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INTERFERON-DEPENDENT MECHANISMS OF DISEASE PATHOGENSIS IN RESPIRATORY SYNCYTIAL VIRUS INFECTION

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INTERFERON-DEPENDENT MECHANISMS OF DISEASE PATHOGENESIS IN RESPIRATORY SYNCYTIAL VIRUS INFECTION

by

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Dedication

For my amazing family, who have supported me throughout this process and continue to support and motivate me. Thank you for believing in me, motivating me, and inspiring me to be more than I thought possible.

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INTERFERON-DEPENDENT MECHANISMS OF DISEASE PATHOGENESIS IN RESPIRATORY SYNCYTIAL VIRUS INFECTION

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Respiratory syncytial virus (RSV) is the major cause of lower respiratory tract infections (LRTIs) in young children and of hospitalization for any reason in the first year of life. Beside supportive management, specific treatments for severe RSV LRTIs are not yet available and the development of an effective vaccine has been a disappointing task for the past five decades. Although pathogen-specific factors are recognized as important contributing factors, the pathogenesis of RSV bronchiolitis and the cellular/molecular mechanisms that determine its clinical severity remain incompletely understood. A hallmark of RSV is its resistance to the canonical antiviral activity of interferon type I (IFN I) and at the same time its paradoxical disease-enhancing properties which have been suggested in recent studies of human infections or experimental animal models. We have also shown that an important component of RSV-mediated pathogenesis is the production of reactive oxygen species (ROS), which combined with viral-induced degradation of NF-E2-related factor 2 (NRF2), a key transcription factor that controls the inducible expression of protective antioxidant enzyme (AOEs) leads to oxidative injury and lung inflammation. We hypothesize that strategies directed to block IFN I activity and to supplement the airway mucosa with AOEs could serve as new therapeutic approaches for RSV infections. In studies that are described in this PhD thesis we demonstrated by the use of mice genetically deficient in IFN I receptor or by neutralizing anti-IFN I receptor antibodies the mechanistic role of IFN I signaling in the process of RSV-induced NRF2 degradation, AOE inhibition, activation of the inflammasome pathway and ultimately airway inflammation and disease. Furthermore, we showed herein that intranasal administration of the antioxidant enzyme catalase, either prior or following experimental RSV infection resulted in remarkable improvement in inflammation and clinical disease in mice. The protective effect of catalase was associated with reduction in the release of inflammatory cytokines/chemokines and airway obstruction.

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RSV	Respiratory syncytial virus
LRTI	Lower respiratory tract infection
AOE	Antioxidant enzymes
IFN I	Type I interferon
NRF2	NF-E2-related factor 2
PBS	Phosphate buffered saline
PG	Polyethylene glycol
PG-CAT	Polyethylene glycol conjugated catalase
BALF	Bronchoalveolar lavage fluid
ROS	Reactive oxygen species
NK	Natural killer cells

Chapter 1 : Introduction

RESPIRATORY SYNCYTIAL VIRUS (RSV)

RSV is a negative-sense single stranded RNA virus, in the order Mononegavirales, family *Pneumoviridae*, and genus orthopneumovirus (1). Other members of the genus include bovine respiratory syncytial virus and murine pneumonia virus (1). RSV is the leading cause of hospitalizations, pneumonia and bronchiolitis in children <2 years of age, and is also a major cause of morbidity and mortality in the elderly (2;3). RSV has been associated with 22% of all lower respiratory infections in children <5 years old, a cause of 3.2 million hospitalizations, and 33.1 million infections world-wide (4). Despite its high disease burden and annual hospitalization costs of ~ 394 million dollars annually, currently there are no vaccines or effective treatments available for RSV infection (5). Even though Synagis (palivizumab) is an FDA approved medication available for RSV, it is limited to prophylaxis in patients with prematurity or congenital cardio/pulmonary diseases (6). There was a vaccine previously available for RSV infection (formalin inactivated), which was discontinued due to enhanced disease and even mortality in patients experiencing their first RSV infection (7). Currently the mainstay of treatment for RSV infection is supportive therapy consisting of intravenous fluids, and supplemental oxygen.

RSV is spread through droplets and fomite contact, and virus remains viable on surfaces for up to six hours (8). RSV infects people through mucosal membranes and causes pulmonary disease ranging from a mild cold to pneumonia and bronchiolitis (8). Severe RSV infection is characterized by tachypnea, chest retractions, and hypoxemia requiring hospitalization for oxygen supplementation, sometimes through mechanical ventilation (8). RSV infection is characterized by severe epithelial barrier damage resulting in cellular debris and edema leading to airway obstruction and poor gas exchange (8;9). Damage to airways also leads to airway hyperreactivity, which has been linked to the development of asthma later in life (10).

RSV infection is associated with enhanced pulmonary neutrophil infiltration, which provides some antiviral function, but causes significant immune mediated damage to the pulmonary epithelium overall (11;12). Mucosal immunity to RSV is incomplete and often patients are infected several times before acquiring protection against RSV reinfection (2).

RSV is a single stranded, non-segmented, negative sense, and enveloped virus, that has ten genes and eleven proteins. Two of these proteins are surface proteins F and G, which are involved in cellular binding and endocytosis of the virus (8). Although the exact mechanism(s) responsible for RSV cellular entry are still being investigated, several candidates have emerged in recent years as possible receptors responsible for RSV entry into the cells, including CX3CR1 (fractalkine) receptor, epidermal growth factor, Toll like receptor 4, nucleolin receptor, and heparin sulfate proteoglycans (13). The other major RSV proteins include the M protein which stabilizes the viral envelope, the small hydrophobic (SH) protein that helps with internalization, the L protein which is the RNA-dependent RNA polymerase, the NS1 / NS2 proteins which inhibit interferon signaling, and the N protein that forms the nucleocapsid (14).

Although the mechanisms of RSV-induced airway disease and associated long term consequences remain incompletely defined, the lung inflammatory response together with oxidative cell damage are likely to play a fundamental role. RSV infection is associated with high pro-inflammatory cytokine/chemokine responses, including interferon (IFN) production, and pathogenic immune infiltration, as well as enhanced reactive oxygen species (ROS) generation, with concomitant reduction in antioxidant enzyme (AOE) expression, leading to oxidative-mediated epithelial cell damage (15-19). Although inflammatory responses and ROS generation might be important to control virus replication, altogether they result in significant damage to the lung, diminishing its ability for oxygen exchange. All of these concepts will be further discussed in the following sections.

EPITHELIAL BARRIER AND AIRWAY FUNCTION

The epithelial barrier in the lung is composed of two types of pneumocytes (type I and type II), and goblet cells. Type I pneumocytes make up the bulk of the epithelial barrier (96% surface area), and type II pneumocytes make up 4% of surface area and are involved in secretion of surfactant (20). Goblet cells are primarily responsible for secretion of mucous and mucociliary clearance (21). Under normal conditions, the epithelial barrier serves to protect the lung environment from external stimuli, however, during RSV infection the epithelial barrier is disrupted due to degradation of epithelial tight junctions, epithelial cell necrosis, and epithelial sloughing, leading to accumulation of debris in the airways (9;22). Epithelium damage combined with enhanced excretion of mucus and edema in the air spaces results in obstruction of the airways (23). In addition, RSV mediates smooth muscle dysfunction through production of IL-4, IL-5, IL-13, and enhanced smooth muscle contraction to methacholine and other stimuli, resulting in enhanced airway reactivity (24) . In mouse models of infection, measurements of airway obstruction and airway hyperresponsiveness (AHR) can be performed using full body plethysmography via measurement of enhanced pause. Although it is not a direct measure of lower airway reactivity, it does provide a correlate of change from baseline breathing patterns and has been used in several models of infection including influenza, West Nile virus, and RSV (17;25;26). In addition, level of total protein in bronchoalveolar lavage fluid (BALF) is used as a measurement of epithelial leakage/vascular leakage, and damage to the epithelium, in conjunction with determination of neutrophil recruitment, or increased levels of other inflammatory products i.e. myeloperoxidase etc. that induce cell damage (27).

INTERFERONS

Interferons are cytokines produced by cells in response to infection, and signal through their receptors to trigger an antiviral state against microbes (28). There are three types of interferons: type I including IFN-α and IFN-β, which signal through the type I interferon receptor (IFN I), type II interferon consisting only of IFN-γ which signals through type II receptor, and type III

interferons consisting of IFN-λ1, IFN-λ2, and IFN-λ3 which signal through the IFN-λ1 receptor (composed of IL-10 receptor 2 and IFN-λ receptor 1) (28). Although type I and III interferons can be produced by many cell types, type II interferon is primarily produced by immune cells (28). All interferons play important but dual roles in RSV infection. Type II interferon is known to reduce inflammation and affect virus replication, but also enhances airway obstruction, and reduces antibody production (29;30). Similarly, type III interferon has an antiviral role in RSV infection, but is also associated with enhanced disease in patients (31;32). For the purpose of this study, we will primarily focus on type I interferons and their signaling during RSV infection.

Type I IFN production is regulated in RSV infection through pattern recognition receptors, including Toll-like receptors such as TLR3 and TLR4, and the RNA helicases such as RIG-I and MDA5 (33). Several studies have shown that administration of type I IFN prior to RSV infection, but not after infection, can induce an antiviral response resulting in significant reduction in viral titers (34;35). IFN I signaling also enhances fibrosis via matrix metalloprotease activation, and enhanced IFN I stimulated genes are associated with reduced lung function (FEV1/FVC) in asthma patients (36;37). In addition, murine studies with elimination of type I interferon receptor signaling result in a slight increase (~0.5 log) in viral titer, compared to a much higher viral replication difference (2 logs) for STAT1 knockout mice, lacking all types of IFN signaling, indicating that type I IFN activity can be in part substituted by other interferons (38). Altogether these results show that interferon type I signaling needs to be further studied in RSV infection in order to clarify its pathogenic or protective role.

CYTOKINES

Several cytokines were measured in the course of the following studies, in order to further understand the innate immune response during RSV infection. These cytokines include IL-1α, IL-1β, IL-6, IL-9, IL-12, and TNF-α. In terms of general infection, the majority of these are pro-inflammatory and pyrogenic cytokines, such as IL-1α, IL-1β, IL-6, and TNF-α (39). IL-9

is a Th2 cytokine, which is produced following IL-4 expression, and is involved in mast cell growth, and TGF-beta production (40). IL-12 is involved in NK cell activation, and is protective in the influenza model of lung infection, reducing inflammation and reducing viral titer via NK proliferation and activation (39;41).

For the course of RSV infection specifically, most of these cytokines have similar roles. IL-1α is involved with neutrophilia and immune pathology, and also serves as a non-classical marker of necrotic/pyroptotic cell death (42). IL-1β, similarly is produced by caspase 1, and inflammosome activation during RSV infection and is a classical marker of pyroptotic cell death (43;44). IL-6 is produced by alveolar macrophages in early response to RSV infection, and is associated with enhanced clinical disease severity in patients as well (45-47). IL-9 is associated with enhanced airway reactivity and is produced by neutrophils during RSV infection (48). In contrast, IL-12 is protective during RSV infection, and blockade results in enhanced airway reactivity, eosinophilia, and mucus production, while also skewing towards a Th2 response (49;50). However, too much IL-12 signaling can also be detrimental, since the IL-12p40 subunit can form homodimers (IL-12p80), which antagonize IL-12 function and dampen the protective functions of IL-12 (51).

CHEMOKINES

Chemokines are secreted molecules primarily involved in chemotaxis of immune cells to sites of injury/infection. Several key chemokines were also studied in the course of the following studies including CCL11 (eotaxin), CXCL1 (KC), CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES). For general infections, CCL11 is well known to recruit eosinophils to the lung and enhance airway reactivity and inflammation in asthma models (39). CXCL1, is a murine IL-8 homolog, and is involved in neutrophil influx (39). Similarly, CCL2 is involved in chemotaxis of inflammatory monocytes (52). CCL3, CCL4, and CCL5 are all involved in macrophage proliferation, NK cell recruitment, and dendritic cell interactions (53). In addition,

several of these chemokines have been implicated in enhancing airway reactivity including CCL11, CXCL1, and CCL5 (54;55).

During the course of RSV infection, these chemokines serve similar roles, however not all of them have been individually studied in RSV infection. Studies have shown that blockade of CCL11 can reduce eosinophil influx, body weight loss, IL-4 and IL-5 production, and reduce CD4+ T cell influx, without altering viral titers (56). CXCL1 has been associated with enhanced disease in RSV-infected patients, as it is elevated in ventilated patients (57). Blockade of CCL2 has not been studied directly in RSV infection, however it is associated with neutrophilia and lung inflammation in a lung LPS model of disease (58). CCL3 has been directly studied in RSV infection, and CCL3 knockout mice had lower levels of lung pathology and had a reduction in CCL5 and CCL2 levels, without any changes in viral titer (15). CCL4 has not been studied very well in murine or clinical models of RSV infection. An *in vitro* model of RSV infection shows that administration of recombinant CCL4 is not anti-viral (59). Lastly, CCL5 inhibition in RSV infection has been shown to reduce eosinophilia, leukotriene levels, and T cell recruitment resulting in reduced airway inflammation (60). Overall these chemokines are involved in modulating the early immune response to RSV, but do not appear to be crucial in antiviral responses.

IMMUNE CELLS

In this study, we have focused on the innate immune response to RSV infection, and several key cell types are critical in establishing this response. These include alveolar macrophages, neutrophils, lymphocytes and eosinophils. Alveolar macrophages are one of the resident cells already present in alveoli, and are responsible for controlling pathology and airway obstruction (61). In addition, they are responsible for the production of type I interferons, IL-6, TNF-α, GM-CSF, CCL4, and CCL5 during RSV infection (61). Alveolar macrophages are major mediators of antiviral effects, although this effect is not dependent on type I interferons (62). In addition, they are also involved in natural killer (NK) cell recruitment and activation (63). Another major cell

type involved in RSV infection is neutrophils. Neutrophils have a dual role in RSV infection, by providing antiviral effect, while also enhancing pathology (12). Neutrophil elastase and IL-9 production have been associated with enhanced airway reactivity and bronchiolitis in RSV infection (12). In addition, massive neutrophil numbers are present in patient tissues obtained post-mortem after RSV infection (22). Another leukocyte cell type recruited to the lungs is eosinophils. Eosinophils also provide antiviral activity against RSV in a MyD88 and nitric oxide dependent manner, however they are also associated with enhanced risk of asthma and increased airway reactivity (64;65). In addition, studies with patients have also shown that RSV infection leads to prolonged eosinophil activation, providing a possible link to asthma development associated with RSV infection (66).

Several lymphocyte cell subsets also play a crucial role in RSV disease pathogenesis including NK cells, innate lymphoid cells (ILC), CD8, and CD4 cells. NK cells are involved in controlling RSV viral replication, but are also associated with enhanced pathology, and depletion results in improvements in body weight and reduced inflammatory cell infiltration (67). For ILC's there are three different classes (ILC1, ILC2 and ILC3). RSV is known to enhance levels of lung ILC2 cells, which start increasing after 3-4 days post infection, and are associated with production of IL-13, enhanced airway reactivity, and enhanced body weight loss, without any effects on viral replication (68). CD4 cells are associated with enhanced disease in RSV infection, with production of IL-9, IL-13, and IL-17, and are also upregulated in patients with severe disease (69;70). Lastly, CD8 cells are involved in providing effective antiviral activity, but are also associated with enhanced immunopathology, mediated by interferon gamma and TNF-α (71). Altogether, although these cells play an important role in clearance of the virus, the clearance comes at a cost of damage to the tissue environment.

REACTIVE OXYGEN SPECIES AND ANTIOXIDANT ENZYMES

Reactive oxygen species (ROS) are produced in the course of RSV infection by the reduction of oxygen into superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH•), and they play a significant role in causing tissue damage and oxidizing DNA, proteins, and lipids (72). Several enzymes are involved in the generation of ROS species including lipoxygenase, xanthine oxidase, cyclooxygenase, and nicotinamide adenine dinucleotide phosphate (NADPH) (72). Due to their volatile nature ROS and enzymes generating them are carefully regulated. In order to balance their effects several antioxidant enzymes (AOE) are also produced that can quench these ROS species. These enzymes include superoxide dismutases (SOD), which converts O_2^- into H_2O_2 . There are three isoforms of SOD, including SOD1 (cytosolic), SOD2 (mitochondrial), and SOD3 (extracellular) (72). Additional AOE, include catalase and glutathione peroxidase, which are able to convert H_2O_2 to CO_2 and H_2O, which are then excreted by cells. Additionally, neutrophil products (myeloperoxidases etc.) are further able to convert H_2O_2 to OH•, which is also a potent mediator of disease (72).

RSV is a potent inducer of ROS species, from epithelial cells, neutrophils, and eosinophils (73-75). In addition, RSV also reduces levels of AOE including SOD, catalase, glutathione peroxidase etc. (18;76). This imbalance leads to increased lung cellular oxidative damage which plays an important pathogenic role, as antioxidant treatment significantly ameliorates RSV-induced clinical disease and pulmonary inflammation (17). Several studies have administered exogenous AOE to determine if re-balancing the antioxidant cellular capacity can ameliorate RSV disease severity. SOD 1 and 2 administration, has been shown to have an antiviral effect in cotton rats infected with RSV, however, their effect on other disease parameters was not studied (77). In addition, EUK mimetics which contain catalase, glutathione peroxidase, and SOD activities, were also successful in decreasing RSV-induced cytokine/chemokine production, and lower ROS levels *in vitro* (76). Although catalase administration has not been directly studied in RSV infection, supplementation in an influenza model was associated with enhanced survival and reduced viral titers (78). Furthermore, catalase

was also shown to be beneficial in models of acute lung transplant injury, pulmonary fibrosis, and hyperoxia mediated injury (79-81).

NUCLEAR FACTOR ERYTHROID 2-RELATED FACTOR 2 (NRF2)

NRF2 is a transcription factor responsible for regulating AOE gene expression through the antioxidant response element (ARE) (82). Although other viral infections result in upregulation of NRF2 i.e. hepatitis B, hepatitis C, human cytomegalovirus etc., RSV uniquely leads to a reduction in levels of NRF2 (83). NRF2 is normally bound to its inhibitor Kelch-like ECH-associated protein 1 (Keap1) in the cytosol, and oxidative stress causes dissociation of Keap1, allowing NRF2 nuclear translocation (82). RSV infection-induced decrease in AOE levels is mediated by inhibition of NRF2 activation. In the course of RSV infection, nuclear NRF2 undergoes several modifications including de-acetylation, SUMOylation (small ubiquitin-like modifier), and SUMO dependent ubiquitination, resulting in proteasome-mediated degradation, which occurs in a promyelocytic leukemia protein-nuclear bodies (PML-NBs)-dependent manner (83;84). Few studies have investigated modulators of NRF2 in RSV infection, including sulforaphanes and butylated hydroxyanisole (BHA). Administration of sulforaphanes was associated with increased levels of NRF2, and reduced neutrophilia, eosinophilia, and a decrease in RSV replication (85). Treatment with BHA strongly reduced lung oxidative damage, neutrophil recruitment, and cytokine/chemokine production during RSV infection (17). These studies show that direct modulation of NRF2 could also be used as a potential therapeutic strategy to ameliorate RSV lung disease.

