with an immune or protective function that are lowly expressed in homeostatic conditions may be epigenetically poised for higher expression upon subsequent infection. These immune function genes may be epigenetically pre-programmed for a rapid response to a variety of immunological challenges such as virus infection in the form of higher cytokine/chemokine production. It will be interesting to also study the effect of Pam2-ODN-induced epithelial ROS in inducing transcriptomic and epigenetic changes in the lungs for the rapid response upon a secondary assault. These are several lines of future investigation in the laboratory to better understand the broad protection afforded by Pam2-ODN treatment.

The discovery that skin epithelial basal stem cells can remember acute inflammation and insults to induce persistent and durable protection against secondary insults is remarkable supporting evidence to our anti-asthma investigations by Pam2-ODN treatment [124]. Therefore, another related mechanism could be that Pam2-ODN induced lung epithelial responses remain persistent and

durable for a long time due to epigenetic training and memory. The support for this hypothesis also comes from multiple studies of trained innate immunity in the hematopoietic system [24, 25, 27]. One could hypothesize that Pam2-ODN treatment before or after viral challenge can induce persisting memory in the lung epithelium of prior insults (virus challenge or Pam2-ODN treatment) that can lead to faster resolution and durability against secondary insults. Comprehensive studies to explore these hypotheses require the use of single-cell sequencing and screening of epigenetic and transcriptional changes in the lungs' epithelium after Pam2-ODN treatment. Further, structural cells such as endothelium and fibroblasts need to be investigated for their involvement in long-term memory since substantial evidence indicating their role in the regulation of systemic leukocyte immune response has now been gathered [123].

Another possible area of investigation is at the IL-33 and group 2 innate lymphoid cells (ILC2) axis. Epithelial IL-33 produced can activate lung dendritic cells and/or ILC2 to initiate a maladaptive type 2 immune response in the airways. The latter series of events may also be an important area of investigation to understand how targeting lung epithelial cells may redirect or reprogram the host leukocyte population to be less inflammatory or maladaptive, especially by the control of lung epithelial cytokines such as IL-33. Indeed, our lab has found that Pam2-ODN treatment also reduces eosinophilic inflammation and airway hyperreactivity in non-pathogenic models of asthma (unpublished data). Therefore, there is clear evidence of immunomodulation that may or may not use the same anti-microbial strategies

used to induce host resistance. Defining resistance and tolerance mechanisms by targeting the lungs' epithelial responses will be an area of future investigation.

Taken together, our findings provide mechanistic insights into the antiviral responses generated by the lung epithelium, prevention of host immunopathology, and asthma-like disease that may inform future therapeutics to target immunomodulation as a means to improve the outcomes of respiratory infections in vulnerable population.

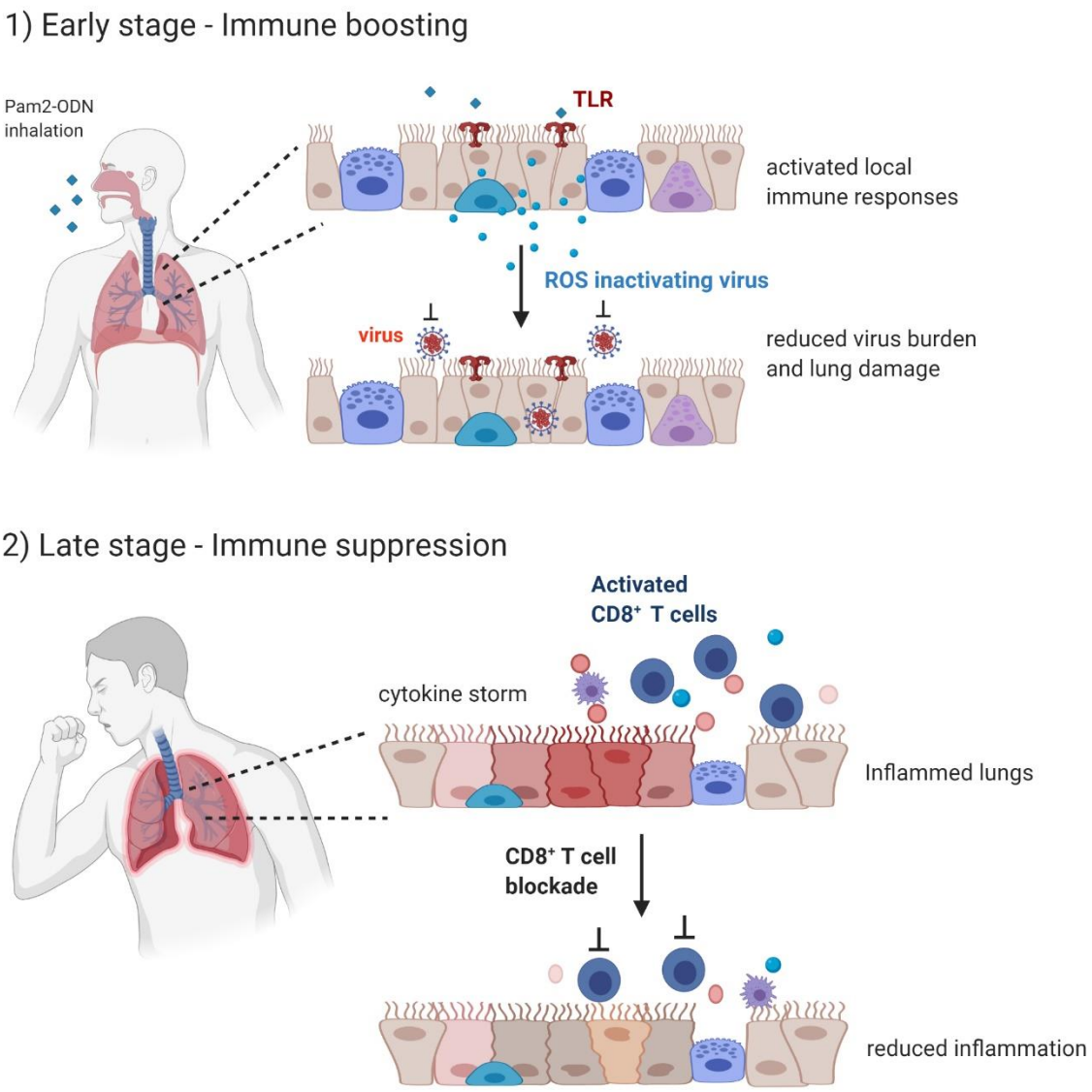

Figure 20: Strategies to improve the outcome of respiratory virus infections.

Two ways to enhance survival of respiratory virus infections – 1) By early stimulation of innate immunity which leads to epithelial ROS inactivating virus particles prior to internalization into epithelial targets, thus reducing viral replication and lung damage and in turn enhancing host survival. 2) By the suppression of host immune response after virus-induced inflammation in the lungs is established, thereby decreasing lung

damage associated with host $CD8^+$ T cells and enhancing host survival. Figure created with BioRender.com.

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