PML AND PML-NUCLEAR BODIES

PML protein is the primary protein involved in formation of PML-NB's, and a member of the tripartitie motif (TRIM) family, which is upregulated by type I and II interferon in viral

infections (86;87). PML knockout mice are unable to form PML-NB's (88). PML is a single gene, composed of 9 exons which are alternatively spliced in humans into seven distinct isoforms (89). The isoforms are identical in their N-terminal region, and are differentially truncated at the C-terminal region (89). All isoforms translocate to the nucleus, except for PML VII (89). The RING finger motif in PML protein, allows formation of PML-NB's due to multimerization (86;90;91). In addition, C-terminal regions are involved in recruitment of other protein components of PML-NB's (92;93). These other components include SP100, DAXX, BLM, SUMO1, and NDP55 (94). PML is upregulated in infection by type I interferon, and has varying roles including tumor suppression, apoptosis, cell cycle regulation, protein-protein interactions, and antiviral activity against herpes simplex virus 1, adenovirus, human cytomegalovirus, HIV, influenza A, etc. (86;87;95-103)

Recent studies have shown that RSV is also able to induce PML-NB formation, through upregulation of PML levels, in a type I interferon-dependent manner (84). PML-NB's recruit SUMOylating enzymes, and SUMO-specific E3 ubiquitin ligase-RING finger protein 4 (RNF4), resulting in SUMOylation, and SUMO dependent ubiquitination of NRF2, leading to its degradation (84). Inhibition of type I IFN production or signaling prevents degradation of NRF2 and results in higher AOE levels (84).

PYROPTOSIS AND INFLAMMASOME ACTIVATION

There are three established forms of cell death caused by infection apoptosis, necrosis, and pyroptosis. Apoptosis is programmed non-lytic cell death, and necrosis and pyroptosis are lytic cell deaths, which release cellular components into the lumen (104). Pyroptosis is cell death caused by caspase 1 activation, which cleaves gasdermin D resulting in pore formation in the cell membrane and release of cellular contents (104). Caspase 1 is also responsible for cleavage of pro-IL-1β to IL-1β, which is sometimes used as a surrogate for caspase 1 activity (104). Inflammasomes are a key mediator of pyroptotic cell death, and are composed of Nod-like receptor family members NLRP1, NLRP3, NLRC4, and the adapter protein ASC (44). In the

course of RSV infection, NLRP3 inflammasome is activated and is responsible for activation of caspase 1 (105). In response to RV infection, the NLRP3 inflammasome is activated via TLR2/MyD88signaling, leading to NF-κB translocation and signaling in the nucleus (105). Intracellular expression of RSV small hydrophobic protein has also been associated with activation of the NLRP3 inflammasome (106). In addition to IL-1β, pyroptosis results in release of high-mobility group box 1 (HMGB1) (107), which in turn has been shown to trigger pyroptosis once it is endocytosed by macrophages (108). Altogether, RSV infection leads to significant cell death via pyroptosis, through the activation of the NLRP3 inflammasome, and caspase 1 pathway. Therefore, caspase 1 levels and HMGB1 can be utilized as markers to determine enhancement/reduction in pyroptosis during RSV infection.

Chapter 2: Role of IFN I Signaling in Innate Immune Responses to RSV Infection

INTRODUCTION

In this chapter we have further studied the role of type I interferon signaling during RSV infection. Type I interferon is involved with generating an antiviral environment during viral infections. However, this is not always the case for each virus type and several recent studies have shown the detrimental role of acute/chronic type I interferon production in viral infections including Zika virus, HIV, HBV, HCV, and LCMV (109-112). In these models, type I interferon activity caused enhanced pathology , chronic low level viremia, and exhaustion of the T cell response, and blockade of type I interferon, ameliorated these disease parameters. Similarly, RSV is poorly responsive to the antiviral activity of type I interferon, which is in fact correlated with enhanced disease severity, neutrophil mediated inflammation and decreased airway function in patients (113-115). In addition, in murine models of

infection complete knockdown of type I interferon signaling only results in a modest increase in viral titer of ~0.5 log, compared to a ~2 log increase following STAT knockdown (38), showing the possibility that other interferons (II and III), play redundant and possibly more antiviral/protective roles. In addition, no changes were observed in immune cell recruitment, eosinophil numbers, or overall changes in pathology with specific type I interferon receptor knockdown (38;116). In contrast, administration of interferon β is associated with enhanced matrix metalloprotease activation, and airway hyperresponsiveness in mice (36). Therefore, in this study we tested the hypothesis that type I interferon is not significantly antiviral, and its blockade is potentially more beneficial in terms of controlling the immune mediated pathology.

We also studied the role of type I interferon in RSV infection, to rectify the contrasting results present in literature regarding the protective/detrimental role of type I IFN in RSV infection. Some studies have found, that although knockout of type I interferon receptor results in a significant reduction in pro-inflammatory cytokines and chemokines, in RIG-I signaling, and neutrophil influx, it also enhanced body weight loss (117). In addition, other studies have also shown that administration of type I interferon only prior to infection (as either IFN-α or Poly IC) results in reduction of viral replication (34;118). However, administration of IFN-β is also associated with enhanced fibrosis and matrix metalloprotease activation, further highlighting a dual role of type I interferon signaling in RSV infection (36). In addition, several clinical cases of patients with defective type I interferon signaling showed unremarkable effects on viral clearance or the course of RSV infection, highlighting a limited role of type I interferon, and possibly redundant roles of other interferons, for RSV protection in humans (119;120).

A key factor in these alternating results is the usage of either C57/Bl6 or 129s mice, both of which have different responses to RSV infection, and unlike BALB/c mice are not the most ideal model for RSV infection (121;122). However, due to the lack of availability of knockout mice in the BALB/c model, studies have been limited to these strains. Therefore, in

our study we have studied two models of type I interferon inhibition side by side: a knockout model in 129s mice and a novel antibody inhibition model in BALB/c mice. We believe that by performing comprehensive studies in both models, we can clearly delineate the role of type I interferon blockade in RSV infection. Based on the limited role of type I interferon signaling on viral titers, and the significant role it plays in detrimental innate immune responses, we have hypothesized that blockade of interferon will result in significant improvements in disease parameters of RSV infection.

Type I interferon has also been associated with enhanced production of promyelocytic leumkemia (PML) protein, and upregulation of PML-nuclear bodies (PML-NB's). These sub-nuclear structures are responsible for degradation of NRF2, and downstream AOE response in RSV (84). However, the direct role of type I interferon has not been studied in a murine model of RSV infection, in relation to enhancement of PML, degradation of NRF2, or downstream AOE response. Since previous studies show that one of the key inducers of PML-NB's is type I interferon signaling, we hypothesized that type I interferon is directly responsible for dysregulation of NRF2-dependent AOE pathways and contributes to inflammation in RSV infection. Therefore, the elimination of type I interferon will improve AOE capacity.

In addition, type I interferon is also linked to upregulation of IL-1β expression and protein levels (123). However, the relations of these results to inflammasome activation, inflammasome assembly, or caspase 1 activation have not been studied. Therefore, in this study we have also hypothesized that IFN I signaling directly leads to upregulation of caspase 1 levels and leads to enhanced proteolytic cleavage and secretion of IL-1β.

MATERIALS AND METHODS

RSV viral preparation

RSV long strain was utilized in all experiments, and was grown in HEp-2 cells. Virus was purified via centrifugation using discontinuous sucrose gradients and stored at -80C prior to usage. Titers of viral pools were determined via a methylcellulose plaque assay. HEp-2 cells were grown to confluency and infected with serial dilutions of viral preparation for 1h. Methylcellulose was added to wells and cells incubated for 5 days at 37C. Cells were then fixed with 10% PFA and stained with crystal violet for visualization and counting of plaques to determine PFU/mL.

Animal studies

All studies were performed under approval from IACUC (protocol # 9001002I) and in accordance with IACUC recommendations. IFN I KO mice were bred at the institutional animal resource center, and age matched WT mice (129S) were purchased from Jackson laboratory (Bar Harbor, ME). Mice were infected with $1x10^7$ PFU/mouse intranasally under light anesthesia with ketamine/xylazine. For the interferon antibody blockade model (IFNAR abx), 14-16 week old BALB/cJ mice were purchased from Jackson laboratory. Mice were treated with 5mg/kg of anti-mouse interferon receptor 1 antibody from leinco technologies (St. Louis, MI) 12-24h prior to infection with $5x10^6$ PFU/mouse. Mice in both groups were observed for body weight loss. BALB/c mice were also observed for clinical disease (0 – no disease, 1 – ruffled neck fur, 2 – ruffled fur whole body, 3 – ruffled and hunched back, 4 – ruffled, hunched back and inactive, 5 – dead). IFNAR abx model was further verified through evaluation of lung mRNA at 6h, D1, D2, D3, and D5 post infection (p.i) with RSV for Interferon-induced GTP-binding protein (Mx1), which is an interferon stimulated gene.

Cytokine and Chemokine Analysis

Bronchoalveolar lavage fluid was collected from at 6h, D1, D2, D3 and D5 p.i with RSV. 1 mL of phosphate buffered saline (PBS) was used to flush the lungs twice through an incision in the trachea. The resulting BALF was centrifuged at 13,000 rpm for ten minutes and supernatant was utilized to measure cytokines/chemokines. Cytokines/chemokines were measured using the mouse cytokine 23-plex assay from Biorad (Hercules, CA), using the Luminex Bioplex HTF system from Biorad. Data was analyzed via spectrophotometry.

Viral Titer

Lungs were collected from mice 5 days p.i with RSV. Lungs were homogenized and centrifuged, and were further diluted serially 1:2 – 1:256. Dilutions were plated onto HepG2 cells and incubated at 37C for one hour. Lysates were replaced with methylcellulose and cells were allowed to incubate at 37C for five days. On D5, cells were fixed in 10% paraformaldehyde and stained with crystal violet to allow visualization of plaques. Plaques were counted for each experimental group to determine viral titer. Viral titers were also obtained through mRNA using expression of viral antigenome using qRT-PCR.

Antioxidant enzyme assays

Antioxidant enzyme expression (catalase and superoxide dismutase 1 (SOD1)) was determined using qRT-PCR, utilizing lungs from infected mice at D2 or D3 p.i for IFN I KO model and IFNAR abx model respectively. In addition, catalase activity was also determined in BALF fluid at D2 p.i and D3 p.i in both models respectively using the catalase activity assay from Cell Biolabs (San Diego, CA) and Cayman Chemicals (Ann Arbor, MI).

Airway Parameters

Both airway obstruction and airway hyperreactivity (AHR) were determined using full body plethysmography with BUXCO apparatus from Data Sciences International (New Brighton, MN). For airway obstruction baseline Penh was determined as a measure of airway function. For AHR, first baseline was determined at D5 p.i with RSV and then mice were administered increasing concentrations of aerosol beta acetyl methacholine from sigma (St. Louis, MO) including 6.25, 12.5, 25, and 50 mg/mL. Resulting changes in Penh were utilized for determining reactivity.

Caspase 1 and HMGB1 measurements

Mice were sacrificed at D1 p.i and total lung lysates were obtained. Lungs were homogenized in PBS supplemented with 10% RIPA buffer, 2% protease inhibitor and 1% phosphatase inhibitor solution. Lungs were homogenized and centrifuged at 13,000 rpm for 15 min. Supernatants were collected and stored at -80C for analysis. Western blots were performed with lung lysates and caspase 1 were determined using rabbit anti mouse caspase 1 from Santa Cruz (Dallas, TX. 12% gels were used from Fisher Scientific (Hampton, NH). A full range rainbow marker was utilized to determine protein sizes. Transfers were performed with 0.45 μM PVDF membranes, overnight at 25V. Blots were blocked overnight in 5% mild-TBST. Blots were imaged and densitometry analysis was performed using UVP imaging software. BALF was obtained at D1 p.i with RSV, and HMGB1 levels were determined using an Elisa kit from IBL international corp (Hamburg, Germany).

Statistics

For body weight loss, clinical disease, airway obstruction and AHR measurements statistics were performed using repeated measures ANOVA, for all other comparisons one-way ANOVA or student's *t*-test were used. Statistics were performed using GraphPad Prism

software (San Diego, CA). A p value <0.05 was considered significant. *** p<0.001, **p<0.01, and *p<0.05.

RESULTS

Prior to performing experiments, both models of interferon blockade were verified for inhibition of type I interferon signaling. As shown in Figure 1, the interferon stimulated gene Mx1 was chosen and quantities were determined in lung tissue from infected and either IFN I KO, or IFNAR abx treated mice using qRT-PCR. For the IFN I KO model only one time point of D1 was used (Fig 1a) as verification (due to colony information availability and prior testing in the previous lab), and for the antibody inhibition model, a full time course of inhibition was determined as shown in Figure 1b. As shown in Figure 1, both model show significant inhibition of downstream Mx1 signaling, and inhibition with the antibody dosage used is identical to that observed with the IFN I KO mice. In addition, the inhibition is maintained throughout the course of type I interferon production (6h – D3) during RSV infection. After the verification of inhibition, the following studies were performed.

IFN I signaling blockade reduces RSV disease severity

After the models of type I interferon inhibition were established, we determined the effect of IFN I blockade on RSV disease parameters including body weight loss, clinical disease score and viral titers. As shown in Figure 2a and 2b, both the IFN I KO mice and the IFNAR abx treated mice had significantly lower body weight loss, in comparison to their WT or PBS/IgG antibody treated infected controls. In addition, as shown in Figure 2c, a significant reduction was also seen in clinical disease score for IFNAR abx treated mice. Clinical disease score was not measured for IFN I KO mice due to limited expression of clinical disease in this mouse model. Only mild ruffling (only for WT – RSV) was observed in these mice overall.

Therefore, clinical disease is only shown for the IFNAR abx model (BALB/c mice). In addition, viral titers were determined for both disease models and although a significant increase of ~ 0.7 log was observed for IFN I KO mice (Figure 3a), only a mild increase of ~ 0.2 log was seen for the IFNAR abx model (Figure 3b), which was non-significant. These results show that although, the IFN I KO model has significantly higher viral titers you still observe a significant amelioration in body weight loss. In addition, for the IFNAR abx model, a significant improvement is observed in body weight loss, and in contrast to IFN I KO mice, IFNAR abx treated mice did not have a significant increase in viral titer.

IFN I signaling blockade improves airway obstruction

Since enhanced type I interferon signaling in RSV infection is associated with reduced airway function in patients (37), we determined whether the models of type I interferon blockade result in any changes to airway function parameters including airway obstruction and airway hyperresponsiveness (AHR). As shown in Figure 4a and 4b, both models of IFN I blockade result in significant improvement in airway obstruction at D1 p.i with RSV. AHR was also measured with increasing dosages of methacholine. As shown in Figure 4c and 4d, IFN I blockade did not result in any significant changes in AHR.

IFN I signaling blockade reduces pro-inflammatory cytokine, chemokine, and growth factor production

Levels of several pro-inflammatory cytokines, chemokines and growth factors were measured for both models of type I interferon signaling blockade. As shown in Figure 5, IFN I KO mice show a significant reduction in key pro-inflammatory cytokines including IL-1α, IL-1β, IL-6, IL-9, IL-12p40, and TNF-α. In addition, levels of IL-10, and IL-12p70 were also reduced (data not shown). Levels of cytokines were also determined for the IFNAR abx

model, and as show in Figure 6, it also resulted in a similar reduction in the same cytokines. In addition, levels of key chemokines were also determined for both models. As shown in Figure 7, IFN I KO mice had a significant reduction in key pro-inflammatory chemokines including CCL11 (eotaxin), CXCL1 (KC), CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES). Similarly, as shown in Figure 8, the same chemokines were also reduced in the IFNAR abx model. In addition, as part of the innate immune response we also determined levels of G-CSF and GM-CSF the two growth factors involved in RSV infection. As shown in Figure 9, both IFN I KO (Fig 9a-9b) and IFNAR abx model (Fig 9c-9d) result in a significant reduction in levels of G-CSF and GM-CSF.

IFN I signaling blockade reduces immune cell influx

Following the reduction in levels of key chemokines and growth factors, we determined whether there were any changes in immune cell influx into the lung. As shown in Figure 10, IFN I KO mice had a significant reduction in number of total cells (Fig 10a), neutrophils (Fig 10b), and lymphocytes (Fig 10c), but no significant changes in levels of macrophages (Fig 10d). Immune cells were also measured for the IFNAR abx model in BALF at similar timings. As shown in Fig 11, IFNAR abx treated mice showed a significant reduction in total cells (Fig 11a), neutrophils (Fig 11b), and macrophages (Fig 11c) only on D3 p.i with RSV. In comparison, lymphocytes (Fig 11d) were significantly reduced for D2 and D3. Therefore, although both models show a significant reduction in immune cell infiltration into airspaces, the difference is more evident in the IFN I KO model.

IFN I signaling blockade reduces caspase 1 levels and pyroptotic marker HMGB1

As shown in previous sections, both IFN I KO and IFNAR abx treated mice have a significant reduction in levels of IL-1β. Therefore, we hypothesized that a reduction could be

due to either reduced expression or activity of caspase 1 and inflammosome activation. Therefore, we tested the levels of pro-caspase 1 in both models of IFN I blockade. As shown in Figure 12a, IFN I KO mice had significantly lower levels of both pro-caspase 1 (45 kda). In addition, density analysis shows a significant reduction in levels for caspase 1 in IFN I KO mice in comparison to WT – RSV mice (Fig 12b). Similarly, western blots were also performed for IFNAR abx mice, and as shown in Figure 13, mice treated with IFNAR abx also had a significant reduction in levels of caspase 1, both visually (Fig 13a), and quantitatively (Fig 13b). We further determined levels of a known pyroptotic marker HMGB1, and as shown in Figure 13c, IFNAR abx treated mice (Fig 13b) showed a significant reduction in levels of HMGB1 in BALF at D1 p.i compared to their infected controls. Additionally, to verify these findings we also determined levels of total protein in BALF and as shown in Figure 14, both models also significantly reduced levels of total protein in BALF. Altogether, these findings show a significant reduction in pyroptosis with IFN I blockade and an overall preservation of the epithelial barrier during RSV infection.

IFN I blockade rescues NRF2 from PML-nuclear body mediated degradation and enhances antioxidant levels

One of the key aspects of RSV mediated disease, is a reduction in antioxidant enzyme levels caused by degradation of the transcription factor NRF2 as previously described. Since, NRF2 is degraded in a PML-NB dependent fashion via proteasomes, we determined the role of IFN I signaling on the PML/NRF2/AOE axis. As shown in Figure 15, both IFN I KO mice (Fig 15a) and IFNAR abx treated mice (Fig 15b) show a significant reduction in levels of PML expression, the main component of PML-NB's. In addition, as shown in Fig 15c-d, IFNAR abx treated mice also have a significant reduction in protein levels of PML as measured via western blots and subsequent densitometry. Next, NRF2 levels were determined for IFN I KO mice, and as shown in Fig 16 a-b, IFN I KO mice maintained levels of NRF2, while WT-

RSV mice had a significant degradation of NRF2. These results were further quantified via densitometry analysis and shown in Figure 16 (b). Lastly, effects of NRF2 protection were determined downstream, and levels of catalase expression were determined for both models. As shown in Figure 17, both models resulted in a significant increase in catalase expression measured via qRT-PCR. Overall, these results indicate that IFN I blockade results in improved antioxidant enzyme expression via reduction of PML-NB mediated NRF2 degradation.

DISCUSSION

Interferon type I signaling is well known for its role in creating an antiviral environment against viral infections. However, in the case of RSV infection, even though type I interferon signaling plays an antiviral role, it is not as robust as other infections (34). In addition, elimination of type I interferon enhances RSV viral titers only mildly (38). Additionally, administration of type I interferon results in enhanced pathology and fibrosis, however elimination does not enhance innate immune responses (36;38;116). Therefore, in this study we determined the exact role of early type I interferon production on RSV disease parameter, and whether knockdown/blockade of type I interferon specifically can ameliorate disease severity, while having minimal effects on viral titer. As shown in our studies, complete knockdown of the type I interferon receptor or inhibition through an anti-type I interferon receptor antibody treatment resulted in significant improvements in RSV infection. In addition, although a significant increase in viral titer is observed with the IFN I KO model, this increase is only ~0.6 log. Additionally, the difference in viral titer is further reduced and non-significant in the IFNAR abx model, most likely due to the transient nature of the blockade provided by antibody treatment. These results are in contradiction with previous studies showing either no changes or enhanced disease (38;123). However, since these

models have not utilized a transient model of blockade in the BALB/c mouse model of RSV, our results show a novel role of type I interferon in enhancing pathology.

Next we determined the effect of IFN I blockade on additional disease parameters, which have not been studied previously, including airway obstruction and AHR. As shown in Figure 4, IFN I blockade results in significant improvement in airway obstruction, but does not alter AHR. We hypothesize that this is due to the reduction in inflammatory mediators in early RSV infection. These include damage to the airway epithelium, production of pro-inflammatory mediators i.e. TNF-α, ROS species, etc (124). All of these result in deposition of cell debris in airways and accumulation of fluid blocking air exchange and worsening lung function (125). However, since AHR is determined later in the course of infection and is related to different mechanisms of disease i.e. leukotriene production, Th2 cytokines, and smooth muscle dysfunction (126-128), it is most likely that these are not effected by IFN I signaling in the same way as airway obstruction. However, since our model of RSV infection is not a potent inducer of Th2 responses it is possible that an OVA-sensitization model needs to be tested in order to fully determine the potential of type I interferon blockade and its role in AHR. Especially since some common mediators of both airway obstruction and AHR, such as TNF-α, CXCL1, CCL5 etc. are reduced with type I interferon blockade (60;129).

In addition to enhancing airway disease; cytokines, chemokines, and the resulting cytokine storm, also play a significant role in disease pathogenesis, and tissue damage. As shown in Figure 5 and 6, in addition to TNF-α, type I interferon blockade also reduces levels of other key pro-inflammatory cytokines including IL-1α, IL-1β, IL-6, IL-9, and IL-12 (p40). Both IL-1α, IL-1β, are related to non-apoptotic cellular death during inflammation and infection. IL-1α is sequestered in nuclear foci in apoptotic cells so release in BALF as observed in our studies, shows that type I interferon enhances necrotic/pyroptotic pathways (42). In addition, IL-1α is also associated with enhanced neutrophilia and tissue pathology (43). Similarly, IL-1β is also associated with enhanced caspase 1, inflammasome activation, and pyroptosis, which will be discussed in further detail (44). IL-6 is also a well-known pro-

inflammatory and pyretic cytokine, which is involved with neutrophil infiltration and is associated with enhanced disease severity in patients infected with RSV (46;47). Additionally, IL-9 is also linked with bronchiolitis in RSV infection, is produced by neutrophils, and associated with enhanced airway reactivity (48). Finally, although IL-12 is also a pro-inflammatory cytokine, during RSV infection it is associated with reduced airway reactivity and mucus production (49). Overall, there is a reduction in all of these cytokines, which play significant roles in RSV disease pathogenesis. Their inhibition via type I interferon inhibition further validates the negative role of type I interferon in RSV infection. In addition, reduction in these cytokines is also as a possible mechanism of disease reduction in IFN I blockade models.

One key cytokine that has been reduced with IFN I blockade is IL-1β. Since IL-1β is cleaved by caspase 1 into its soluble form, and since caspases are an integral role of inflammasome activation, we have further studied the role of IFN I signaling on caspase 1 levels. As shown in Fig 12-13, IFN I blockade results in a significant reduction in levels of caspase 1, which explain the reduction observed in IL-1β. In addition, IFN I blockade results in lower levels of total protein (Fig 14) and HMGB1 released into BALF fluid. Both the activation of caspase1 and release of HMGB1 are known markers of pyroptosis and have been studied previously in RSV infection (104;107;108). Altogether these results suggest that the activation of the inflammasome is reduced with blockade of IFN I signaling, and additionally there is less epithelial cell death through pyroptosis.

Our results also show a significant reduction in key pro-inflammatory chemokines including CCL11, CXCL1, and CCL2-5. Similar to the cytokines, these chemokines are also involved in RSV mediated pathology and are involved in influx of neutrophils (CXCL1, CCL2, and CCL3), eosinophils (CCL11, and CCL5), and NK cells (CCL3) (56;58;130-132). Additionally, several of them are also associated with pulmonary damage and airway reactivity including CCL2, CCL3 and CCL5 (15;60;133). In addition, levels of growth factors G-CSF and GM-CSF were also reduced in both models of IFN I blockade. G-CSF is

related to enhanced disease i.e. neutrophilia (134;135). Altogether, a reduction in all of these chemokines further improves RSV disease severity, provide an explanation for the reduction in airway obstruction, and also explain the reduction in immune cell infux.

Altogether both models of IFN I blockade have shown a significant reduction in several key cytokines, chemokines, and growth factors, and their effect is further evident in immune cell differentials. As shown in Fig 10-11, both models of IFN blockade result in significant reduction in both neutrophils and lymphocytes. Neutrophils have a dual role in RSV infection, providing antiviral function, but also causing a lot of immune-mediated pathology, and airway hyperresponsiveness (11;12). Additionally, at this stage of infection lymphocytes are composed primarily of NK cells and innate lymphoid cells (ILC's). RSV infection NK cells have been associated with a pro-inflammatory phenotype and enhanced lung injury (67;136). Additionally, ILC 2s which are a smaller population early on are also associated with enhanced disease and airway reactivity (68). Therefore, a reduction in these populations is also a negative effect of IFN I signaling and can be ameliorated with IFN I blockade.

Lastly, we looked at the role of IFN I signaling on antioxidant enzyme response in RSV infection. Antioxidant enzymes are important in the course of RSV infection to eliminate ROS species generated. However, RSV is known to inhibit the expression of antioxidant enzymes including SOD enzymes, catalase, glutathionine peroxidases etc. (76). Our previous studies have shown that RSV mediates this through degradation of the transcription factor NRF2 (83;84). In addition, our recent study has shown that NRF2 degradation occurs via interaction with PML-nuclear bodies, which are known to be generated through type I interferon signaling. Therefore, we have studied these parameters with IFN I blockade. Our results show a clear reduction in PML protein upregulation with IFN I blockade (Fig 15), followed by protection of NRF2 levels (Fig 16), and enhanced AOE-catalase levels (Fig 17). Altogether, these results strongly link type I interferon specifically with reduction in antioxidant enzyme responses during RSV, and provide further evidence that blockade of IFN I can provide protection from ROS mediated damage.

In conclusion, our study highlights the key role of type I interferon in immune mediated damage observed in RSV infection. We have shown that type I interferon blockade can improve several disease parameters, dampen the cytokine storm, and even enhance antioxidant protection. Since interferon administration is used as a treatment for some infections, it is important that its role is studied further, and our studies establish that it is not beneficial in the case of every infection, and especially not RSV. In addition, for severe cases of infection, inhibition of type I interferon can potentially provide significant improvement in clinical parameters and reduce disease severity. Compared to the viral infection, the damage from the immune response is often more severe in RSV infection, and is the cause of enhanced disease severity and reduced air exchange. We have shown in this study that the antiviral role of type I interferon in RSV infection is minimal, and therefore RSV is an excellent candidate for type I interferon signaling blockade as a potential therapeutic.

Chapter 3: Effects of Exogenous Catalase Supplementation on RSV Disease Parameters

INTRODUCTION

The previous chapter discussed the role of IFN I signaling in RSV lung disease and antioxidant defenses. In this, chapter we will further discuss how supplementation of exogenous antioxidant enzymes (AOEs) can reverse the negative effects of IFN I signaling, and therefore serve as a potential therapeutic approach for RSV infections. For this study, several antioxidant enzymes were considered along with several methods of enzyme delivery including superoxide dismutase, heme oxygenase 1, and catalase. Superoxide dismutase 1 (SOD1), is the enzyme responsible for conversion of superoxide anion to hydrogen peroxide (H_2O_2). Previous work with RSV infection had shown an antiviral effect of SOD1 administration in RSV infection (77). Similarly, administration of heme oxygenase 1 (HO1) inducers results in improvement in RSV related body weight loss, inflammation and reduction in viral replication in the mouse model (137). Catalase had not been previously studied for RSV infection, however, its exogenous administration in other models of lung disease including hydrogen peroxide mediated injury, fibrosis, and infection with influenza (H1N1) was associated with improvements in disease parameters (78;138;139). In the H1N1 influenza model, treatment was performed using a polyethylene glycol (PG) conjugated form of catalase, which has increased half-life and enhanced bioavailability in comparison to the non-conjugated catalase treatment (140). This provided an advantage in terms of modality of treatment, since fewer and lower dosages of catalase were required to achieve a therapeutic benefit.

As described previously RSV infection results in significant upregulation of ROS, which cause lung inflammation without contributing to host innate anti-viral responses (17). In addition, RSV causes degradation of NRF2 in a proteasome dependent manner, reducing

the expression of AOEs (84). One of these AOEs is SOD1, which is reduced both *in vitro* and *in vivo* models of RSV (18;76). Therefore, we performed initial studies with SOD1 exogenous supplementation, using either adenovirus-mediated expression, or PG-SOD1 administration. Both treatments resulted in improvements in some disease parameters i.e. disease score and pathology, however several others did not show any significant improvement (data not shown). The result could have been due to the low levels of SOD1 expression/treatment, due to infectivity of adenoviral vectors (primarily in larger airways), and low activity of SOD1 enzyme in PG-SOD1 treatment available (1,350 U vs. 40,000 U compared to catalase).

Although SOD1 levels are lower, levels of SOD2 are higher in airway epithelial cells and unchanged in mouse conductive airways following RSV infection (18;76). A stable or increased total SOD activity together with a progressive decrease in catalase, glutathione peroxidase, and glutathione S-transferase expression and activity suggests that RSV infection likely results in intracellular H_2O_2 production, which is not detoxified by AOEs, leading to the generation of highly reactive free radicals, such as the hydroxyl radical. Based on this knowledge, we hypothesized that catalase would be a better target for treatment of RSV infection. Administration of catalase via adenoviral approaches was not feasible, due to the 36 kB size of catalase gene, and restriction of adenoviral vector foreign gene of 9 kB for proper growth and maintenance of vector/gene. However, catalase was available in pegylated form with 40,000 U/activity, therefore direct administration of catalase was selected.

MATERIALS AND METHODS

Animal studies

14-16 week old BALB/c mice were purchased from Jackson laboratory (Bar Harbor, ME). For the standard model mice were treated intranasally 24h prior and 24h after RSV infection with 62.5µg (2.84 mg/kg) of exogenous catalase conjugated to polyethylene glycol (PG-CAT) from sigma (St. Louis, MO) in 50µL of PBS. Mice were then infected with $5x10^6$ PFU/mouse intranasally under light anesthesia with ketamine/xylazine. For controls mice were treated with either PBS or 48.2µg (2.19 mg/kg) of polyethylene glycol alone (PG). Uninfected controls were treated with PBS alone. Mice in both groups were observed for body weight loss, and clinical disease (as described previously- Materials and Methods chapter 2). For experiments with varying dosages of treatment mice were treated with either 31.25 µg (0.5x) or 125 µg (2x) of PG-CAT.

For treatment models mice were infected with RSV with $5x10^6$ PFU/mouse. Mice were treated with 62.5µg of PG-CAT intranasally at 3h and D3 post infection (p.i). Mice were observed for clinical disease parameters. Airway function parameters, cytokine/chemokine analysis, and immune cell population were also determined as described previously.

Catalase Activity Assay and polymorphism analysis

Catalase activity was determined in BALF samples to verify functionality of exogenous catalase treatment. Mice were treated as described previously at D(-1) and D1 p.i with RSV and BALF was obtained and catalase activity assay from Cell Biolabs was utilized to determine activity as described previously. Samples were protein/volume normalized prior to determining activity. In addition, catalase activity was also determined in nasopharyngeal secretions obtained from patients with a flush of 1mL PBS under vacuum from nares. Resulting fluid was centrifuged to obtain the acellular fraction, and catalase activity was determined for this fraction in protein/volume normalized samples.

For single nucleotide polymorphism (SNP) analysis, NPS samples were obtained from patients as described, and DNA was extracted from samples for genotype analysis.

Genotyping was performed for SNP rs1001179 using TaqMan ® assay C_11468118_10 (Applied Biosystems, Foster City, CA). PCR fluorescence detection was done with ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA), and data was analyzed with ABI Prism 7900 allelic discrimination software.

Hydrogen peroxide and hydroxyl antioxidant capacity assays

Hydrogen peroxide (H_2O_2) levels and hydroxyl radical antioxidant capacity (HORAC) were determined in BALF samples using the FLUOStar Optima (BMG Labtech, Cary, NC), and the hydrogen peroxide/peroxidase assay kit and the (HORAC) activity assay kit, respectively, both from Cell Biolabs (San Diego, CA).

Neutrophil elastase assay and total protein concentration

Neutrophil elastase levels were determined in BALF fluid as a measure of neutrophil activity. A neutrophil elastase Elisa was purchased from R&D systems (Minneapolis, MN). In addition, total protein concentrations were determined as a measure of epithelial barrier function utilizing the Biorad (Hercules, CA) protein concentration solution using a BSA standard.

Pulmonary Histopathology

Lungs were excised from mice at D3 and D7 p.i. RSV and stored in 10% neutral buffered formalin prior to paraffin embedding and sectioning. Pathology was observed using H&E stain under light microscopy. Images were taken at sites representing bronchi, bronchioles and alveolar structure for evaluation of pathology. Slides were blinded and assessed by an independent trained pathologist to obtain the pathology scores at Iowa State University.

Tumor Necrosis Factor alpha receptor (TNFR) infection model

14-16 wk Balb/cJ mice were given 80 μg of anti-tumor necrosis factor alpha receptor 1 (TNFR1) antibody from Leinco Technologies (St. Louis, MO), and control mice were given either PBS or IgG isotype control from Bio X Cell (West Lebanon, NH). Mice were infected 24h later with $5x10^6$ PFU/mouse of RSV, and uninfected controls were given PBS. Airway obstruction was determined at D1 p.i with RSV as previously described. Cytokines and chemokines were determined at D2 and mice, and mice were followed until D5 for body weight loss and clinical disease as previously described.

Statistics

For body weight loss, clinical disease, airway obstruction and AHR measurements, statistics were performed using repeated measures ANOVA, for all other comparisons one-way ANOVA or student t-test were used. Statistics were performed using GraphPad Prism software (San Diego, CA). A p value <0.05 was considered significant. *** $p<0.001$, **$p<0.01$, and *$p<0.05$. Statistics for SNP data were performed using exact test goodness of fit test via a binomial test.

RESULTS

Ongoing studies in the lab, concurrent with this aim had tested the role of several single nucleotide polymorphisms (SNP's) in antioxidant genes as well as in NRF2. Of all the genes tested, catalase SNP (rs1001179), which is known to enhance catalase expression (141;142), was the most protective in patient populations during RSV infection. For these experiments over the course of three RSV seasons (2013 – 2017) nasopharyngeal secretions were

obtained under vacuum with PBS. Samples were tested for RSV infection via a Luminex NxTAG^{TM} Respiratory Pathogen Panel (Luminex Molecular Diagnositcs), with confirmation of RSV infection DNA was extracted from samples and SNP genotyping was performed. Patients were grouped in mild, moderate and severe categories based on oxygen supplementation and admission to intensive care unit. As shown in Table 1, none of the patients with the CT/TT polymorphism were represented in the severe patient group. In addition, CT/TT patients were also significantly less in moderate and severe groups combined (in Caucasian and Hispanic patients). This data along with previous studies that had shown a severe reduction in catalase protein levels in patients requiring ventilation, provided strong evidence for the protective role of catalase in RSV infection (18). Therefore, we determined whether catalase activity could also be used as a marker of disease severity in RSV patients. As shown in Figure 1, even outside the CT/TT polymorphism categorization, patients with mild disease correlated with higher catalase activity in NPS samples. These studies further supported the administration of exogenous catalase as a potential therapeutic treatment for RSV infection. Results from those studies are discussed below.

Optimization of exogenous catalase treatment

Several different schedules of PG-CAT treatment were tried prior to establishment of one optimal lowest treatment protocol for the majority of experiments. High treatment protocols had shown an antiviral effect in RSV infection as shown in Figure 2a and 2b, similar to HIN1 influenza model (78). The optimal treatment model (D-1 and D1 treatment), does not decrease RSV viral titer, as shown in Fig 2c, however it was selected due to its beneficial effects for other RSV disease parameters. Next, catalase activity was determined in BALF obtained from treated mice, as shown in Figure 3, catalase treatment resulted in a significant increase in catalase activity even 24h post administration.

Exogenous catalase treatment improves RSV disease parameters

Once treatment protocols were established, RSV infected mice were treated with exogenous catalase to determine effects on disease parameters. As shown in Figure 4, catalase administration results in significant attenuation of body weight loss (Fig 4a) in treated mice compared to RSV infected controls. In addition, as shown in Fig 4b, PG-CAT treatment results in a significant decrease in clinical disease score. No significant changes were observed with PG-RSV treatment, verifying that results were specifically due to catalase activity. Two additional dosages of treatment were also utilized 0.5x (1.42 mg/kg) and 2x (5.68 mg/kg), and as shown in Figure 5a and 5b, both treatments similarly resulted in significant improvement in body weight loss and clinical disease as well, respectively. Among these treatments only 2x, resulted in a significant reduction in RSV viral titer, as shown in Fig 5c.

Exogenous catalase treatment improves lung function parameters in RSV infection

In addition, to disease parameters the effect of exogenous catalase treatment was also determined for lung parameters specifically. As shown in Fig 6a, PG-CAT treatment results in a significant improvement in airway obstruction at D1 p.i with RSV. This improvement is related to a significant reduction in total protein in BALF (Fig 13d) obtained from PG-CAT treated mice, an indicator of improved epithelial barrier function and reduced vascular permeability. In addition, PG-CAT treatment resulted in a significant reduction in clearance of airway hyperresponsiveness (AHR) measured at D8 p.i, as shown in Fig 6c. No difference was observed in peak AHR at D5 p.i (data not shown).

In addition, to lung function parameters lung pathology was also studied with PG-CAT treatment. As shown in Fig 7, PG-CAT treatment results in a significant improvement in epithelial/alveolar necrosis, interstitial pneumonia and consolidation (f-h), in comparison

to RSV infected controls (c-e), and PG-RSV control (b). Overall, PG-CAT treatment results in significant improvement in lung function parameters.

Exogenous catalase treatment model

In previous studies, mice were given one dose of PG-CAT treatment 24h prior to RSV infection. Therapeutics are often tested with pre-treatment models due to the fast course of RSV infection especially with intranasal administration directly to lower airways. Normally, in patients the virus infects via upper airways and takes longer to cause symptoms, leaving additional room for treatments to work. In addition, an exogenous treatment such as catalase can also be given as a prophylaxis in children to prevent severity of RSV infection. However, an effective treatment being tested *in vivo* should also provide therapeutic benefits with a post –infection treatment model. These effects represent benefits that can still occur with treatment administered several days post-infection, with a possibility of providing benefits even at the peak of infection.

Therefore, several different treatment schedules were also tested for RSV infection. As shown in Figure 8, PG-CAT treatment given at 3h and D3 p.i with RSV resulted in significant improvement in airway obstruction (Fig 8a) and also airway hyperresponsiveness (Fig 8b). However, no changes were observed in body weight loss, clinical disease, or viral titer levels (Fig 9a-c). This further supports that even late administration of PG-CAT can significantly improve airway parameters.

Effect of exogenous catalase treatment on innate immune responses

Next, the effect of PG-CAT treatment on cytokine/chemokines was determined. As shown in Fig 10, PG-CAT treatment resulted in a significant decrease in TNF-α, and a significant increase in IL-12p40. In addition, levels of key chemokines including CXCL1

(KC), CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β) and CCL5 (RANTES) were reduced. As shown in Fig 11, levels of growth factors including GM-CSF and G-CSF were also reduced. These results indicate a reduction in the cytokine storm, and a possible switch to a stronger Th1 response.

Immune cell differentials were also performed at several time points including D1, D2, D5 and D8. As shown in Fig 12, no changes were observed in numbers of total cells (Fig 12a), macrophages (Fig 12b), or lymphocytes (Fig 12c). Neutrophil numbers were also determined, and although the total neutrophil counts were not different (Fig 12d), these neutrophils displayed prematurity (banded nuclei, sequestration of granules etc.), and therefore neutrophil elastase secretion was determined as a measure of neutrophil activity. As shown in Fig 12e, levels of neutrophil elastase were significantly reduced at D2 p.i with RSV, indicating a decrease in neutrophil secretory activity. These results show that, in addition to providing ROS scavenging ability, exogenous catalase treatment is also able to attenuate the innate immune responses caused by enhanced H_2O_2 levels.

Mechanism of exogenous catalase protection and role of TNF-α

In order to determine the underlying mechanisms related to catalase mediated improvements in RSV infection, we further followed a bi-directional approach of studying both the ROS quenching, and if an early reduction in TNF-α could be responsible for the downstream improvements in airway parameters and reduction in chemokines/growth factors. For quenching of the ROS through catalase activity, we first determined effects of exogenous catalase D2 p.i with RSV, and as shown in Fig 13a, exogenous treatment still provided a significant increase in catalase activity even D2 p.i and D1 post-treatment. In addition, levels of H_2O_2 were also reduced with catalase treatment as shown in Fig 13b. Both of these results show that exogenous treatment provided the antioxidant benefit it was expected to. In addition, we determined the hydroxyl radical antioxidant capacity (HORAC) in BALF from

treated and un-treated mice. As shown in Figure 13c, HORAC capacity was also enhanced with exogenous catalase treatment, showing that the treatment increases ROS scavenging capacity. In addition, as shown in Figure 13d levels of total protein in BALF was reduced as well indicating that PG-CAT treatment resulted in a reduction in vascular permeability/epithelial barrier damage, showing that cells are less damaged with the treatment. Altogether, these results show that improvements linked to catalase treatment are due to enhanced antioxidant capacity.

Additionally, we also looked at the role of TNF-α in reducing disease severity. For this purpose an antibody mediated neutralizing model of TNF- receptor 1 was established. As shown in Fig 14, TNFR1 blockade resulted in a significant reduction in body weight loss (Fig 14a), clinical disease (Fig 14b), and airway obstruction at D1 p.i with RSV (Fig 14c). No changes were observed in RSV viral titer p.i (Fig 14d). In addition, TNFR1 blockade also resulted in a significant reduction in several chemokines/growth factors downstream (similar to catalase treatment) as catalase treatment as well, as shown in Fig 15a, including G-CSF, CCL3 (MIP-1α) and CCL5 (RANTES). In addition, as shown in Figure 15b, TNFR1 treatment also reduced levels of IL-6. Together these results show that enhancing ROS quenching abilities, and reducing TNF-α levels both play a role in reducing disease severity in RSV infection.

DISCUSSION

In this study we have determined the effect of exogenous catalase administration on disease parameters in RSV infection. Catalase was selected due to its ability to scavenge H_2O_2 and form innocuous products i.e. water and CO_2, and due to the significant loss in catalase activity during RSV infection (18;76). Our initial studies showed a correlation between reduction in catalase activity and clinical disease severity in clinical samples obtained from RSV infected patients (Figure 1). Although links have been made to reduction

in catalase in patients previously a functional assay for activity in nasopharyngeal secretions has not been performed (18). This data also showed that catalase activity could be used as a biomarker for disease severity and an indication for hospitalization in patients as well. In addition, a pegylated form of catalase was selected for treatment specifically due to its enhanced/prolonged bioactivity in comparison to non-conjugated catalase (140). Pegylated-catalase showed a significant advantage in a lethal murine model of H1N1 infection in comparison to non-pegylated catalase, in terms of enhancing survival (78). Since, the role of exogenous catalase treatment has not been previously studied in RSV infection in this study we determined whether pegylated-catalase treatment could be an effective therapeutic option for RSV infection. As shown, in our data treatment with PG-CAT resulted in a significant reduction in body weight loss, clinical illness score, and improvements in airway function parameters (obstruction and hyperreactivity). In addition, these benefits were obtained with only two dosages of treatment. PG-CAT treatment also showed some capacity to reduce viral titer with increased dosages as well, however at the low/limited dosages we used no changes were observed in viral titer. Previous studies with RSV have shown that although treatment with some antioxidant treatments results in a reduction in viral titers e.g. N-acetyl cysteine, heme oxygenase 1, and salen-manganese complexes (EUK-8 and EUK-189), treatment with other antioxidant treatments can also enhance viral titers e.g. β-hydroxy acid (17;137;143;144). In addition, these models require continuous/repeated treatments, unlike our model of limited treatment. In addition, although a majority of the experiments are conducted with pre-treatment, our treatment model also shows a significant improvement in airway function parameters with only two administrations (3h and D3 p.i) as shown in Figure 8. This data further supports that administration of catalase even after establishment of the infection, can still limit the severity of lung disease.

RSV infection results in significant damage to the structural lung environment. In infants severe RSV infection resulted in interstitial pneumonia, necrosis of lung parenchyma, damage to bronchial mucosa, neutrophil infiltration, pulmonary edema, and airway

obstruction caused by inflammation (22;145). Therefore, we determined if improvements in airway parameters shown in Figure 6, also translated to improvements in airway pathology. As shown in Figure 7, exogenous catalase treatment significantly reduced epithelium/alveolar necrosis, interstitial pneumonia, alveolar exudate, and consolidation of airspaces. These results combined with the improvements in airway function parameters with both pre-treatment and treatment models show that catalase administration has the potential to prevent epithelial damage, reduce necessity for supplemental oxygen/ventilation, and possibly prevent the onset of asthma/wheezing associated with RSV (146).

Although, PG-CAT treatment has been studied in several other models of pulmonary disease including asbestos and radiation mediated pulmonary fibrosis, hyperoxia, and influenza (H1N1) infection, the underlying mechanism responsible for protection have not been studied previously. Our study is the first to provide potential mechanisms for the improvements observed in disease parameters consisting of both the AOE activity provided as well as downstream effects on early innate immune responses. As shown in Figure 10, catalase treatment reduced key pro-inflammatory cytokines and chemokines. Previous studies, have shown that these cytokines/chemokines are associated with key disease parameters in RSV infection e.g. TNF-α is associated with enhanced body weight loss, clinical disease, pathology and airway reactivity (147;148), CCL11 is also associated with enhanced eosinophilia and airway reactivity (56), similarly CXCL1 is associated with enhanced neutrophil influx and activation (130), CCL2 is also similarly related to airway inflammation and hyperresponsiveness (133), CCL3 is related to enhanced airway pathology (15), and CCL5 is also similarly involved in RSV mediated airway hyperresponsiveness and immune cell recruitment (60). All of these are involved with enhancement of disease severity with RSV infection, especially airway reactivity. Reduction in all of these plays a significant role in improvements observed with catalase treatment. In addition, they also provide an underlying mechanism of improvements observed in airway function parameters. Following the reduction of key cytokines/chemokines, we also determined effects on growth factors.

Levels of G-CSF and GM-CSF were significantly reduced with catalase treatment. Although G-CSF is known to have an antiviral role in influenza infection, it is also linked with enhanced neutrophil recruitment and activity (134;135). Therefore, although further studies are needed in RSV infection to determine the exact role of these growth factors their reduction is also beneficial in this model.

Downstream from chemokine/cytokine responses immune cell differentials were also performed with catalase treatment during RSV infection. Although the significant reduction in cytokines/chemokines did not result in significant changes in recruitment of neutrophils, lymphocytes, macrophages/monocytes, or overall total number of cells (as shown in Figure 12), an alteration in morphology was noticed in the recruited neutrophils i.e. banded nuclei and sequestration of granules. Therefore, levels of neutrophil elastase were determined and as shown in Figure 12e, catalase treatment significantly reduced levels of neutrophil elastase in BALF. These results show that although a reduction in cytokines/chemokines did not alter influx of neutrophils their activity was significantly reduced by the treatment. Previous studies have shown that neutrophil elastase release is directly linked to expression of CXCL1 and TNF- α in a synergistic fashion (149). Since, we observe a reduction in both of these mediators the resulting effect on neutrophils was expected. In addition, for overall infection a reduction in release of neutrophil products is also a positive factor, especially since neutrophil elastase is directly related to enhanced airway reactivity and enhanced epithelial damage (11;12).

In order to determine the underlying mechanisms of protection provided by catalase treatment we further verified the role of two parameters in this study: ROS scavenging, and TNF-α. First, we looked at the expected activity of catalase as an antioxidant enzyme and determined the capacity of catalase to decrease ROS species, enhance antioxidant capacity, and increase ROS quenching ability. All of these factors were verified as shown in Figure 13, as catalase treatment not only provided enhanced catalase activity, which lowered overall H_2O_2 levels in the lung, and also enhanced the HORAC capacity of samples (the direct

ability to eliminate hydroxyl radicals). Hydroxyl radicals are generated in the interaction of H_2O_2 with neutrophil products and also enhance viral disease (72). In addition, since H_2O_2 is directly linked with exacerbation of lung disease, enhanced airway reactivity, and epithelial/vascular leakage through damage to the epithelial cytoskeleton (150;151). Therefore, a reduction in levels of H_2O_2 is likely a key factor in reduction of disease parameters.

Secondly, we have also further studied the role of catalase treatment on TNF-α, and downstream effects of reduction in TNF-α on RSV infection. TNF-α is one of the most abundant cytokines produced in the course of RSV infection, and has been related to severe RSV bronchiolitis in patients, epithelial necrosis, and enhanced disease in asthma models (148;152-154). Therefore, we pursued the hypothesis that catalase mediated reduction in TNF- α signaling also plays a significant role in reducing disease severity. As shown in Figure 14 and 15, direct inhibition of TNF receptor 1 signaling resulted in similar reductions in airway obstruction, body weight loss, and clinical disease score as PG-CAT treatment. In addition, levels of similar cytokines/chemokines were also reduced with TNFR1 inhibition. These results show that catalase mediated reduction in TNF-α is also a major mediator of improvement in disease parameters. Additionally, it is possible that TNF-α is able to master regulate these downstream effects, since TNF-α is reduced early with catalase treatment and reduction in other cytokines/chemokines occurs later. Additional studies are currently underway to show the mechanism of TNF-α regulation via catalase as well. Initial evidence shows regulation of TNF-α at the mRNA level, most likely as a result of either NF-kB or AP-1 signaling (data not shown). Further studies are being performed to further characterize this aspect of catalase treatment.

Lastly, involvement of neutrophils in PG-CAT mediated protection was also studied. As shown in Fig 12, PG-CAT treatment resulted in a significant reduction in neutrophil elastase levels. Neutrophils are associated with enhanced pathology during RSV infection, and neutrophil products i.e. elastase and CXCL1 are also known to enhance disease in several

models of lung injury including smoke inhalation, pneumonia, acute edematous injury, and bleomycin-induced acute lung injury (155-158). Neutrophil elastase release is associated with synergistic activity of TNF-α, and CXCL1, which are both reduced in PG-CAT treatment, providing an explanation for reduction in neutrophil activity despite no effects on influx of neutrophils into alveolar spaces.

In summary, our studies have shown that catalase function can both serve as a biomarker for disease severity in RSV patients, and also as a potential treatment in patients. Administration of catalase significantly improves disease parameters associated with RSV infection. Our studies show that patients with lower catalase function are more likely to be grouped in a moderate or severe category of RSV infection. Administration of PG-catalase for these patients can potentially decrease disease severity, ventilation requirements, and onset of airway reactivity. In addition, treatment can dampen the pathogenic immune response by reducing levels of pro-inflammatory cytokines such as TNF-α and neutrophil products such as elastase. In addition, since PG is already an FDA approved medication (miralax) and catalase is also an endogenous enzyme present in humans, approval of PG-catalase as a potential treatment is more feasible. In addition, catalase in this form can also be potentially aerosolized as a nebulizer, or even supplemented in formula for children. Our preliminary studies have shown that aerosolization of the PG-CAT compound does not decrease activity of the enzyme (>95% activity retention), and therefore nebulization of PG-CAT is a feasible delivery route. In addition, PG-CAT is commercially available in a powder form and could also be easily supplemented in formula to mimic protection provided to children through breastfeeding. In conclusion, this study confirms the therapeutic potential of PG-catalase treatment for RSV infection, and its role in reducing RSV disease severity.

Chapter 4: Summary

Acute bronchiolitis and other forms of viral LRTIs are the leading cause of hospitalization in the first year of life and have implications for long-term respiratory health. RSV is the causative agent in most of the cases, but unfortunately effective vaccines are not yet available. RSV is also a major respiratory pathogen associated with severe morbidity and mortality in older adults. Given that RSV infections recur throughout life there is a great and unmet need to develop pharmacologic approaches to prevent or treat these infections and associated airway inflammation. The current evidence-based guidelines for the management of RSV LRTIs are based on supportive management in the inpatient settings (oxygen and intravenous fluids). In view of the paucity of therapeutic alternatives, it is essential to understand the existing challenges to the development of preventive and treatment options for RSV bronchiolitis.

The pathogenesis of RSV bronchiolitis and the cellular/molecular mechanisms that determine its clinical severity remain incompletely understood. Numerous studies over the past five decades, have revealed that distinct host pathways including innate immunity (impaired or excessive), unbalanced Th1 or Th2 immune responses, neurogenic inflammation, epithelial-derived inflammatory cytokines and pro-oxidative mediators may be involved in the obstruction of the small airways and associated clinical manifestations that are characteristic of RSV bronchiolitis. We have identified an IFN type I-oxidative axis that contributes to clinical disease and lung pathology, and could represent a target of new therapeutic strategies in RSV-associated LRTIs. Type I IFNs are a major component of innate host defense against viral infections. At the early phase of viral infection, recognition of pathogen-associated molecular patterns induces rapid production of type I IFNs by various cell types leading to both autocrine and paracrine expression of IFN-stimulated genes that limit viral replication and spread. As such, loss of IFN type I signaling usually causes uncontrolled viral replication in human natural infections and in experimental animal models.

However, in addition to its protective effects, type I interferons have been increasingly recognized as important mediators of host pathogenic responses during viral infections, including enhanced cytokine production and inflammation, epithelial cell damage by apoptosis, and suppression of (secondary) antibacterial responses. Importantly, in vitro cellular models and experimental infections in animals suggest that the balance between protection and/or exacerbated disease induced by type I IFNs may be function of timing and concentration of IFNs as well as presence of virus that may have escaped the first waves of antiviral responses or is relatively resistant to IFN activity.

RSV is poorly responsive to the canonical antiviral activity of IFN I, both in vitro and in vivo. On the other hand, there are suggestions that IFN type I response are associated with disease severity in naturally acquired RSV infections and in experimental murine models. Few clinical studies have shown that both (airway) mucosal and systemic IFN I responses are associated with severity of LRTI in infants and young children. We have shown in our studies that lack of IFN I signaling (IFN I KO) or blocking IFN I receptor by neutralizing antibodies (IFNAR abx) significantly improved clinical disease and airway inflammation in experimental RSV infection, without a significant increase in viral replication. In addition, to type I IFN more information has also accumulated in regards to type III IFNs, which have a mucosal-restricted receptor distribution airways and gut epithelial cells. They also display a great deal of redundancy in their activity with type I IFNs, in term of antiviral properties, thus explaining the apparent lack of enhanced viral titers in mice with deficient type I signaling (but intact IFN type III signaling). Particularly as observed in the transient model of IFN I receptor blockade using neutralizing antibodies. We have shown herein that the disease-protective effect of type I interferon blockade was associated with reduction in the early cytokine/chemokine storm induced by RSV infection, protecting the epithelial barrier function and lung integrity. Overall influx of neutrophils and lymphocytes was also reduced in the lungs of mice with IFN I blockade, leading to improved airway function. Linking the IFN I signaling pathway to the oxidative/antioxidant balance in RSV infection, we

discovered that IFN I signaling caused a reduction in antioxidant gene expression in the lung, via IFN I-induced PML-NB's and degradation of the transcription factor NRF2. This detrimental effect of IFN I was blocked by treatment with anti-IFN I receptor antibodies, with restoration of protective antioxidant responses. These results are summarized in illustration 1.

Over the past decade, we and others have characterized oxidative injury of the airway mucosa as a central pathogenic mechanism of RSV-induced disease and shown that this process is mediated by a disruption of the ROS/antioxidant balance in favor of the former, in vitro as well as in vivo, due to an impairment of the antioxidant defense system. Central to the studies presented in this dissertation are our initial discovery that RSV inhibits expression of AOE, particularly SOD1 and catalase in airway epithelial cells, which are the major site of viral replication. Subsequent investigations of mouse BAL by two-dimensional gel electrophoresis, MALDI-TOF-MS spectrometry, western blot and bioactivity, revealed that RSV infection induced a significant decrease in the expression and activity of AOEs, including catalase, which correlated with parameters of clinical disease and airway inflammation. In this dissertation, we show that RSV-infected infants with more severe clinical disease had lower levels of catalase activity in nasopharyngeal secretions compared to those with milder disease. Moreover, we found in genetic studies that a SNP in the promoter region of the catalase gene, rs1001179, which is associated with higher enzyme expression, was significantly underrepresented among patients with moderate to severe RSV bronchiolitis, suggesting a protective effect against disease severity. Catalase is an antioxidant enzyme present in most aerobic cells that catalyzes the dismutation of hydrogen peroxide to water and oxygen. In the lung, catalase activity increases in parallel with the its embryologic development and at full maturity it is specially localized in alveolar type II pneumocytes and alveolar macrophages, both cell types being a primary target of respiratory viral entry and replication.

To determine whether increasing the levels of catalase in the airways would confer protection against RSV infection, we administered PG-CAT to mice infected with RSV. This catalase formulation was selected based on its enhanced bioavailability and prolonged activity in biological fluids thus resulting in lower dose requirements. Our data in RSV-infected mice demonstrate that two single doses of intranasal PG-CAT effectively increased levels of lung catalase and its antioxidant capacity, and reduced disease, as body weight loss, clinical illness score, and airway obstruction were all significantly improved compared to untreated control littermates. This protective effect of PG-CAT was observed even when administered to mice at time points after RSV inoculation, suggesting a possible therapeutic application. Although the exact mechanism(s) underlying the disease-improving effect of PG-CAT treatment remains to be established, our data suggest a few possibilities. First, PG-CAT administration was associated with significant reduction in overall levels of H_2O_2 and in a significant increase of airway antioxidant capacity in mice infected with RSV. Also, PG-CAT treatment resulted in significant inhibition of several RSV-induced inflammatory cytokines and chemokines, with a pattern of these reduced mediators that was similar but not identical to that observed in mice with a deficiency in the IFN I signaling pathway. Among these inflammatory cytokines, TNF-α was significantly reduced in PG-CAT treated mice. TNF-α been correlated with enhance body weight loss, clinical disease, pathology and airway hyperreactivity in RSV-infected mice and children. Although PG-CAT treatment did not affect the recruitment of inflammatory cells into the lung, it significantly reduced neutrophil activation, as evident by the reduction in neutrophil elastase. These results are further summarized in illustration 2. Despite this significant effect on innate immune responses, PG-CAT treatment did not result in increased RSV replication in the lung, rather we observed a trend for reduction in viral titers when used at higher doses.

In conclusion, the results of the studies presented in this PhD dissertation are important and timely because they have the potential to rapidly translate to new therapeutic or prophylactic approaches for viral-associated wheezing, in particular the most severe forms of

airway disease that lead to hospitalization in young infants and represent a major risk factor for the development of asthma later in life. These new therapeutic strategies can be based on antibody-based strategies to bock IFN I or supplementation, small molecule mediated inhibition of IFN I receptor, nutritional regimens with anti-oxidant nutrients or new drugs that would boost expression/activity of the anti-oxidant enzyme catalase, and even aerosol administration of catalase. In addition, catalase is also present in breast milk, and breastfeeding protects infants in the first three months of life. Therefore, in addition to other strategies, formula given to infants can also be fortified with catalase to enhance protection from RSV. Overall, these studies have focused primarily on the innate immune responses in knockdown/blockade of type I interferon, and PG-CAT administration. Future studies will also focus on the effects on adaptive immune response, mucosal immunity, and co-administration of these immune modulators with antiviral therapies i.e. Synagis.

Appendix

FIGURES CHAPTER 2.

Figure 1.

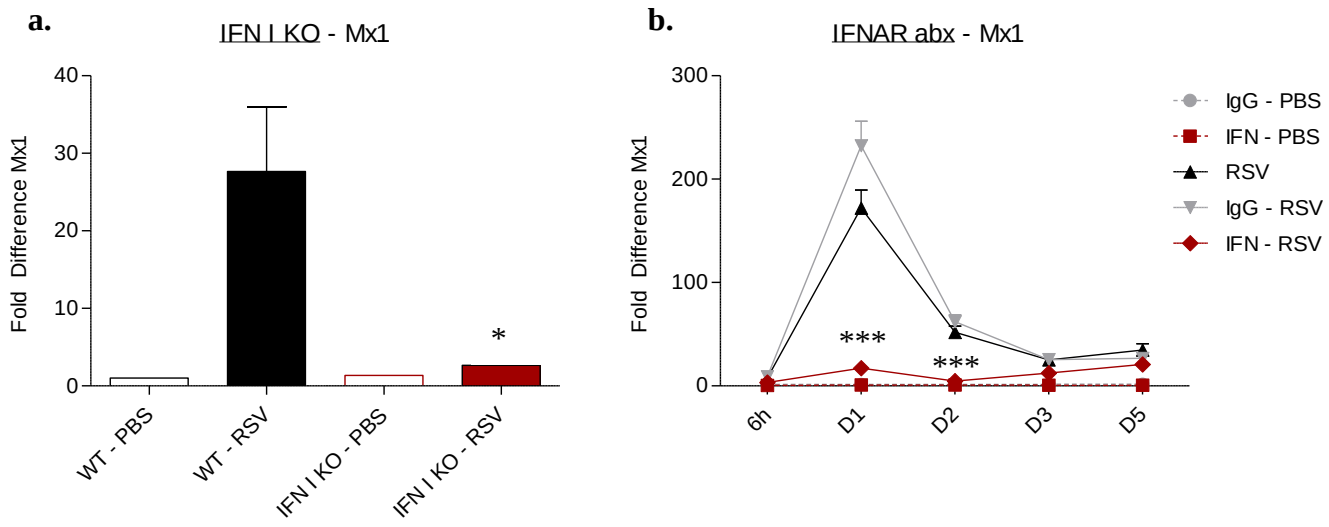
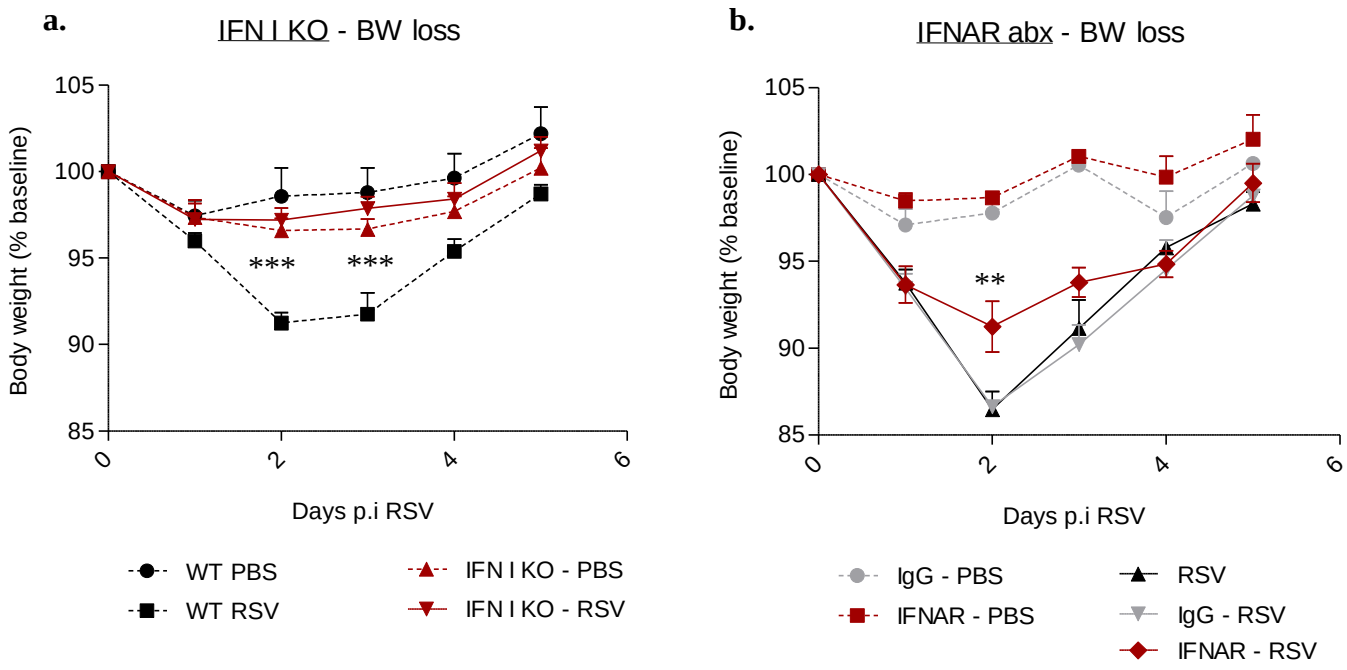


Figure 1. IFN I receptor blockade results in inhibition of interferon stimulated gene Mx1. (a) 14-16 wk 129s mice (IFN I KO and WT age matched controls) were infected with $1x10^7$ PFU/mouse of RSV and sacrificed at D1 p.i. Lungs were harvested for extraction of mRNA and qRT-PCR was performed for Mx1 gene. IFN I KO mice had a significant reduction in Mx1 expression in comparison to WT-RSV. (b) 14-16 wk BALB/c mice were treated i.p. with 150 µg of either PBS/IgG isotype (RSV/IgG RSV) or IFNAR antibody (IFN-RSV) 12h prior to infection with $5x10^6$ PFU/mouse of RSV and sacrificed at 6h, D1, D2, D3 and D5 p.i. Lungs were harvested for mRNA and qRT-PCR analysis for Mx1 gene. As shown, IFN RSV mice also had a significant reduction in Mx1 levels p.i with RSV. Significance was determined using one way ANOVA (a) and repeated measures ANOVA (b). For figure 1b, significance is shown as a comparison to both infected controls utilizing the lowest significance for conservative analysis. * $p<0.05$, and *** $p<0.001$.

Figure 2.



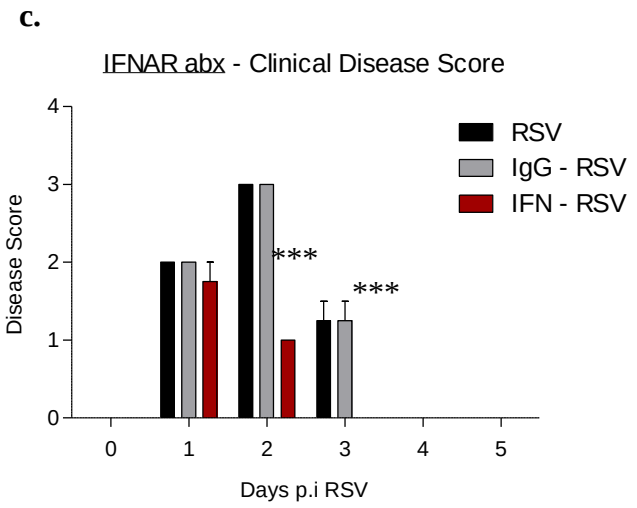


Figure 2. IFN I receptor blockade ameliorates RSV disease severity. (a) 14-16 wk 129s mice (IFN I KO and WT age matched controls) were infected with $1x10^7$ PFU/mouse of RSV and observed till D5 p.i. Body weight was measured daily and percentage of weight loss is presented. IFN I KO mice had significant improvement in body weight in comparison to WT-RSV. (b) 14-16 wk BALB/c mice were treated i.p. with 150 µg of either PBS/IgG isotype (RSV/IgG RSV) or IFNAR antibody (IFN-RSV) 12h prior to infection with $5x10^6$ PFU/mouse of RSV and observed daily till D5 p.i. Body weight loss (a) and clinical disease score (b) was determined daily. As shown, IFN RSV mice had a significant reduction in both parameters. Significance was determined using repeated measures ANOVA (a-c). For figure 2 (b-c), significance is shown as a comparison to both infected controls utilizing the lowest significance for conservative analysis. ** $p<0.01$, and *** $p<0.001$.

Figure 3.

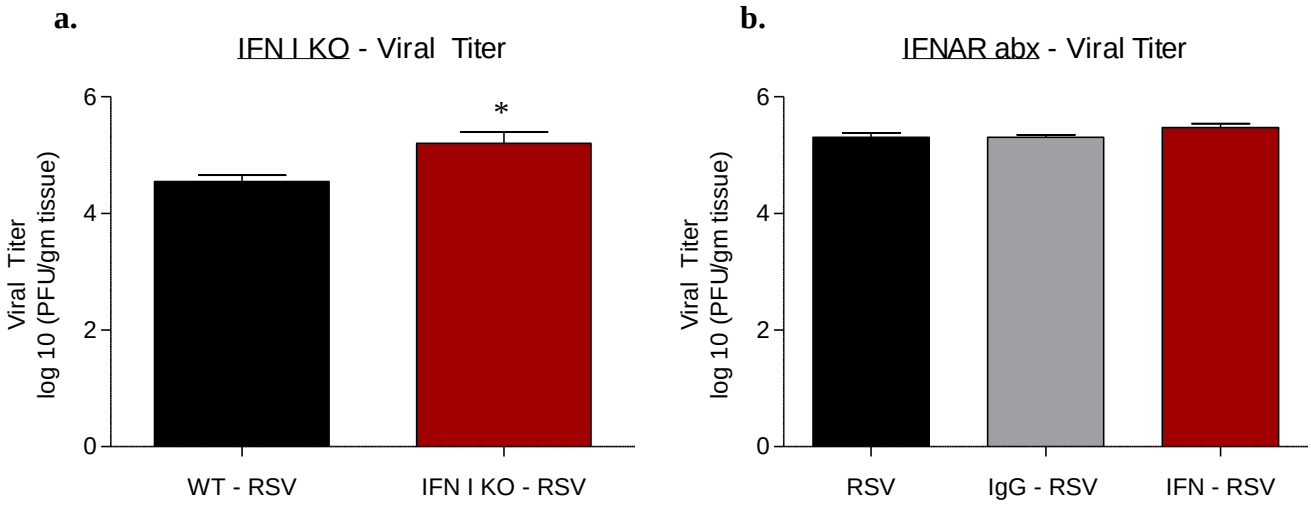


Figure 3. IFN I receptor blockade mildly enhances RSV viral titers. (a) 14-16 wk 129s mice (IFN I KO and WT age matched controls) were infected with 1x107 PFU/mouse of RSV and sacrificed at D5 p.i. Lungs were harvested and plaque assays were performed. IFN I KO mice had a significant increase in viral titer ~ 0.6log in comparison to WT-RSV. (b) 14-16 wk BALB/c mice were treated i.p. with 150 μg of either PBS/IgG isotype (RSV/IgG RSV) or IFNAR antibody (IFN-RSV) 12h prior to infection with 5x106 PFU/mouse of RSV and sacrificed at D5 p.i. Lungs were harvested at D5 p.i and plaque assay for viral titers were performed. As shown, IFN RSV mice had a non-significant increase of 0.2log in viral titer. Significance was determined using student's t-test (a) and one way ANOVA (b). For figure 3 (b), significance is shown as a comparison to both infected controls utilizing the lowest significance for conservative analysis. * $p<0.05$.

Figure 4.

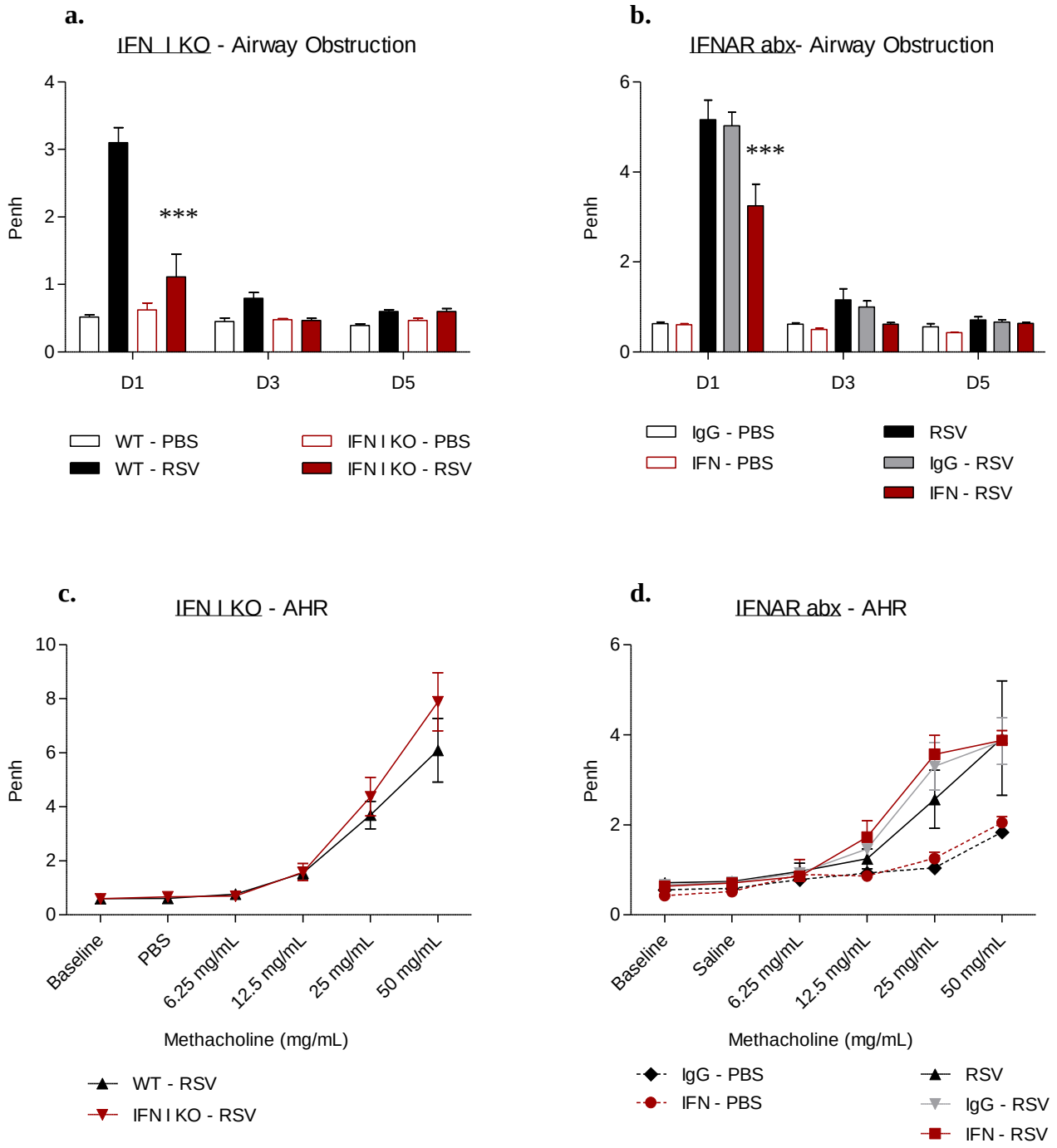


Figure 4. IFN I receptor blockade reduces airway obstruction but does not alter airway hyperresponsiveness (AHR). (a) 14-16 wk 129s mice (IFN I KO and WT age matched controls) were infected with $1x10^7$ PFU/mouse of RSV and airway obstruction was determined at D1, D3 and D5. IFN I KO mice had significantly lower airway obstruction D1 p.i with RSV. (b) 14-16

wk BALB/c mice were treated i.p. with 150 μg of either PBS/IgG isotype (RSV/IgG RSV) or IFNAR antibody (IFN-RSV) 12h prior to infection with $5x10^6$ PFU/mouse of RSV and airway obstruction was determined at D1, D2 and D5. As shown, IFN RSV mice had a significant reduction in airway obstruction at D1 p.i with RSV. (c) IFN I KO mice were infected as described and AHR was determined at D5 p.i with increasing concentrations of β-acetyl methacholine. As shown, there was no significant difference between infected groups. (d) IFNAR abx mice were treated and infected and AHR was determined. Although RSV infected groups had a significant increase in AHR compared to non-infected controls. No significant difference was observed between infected groups. Significance was determined using repeated measures ANOVA. Significance is shown for IFN I KO RSV and WT RSV (a) and both PBS-RSV and IgG – RSV vs. IFN – RSV (b). *** $p< 0.001$.

Figure 5.

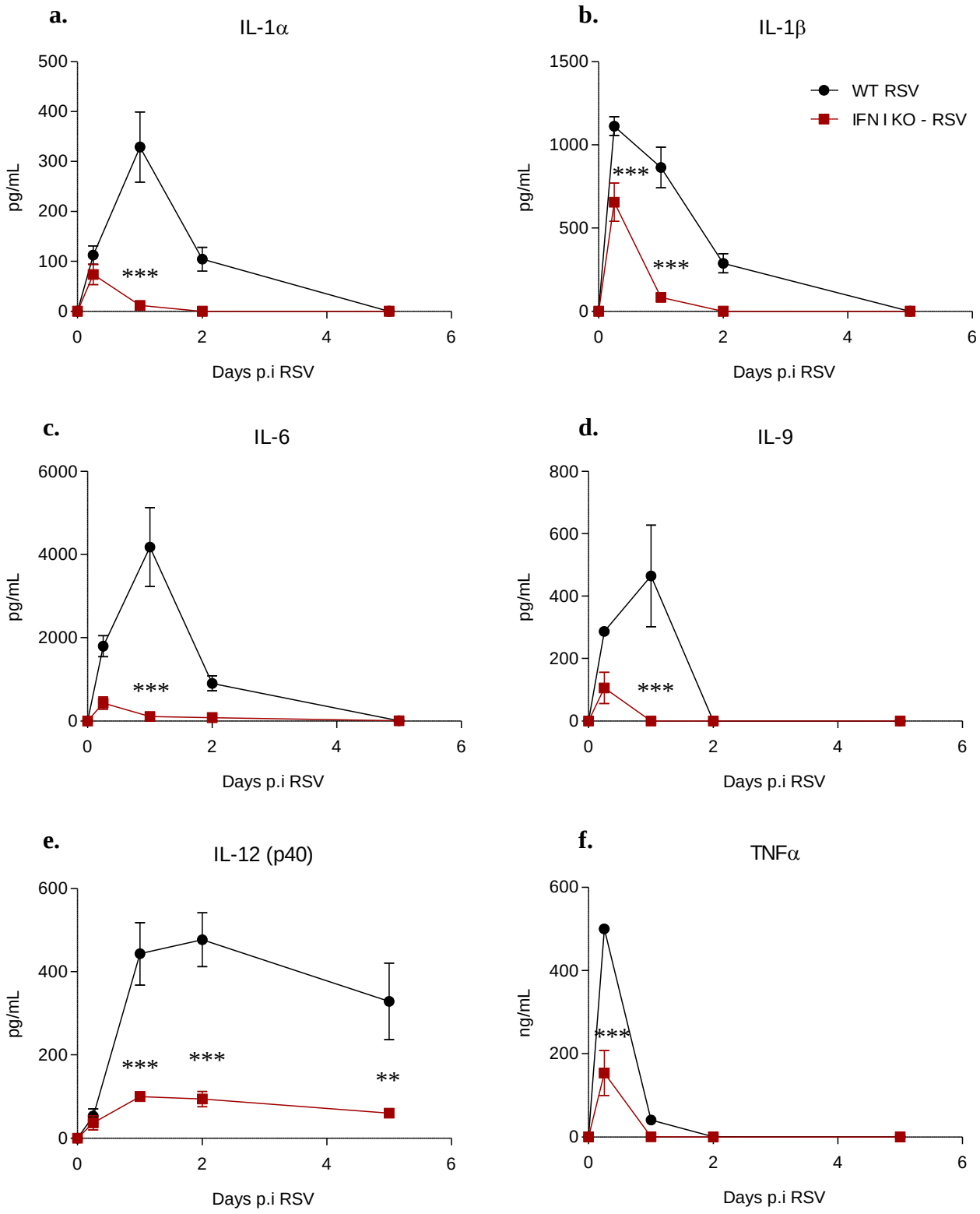


Figure 5. IFN I receptor knockout reduces early innate cytokine production. 14-16 wk 129s mice (IFN I KO and WT age matched controls) were infected with 1x107 PFU/mouse of RSV and

sacrificed at 6h, D1, D3 and D5 to collect BALF. A 23-plex array was run for cytokines/chemokines. IFN I KO mice had significantly lower levels of (a) IL-1α, (b) IL-1β, (c) IL-6, (d) IL-9, (e) IL-12 (p40), and (f) TNF-α. Significance was determined using repeated measures ANOVA. *** $p < 0.001$.

Figure 6.

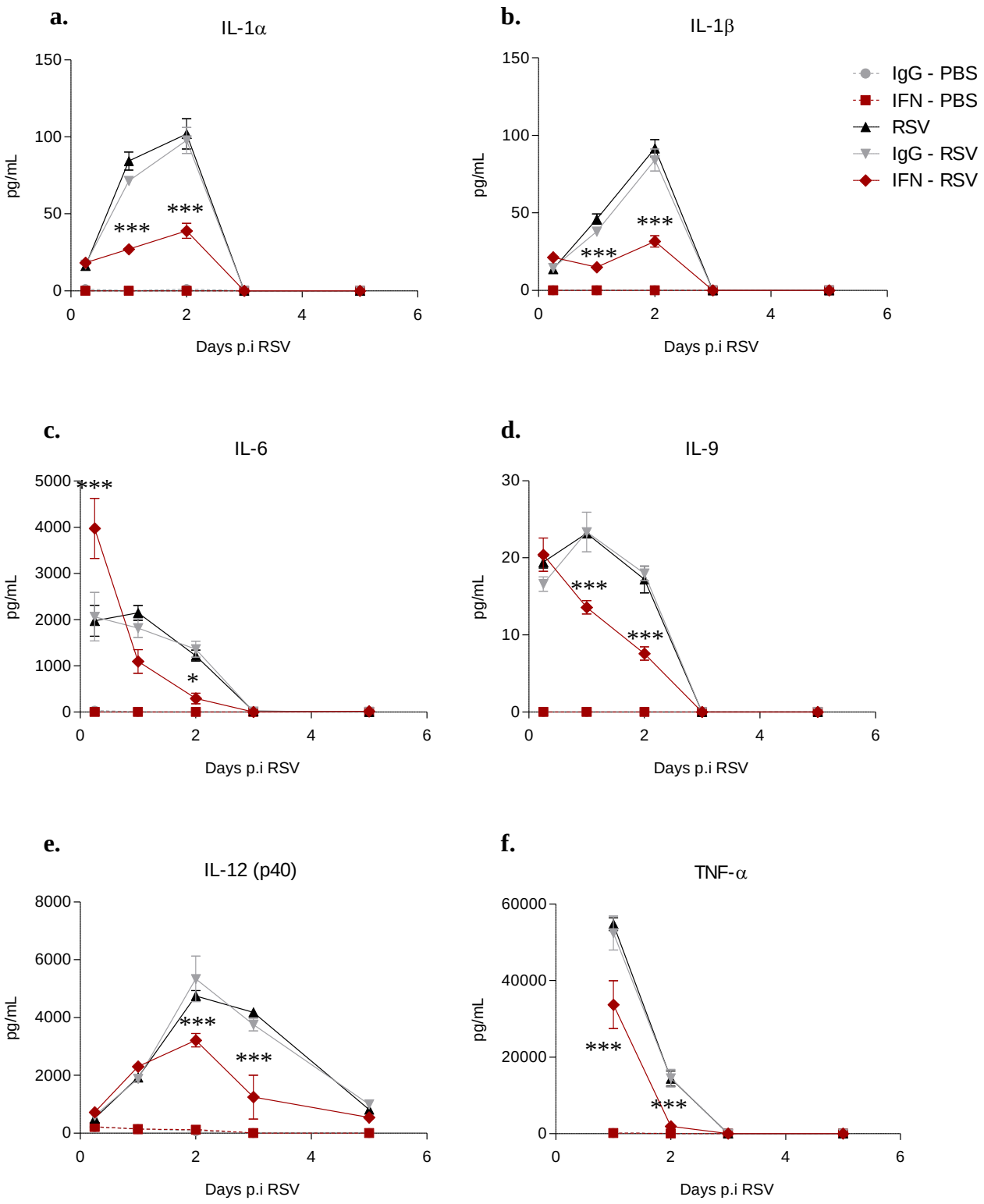

a.
IL-1α
pg/mL
Days p.i RSV
b.
IL-1β
IgG - PBS
IFN - PBS
RSV
IgG - RSV
IFN - RSV
c.
IL-6
d.
IL-9
e.
IL-12 (p40)
f.
TNF-α
***
*

Figure 6. IFN I receptor antibody blockade reduces early innate cytokine production. 14-16 wk BALB/c mice were treated i.p. with 150 μg of either PBS/IgG isotype (RSV/IgG RSV) or IFNAR antibody (IFN-RSV) 12h prior to infection with $5x10^6$ PFU/mouse of RSV and sacrificed at 6h, D1, D3 and D5 to collect BALF. A 23-plex array was run for cytokines/chemokines. IFN I abx mice had significantly lower levels of (a) IL-1α, (b) IL-1β, (c) IL-6, (d) IL-9, (e) IL-12 (p40), and (f) TNF-α. Significance was determined using repeated measures ANOVA. * $p<0.05$, and *** $p< 0.001$.

Figure 7.

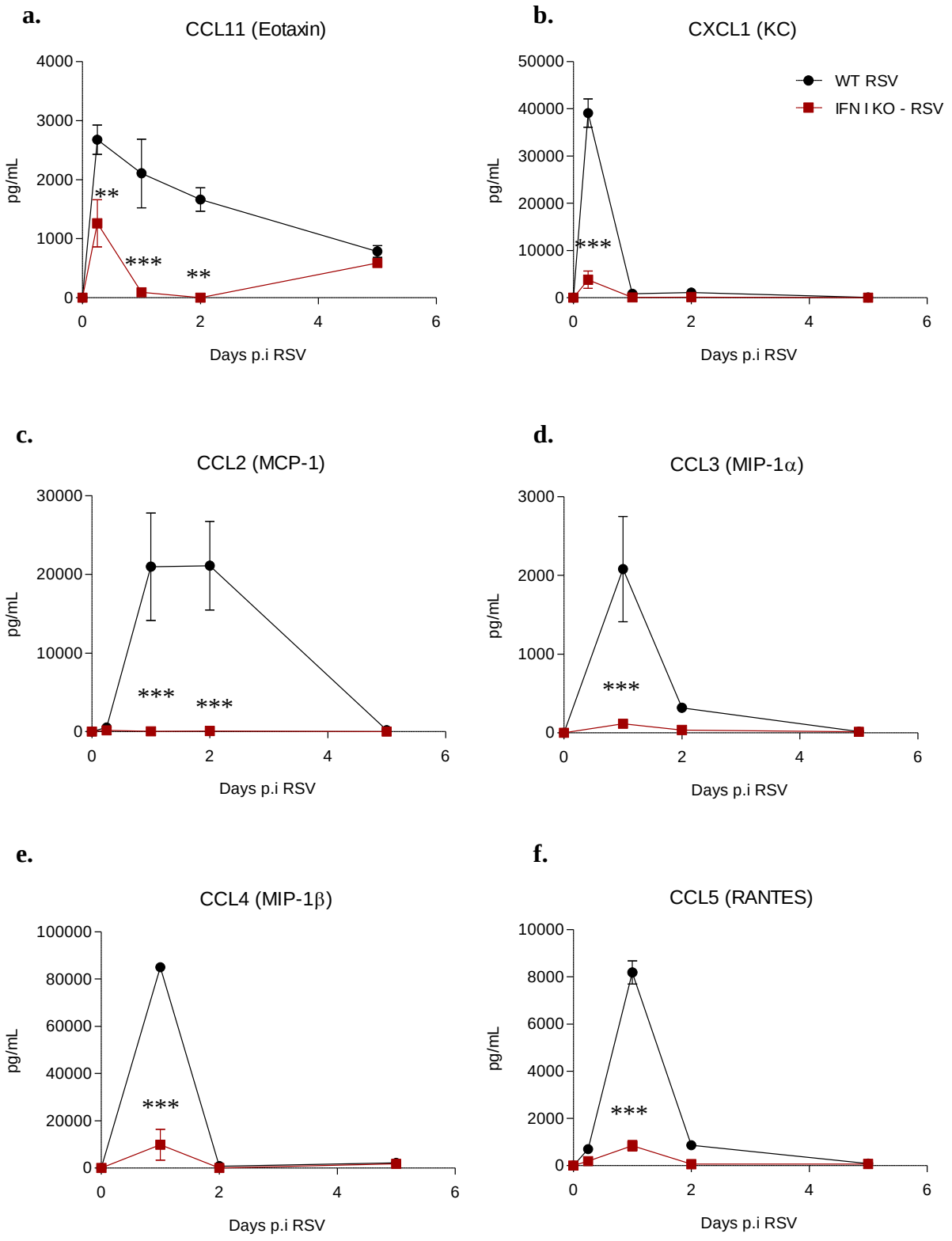

a.
CCL11 (Eotaxin)
pg/mL
Days p.i RSV
b.
CXCL1 (KC)
WT RSV
IFN I KO - RSV
pg/mL
Days p.i RSV
c.
CCL2 (MCP-1)
pg/mL
Days p.i RSV
d.
CCL3 (MIP-1α)
pg/mL
Days p.i RSV
e.
CCL4 (MIP-1β)
pg/mL
Days p.i RSV
f.
CCL5 (RANTES)
pg/mL
Days p.i RSV

Figure 7. IFN I receptor knockout reduces early innate chemokine production. 14-16 wk 129s mice (IFN I KO and WT age matched controls) were infected with 1x107 PFU/mouse of RSV and sacrificed at 6h, D1, D3 and D5 to collect BALF. A 23-plex array was run for cytokines/chemokines. IFN I KO mice had significantly lower levels of (a) CCL11, (b) CXCL1, (c) CCL2, (d) CCL3, (e) CCL4, and (f) CCL5. Significance was determined using repeated measures ANOVA. ** $p<0.01$, and *** $p< 0.001$.

Figure 8.

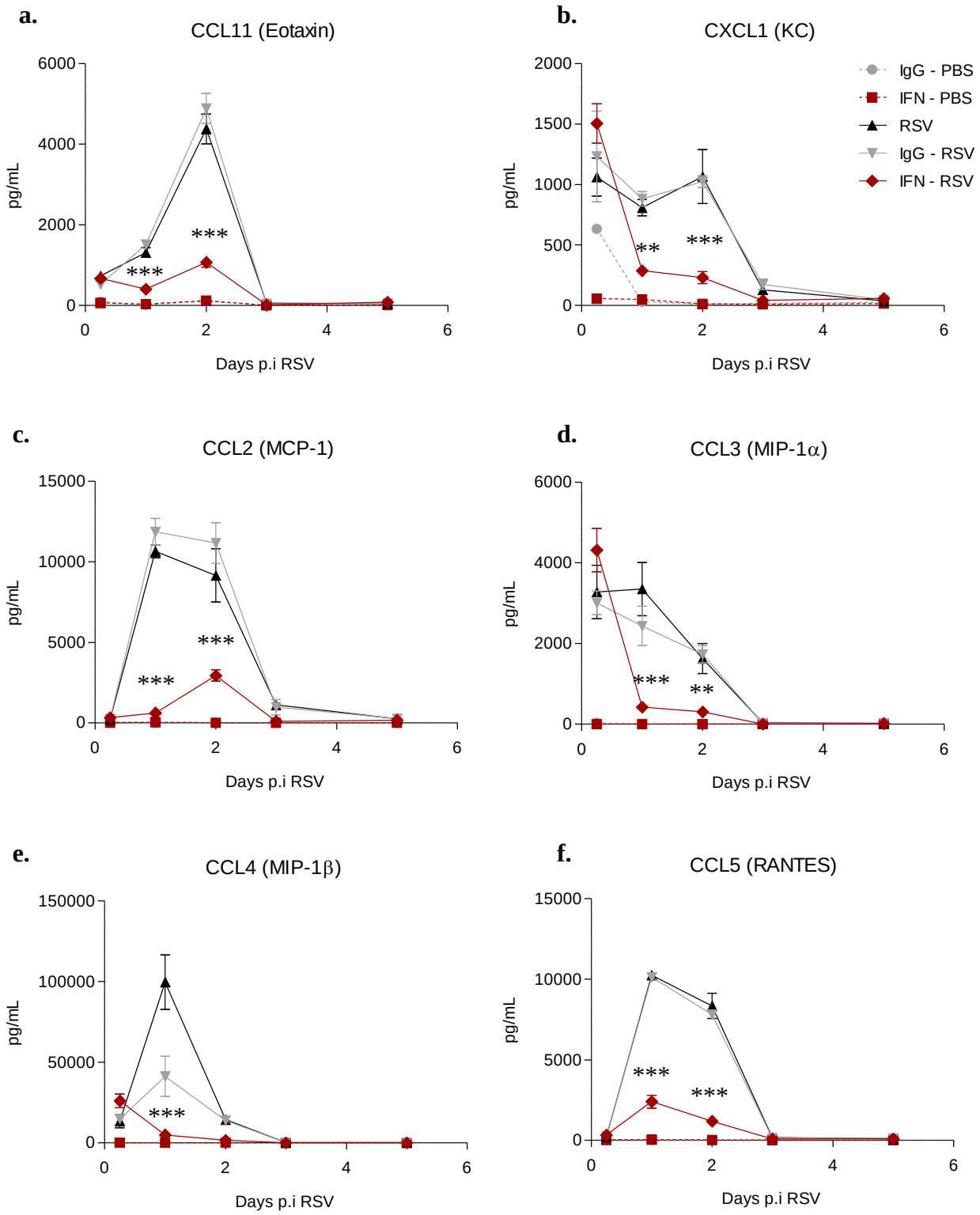

a.
CCL11 (Eotaxin)
pg/mL
Days p.i RSV
b.
CXCL1 (KC)
IgG - PBS
IFN - PBS
RSV
IgG - RSV
IFN - RSV
c.
CCL2 (MCP-1)
d.
CCL3 (MIP-1α)
e.
CCL4 (MIP-1β)
f.
CCL5 (RANTES)

Figure 8. IFN I receptor antibody blockade reduces early innate chemokine production. 14-16 wk BALB/c mice were treated i.p. with 150 μg of either PBS/IgG isotype (RSV/IgG RSV) or IFNAR antibody (IFN-RSV) 12h prior to infection with $5x10^6$ PFU/mouse of RSV and sacrificed at 6h, D1, D3 and D5 to collect BALF. A 23-plex array was run for cytokines/chemokines. IFN I abx mice had significantly lower levels of (a) CCL11, (b) CXCL1, (c) CCL2, (d) CCL3, (e) CCL4, and (f) CCL5. Significance was determined using repeated measures ANOVA. * $p<0.05$, and *** $p< 0.001$.

Figure 9.

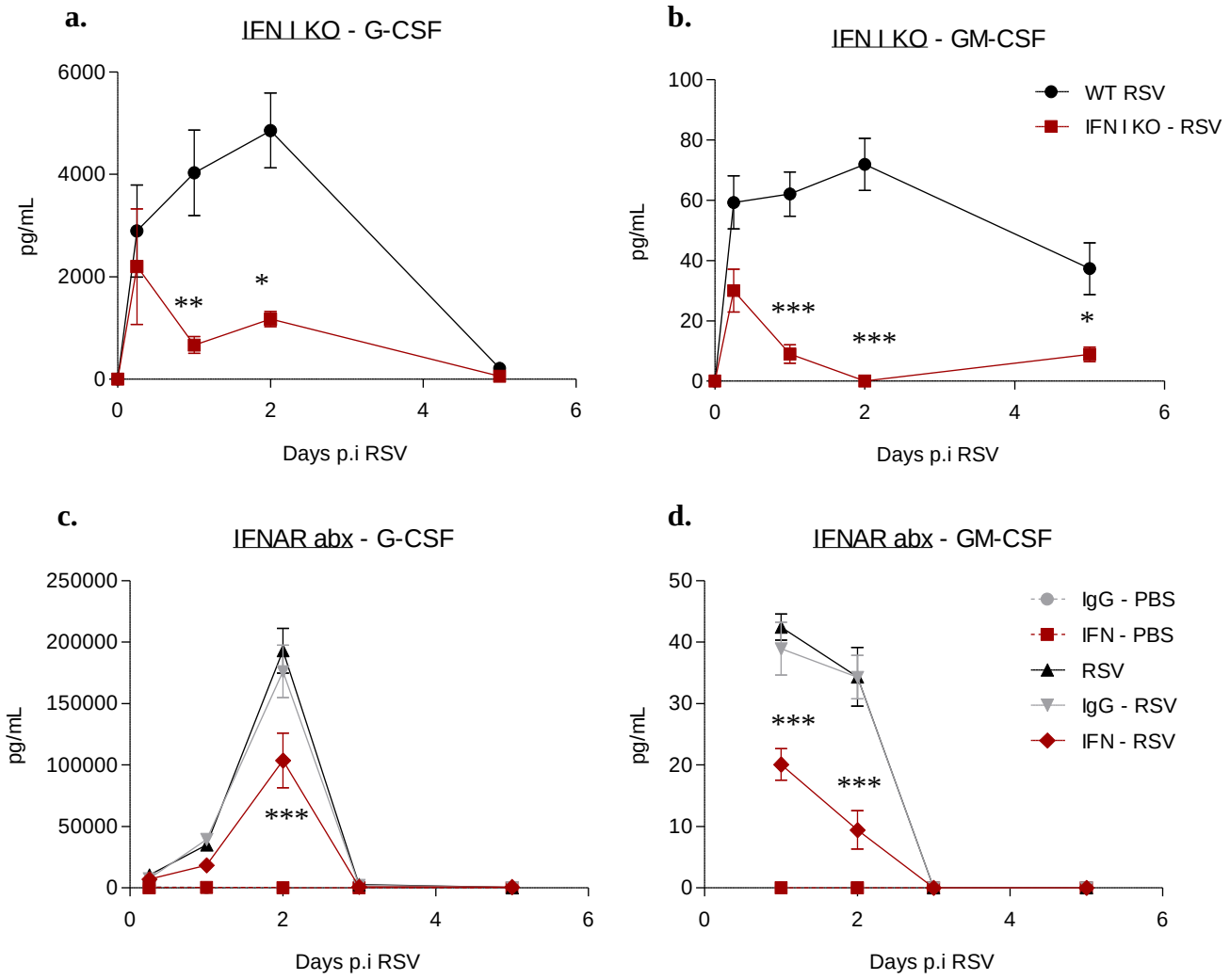


Figure 9. IFN I receptor signaling blockade reduces growth factor production. 14-16 wk 129s mice (IFN I KO and WT age matched controls) were infected with 1x107 PFU/mouse of RSV and sacrificed at 6h, D1, D3 and D5 to collect BALF. A 23-plex array was run for measurement of growth factors. IFN I KO mice had significantly lower levels of (a) G-CSF, and (b) GM-CSF. 14-16 wk BALB/c mice were treated i.p. with 150 µg of either PBS/IgG isotype (RSV/IgG RSV) or IFNAR antibody (IFN-RSV) 12h prior to infection with 5x106 PFU/mouse of RSV and sacrificed at 6h, D1, D3 and D5 to collect BALF. As shown, IFN I abx mice also had significantly lower levels of (a) G-CSF, and (b) GM-CSF. Significance was determined using repeated measures ANOVA. * $p<0.05$, ** $p<0.01$, and *** $p< 0.001$.

Figure 10.

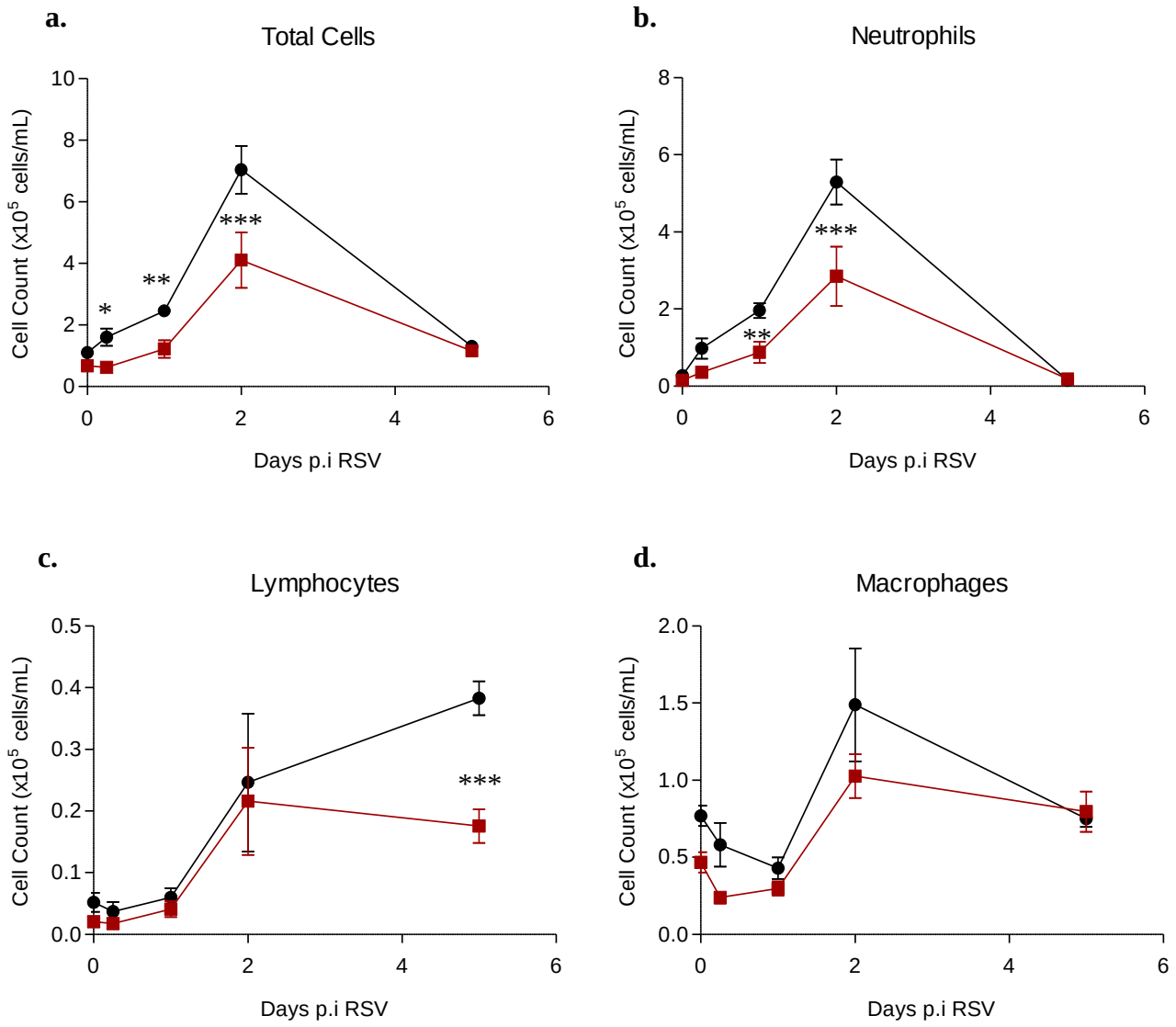


Figure 10. IFN I receptor knockdown reduces innate immune cell infiltration. 14-16 wk 129s mice (IFN I KO and WT age matched controls) were infected with $1x10^7$ PFU/mouse of RSV and sacrificed at 6h, D1, D3 and D5 to collect BALF. Cells from BALF were fixed on slides, stained, and cell differentials were determined. IFN I KO mice had significantly lower levels of (a) total cells, (b) neutrophils, and (c) lymphocytes. (d) No significant changes were observed in macrophage/monocyte counts. Significance was determined using repeated measures ANOVA. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

Figure 11.

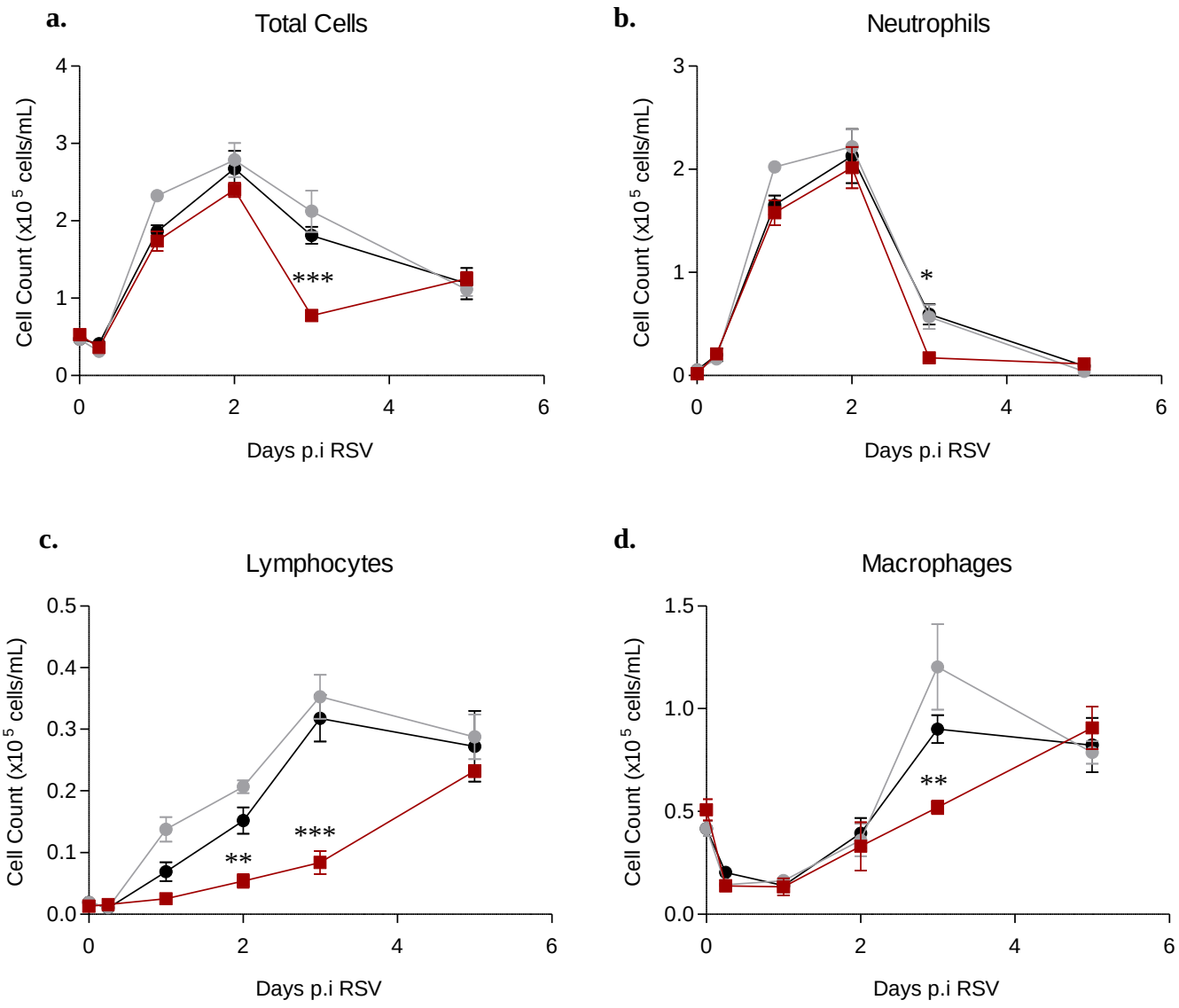
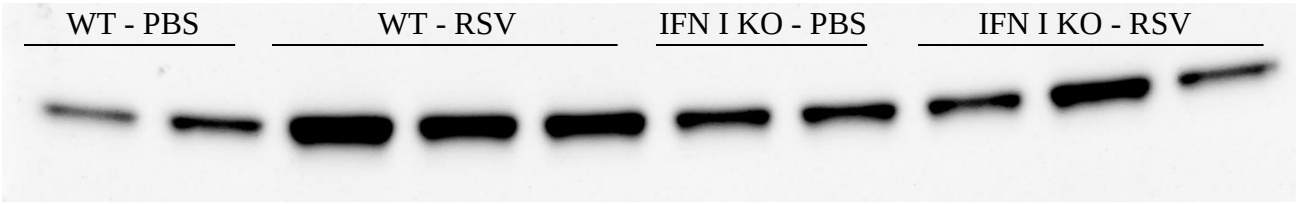


Figure 11. IFN I receptor antibody blockade reduces immune cell infiltration. 14-16 wk BALB/c mice were treated i.p. with 150 µg of either PBS/IgG isotype (RSV/IgG RSV) or IFNAR antibody (IFN-RSV) 12h prior to infection with $5x10^6$ PFU/mouse of RSV and sacrificed at 6h, D1, D3 and D5 to collect BALF. BALF cells were fixed on slides and stained prior to counting. IFNAR abx mice had significantly lower levels of (a) total cells, (b) neutrophils, (c) lymphocytes, and (d) macrophage/monocyte counts. Significance was determined using repeated measures ANOVA. Comparisons are to both PBS-RSV and IgG – RSV and the lowest significance is used. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

Figure 12.

a.



b.

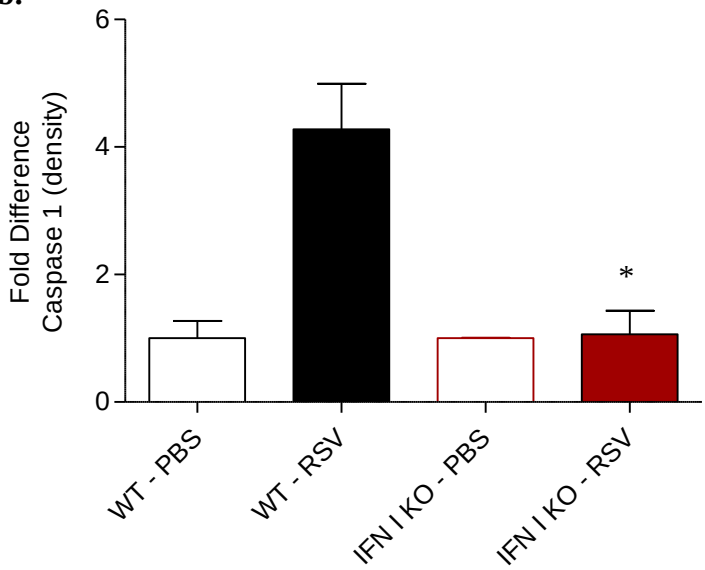


Figure 12. IFN I receptor knockdown reduces levels of caspase 1. 14-16 wk 129s mice (IFN I KO and WT age matched controls) were infected with 1x107 PFU/mouse of RSV and sacrificed at D1 p.i with RSV. Lungs were collected and total lysates were prepared. IFN I KO mice had significantly lower levels of caspase 1 in (a) western blot assays, and (b) density analysis of the western blot. Significance was determined using one way ANOVA, and comparison is shown between WT-RSV and IFN I KO - RSV. * $p<0.05$.

Figure 13.

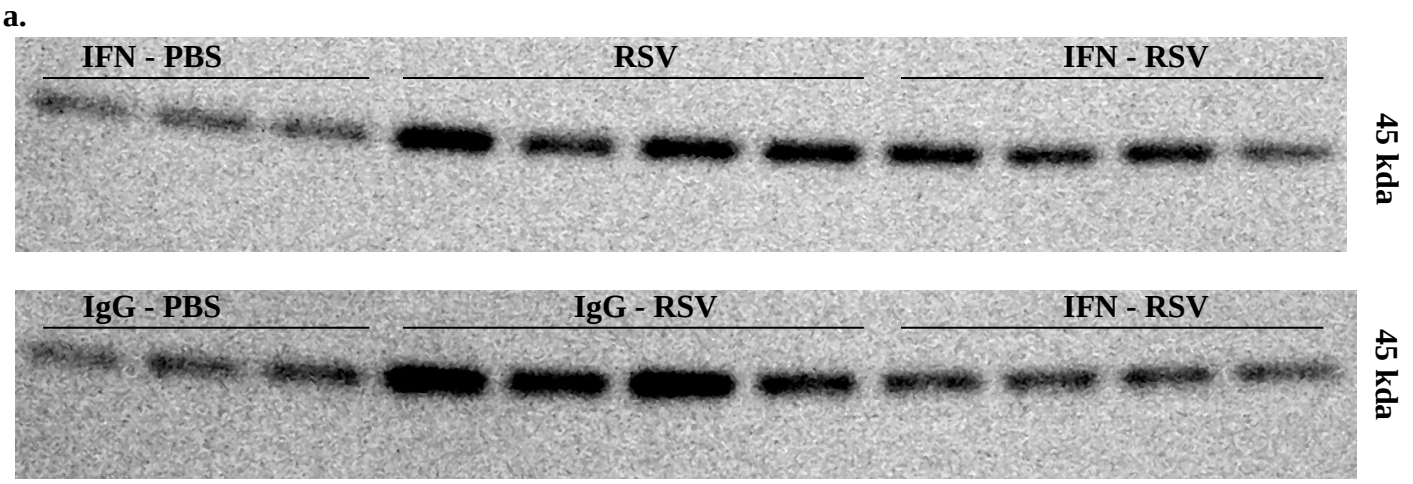

a.
IFN - PBS
RSV
IFN - RSV
45 kda
IgG - PBS
IgG - RSV
IFN - RSV
45 kda
b.
Fold Difference Caspase 1 (density)
3
2
1
0
*
IgG - PBS
IFN - PBS
RSV
IgG - RSV
IFN - RSV
c.
HMGB1 conc (pg/mL)
150
100
50
0
***
IgG - PBS
IFN - PBS
RSV
IgG - RSV
IFN - RSV

Figure 13. IFN I receptor antibody blockade reduces caspase 1 and HMGB1 levels. 14-16 wk BALB/c mice were treated i.p. with 150 μg of either PBS/IgG isotype (RSV/IgG RSV) or IFNAR antibody (IFN-RSV) 12h prior to infection with 5x106 PFU/mouse of RSV and sacrificed at D1 p.i with RSV and lungs were collected. IFNAR abx mice had significantly lower levels of caspase 1 in (a) western blot assays, and (b) density analysis of the western blot. (c) In addition, BALF obtained from IFNAR abx treated mice, also had a significant reduction in HMGB1 levels. Significance was determined using one way ANOVA. Comparisons are given as a cumulative of both PBS-RSV and IgG-RSV vs. IFN-RSV, using the lowest significance. * $p<0.05$, and *** $p<0.001$.

Figure 14.

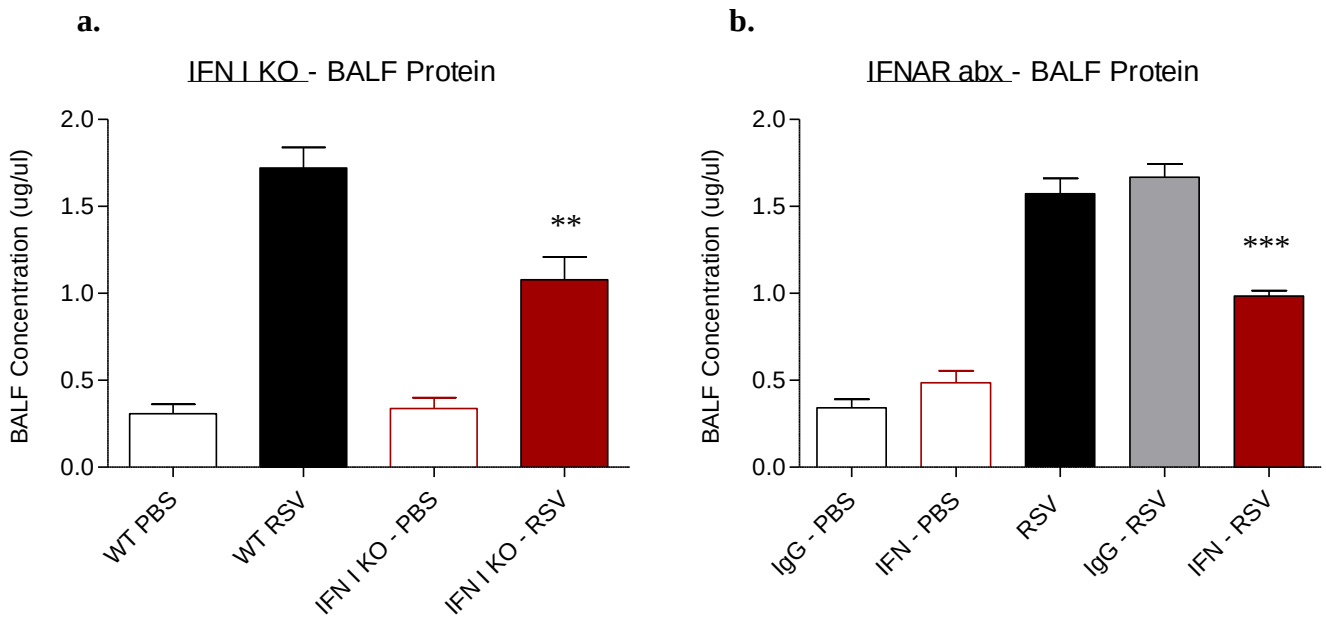


Figure 14. IFN I receptor signaling blockade reduces epithelial barrier/vascular leakage. (a) 14-16 wk 129s mice (IFN I KO and WT age matched controls) were infected with 1x107 PFU/mouse of RSV and sacrificed at D2 p.i RSV to collect BALF. Total protein levels were determined in BALF. IFN I KO mice had significantly lower levels of total protein. (b) 14-16 wk BALB/c mice were treated i.p. with 150 μg of either PBS/IgG isotype (RSV/IgG RSV) or IFNAR antibody (IFN-RSV) 12h prior to infection with 5x106 PFU/mouse of RSV and sacrificed D2 to collect BALF. As shown, IFN I abx mice also had significantly lower levels of total protein. Significance was determined using one way ANOVA. ** $p<0.01$, and *** $p< 0.001$.

Figure 15.

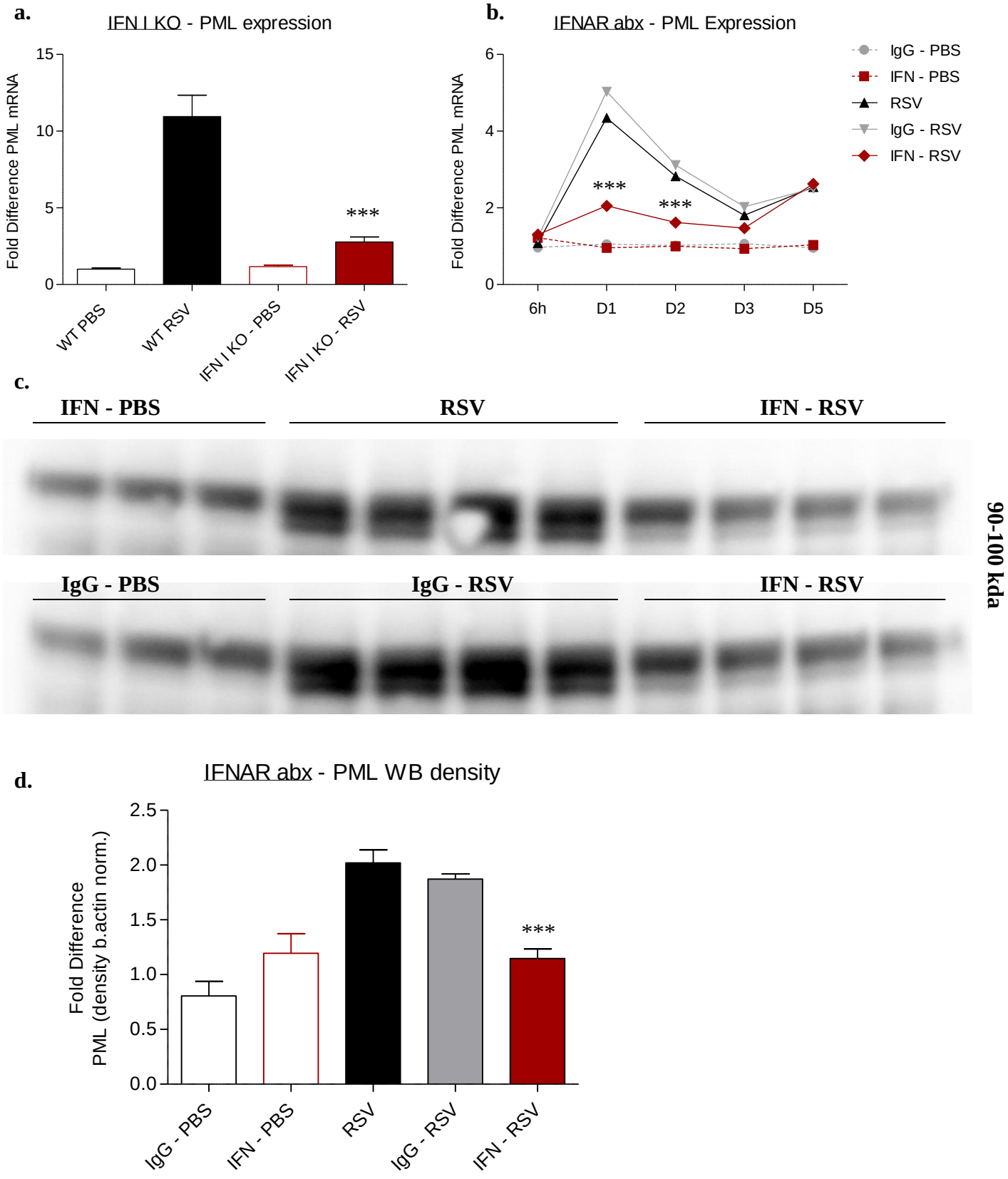
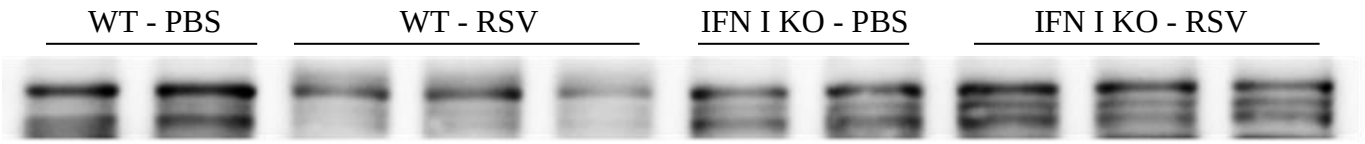

a.
IFN I KO - PML expression
Fold Difference PML mRNA
15
10
5
0
***
WT PBS
WT RSV
IFN I KO - PBS
IFN I KO - RSV
b.
IFNAR abx - PML Expression
Fold Difference PML mRNA
6
4
2
0
***
***
6h
D1
D2
D3
D5
IgG - PBS
IFN - PBS
RSV
IgG - RSV
IFN - RSV
c.
IFN - PBS
RSV
IFN - RSV
IgG - PBS
IgG - RSV
IFN - RSV
90-100 kda
d.
IFNAR abx - PML WB density
Fold Difference
PML (density b.actin norm.)
2.5
2.0
1.5
1.0
0.5
0.0
***
IgG - PBS
IFN - PBS
RSV
IgG - RSV
IFN - RSV

Figure 15. IFN I receptor signaling blockade reduces PML levels. 14-16 wk 129s mice (IFN I KO and WT age matched controls) were infected with 1x107 PFU/mouse of RSV and sacrificed at D1 p.i to collect lungs for mRNA extraction. qRT-PCR analysis was performed and IFN I KO mice had significantly lower levels of (a) PML mRNA. 14-16 wk BALB/c mice were treated i.p. with 150 μg of either PBS/IgG isotype (RSV/IgG RSV) or IFNAR antibody (IFN-RSV) 12h prior to infection with 5x106 PFU/mouse of RSV and sacrificed at 6h, D1, D3 and D5 to collect lungs for qRT-PCR analysis. (b) IFN I abx mice also had significantly lower levels of PML mRNA at D1 and D2 p.i (c) Lung lysates were used for PML western blots and IFNAR abx mice also had significant reduction in PML protein levels. (d) Densitometry analysis of western blots also shows a significant reduction in PML protein levels in IFNAR abx mice. Significance was determined using one way ANOVA (a,d), and repeated measures ANOVA (b). *** $p < 0.001$.

Figure 16.

a.



b.

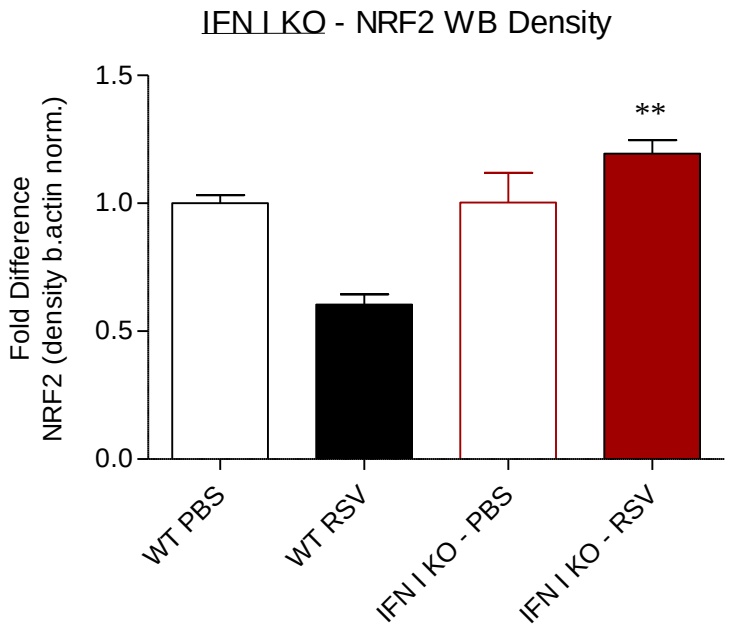


Figure 16. IFN I blockade protects NRF2 levels in RSV infection. 14-16 wk 129s mice (IFN I KO and WT age matched controls) were infected with 1x107 PFU/mouse of RSV and sacrificed at D2 p.i to collect lungs lysate preparation. (a) IFN I KO mice had maintenance of their NRF2 protein levels, whereas their WT RSV counterparts had significant degradation, as shown in the western blot image. (b) Densitometry analysis from western blots also shows a protection of NRF2 levels with IFN I KO as well. Significance was determined using one way ANOVA. ** $p < 0.01$.

Figure 17.

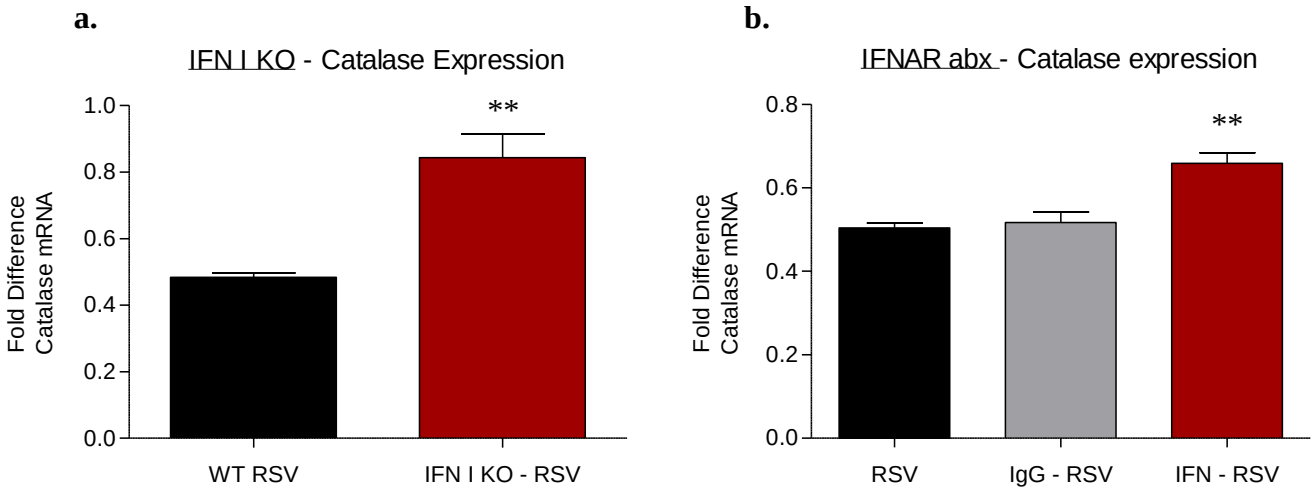


Figure 17. IFN I receptor signaling blockade enhances catalase expression. (a) 14-16 wk 129s mice (IFN I KO and WT age matched controls) were infected with 1x107 PFU/mouse of RSV and sacrificed at D2 p.i to collect lungs for mRNA extraction. qRT-PCR analysis was performed and IFN I KO mice had significantly higher levels of catalase mRNA. (b) 14-16 wk BALB/c mice were treated i.p. with 150 µg of either PBS/IgG isotype (RSV/IgG RSV) or IFNAR antibody (IFN-RSV) 12h prior to infection with 5x106 PFU/mouse of RSV and sacrificed at D3 to collect lungs for qRT-PCR analysis. (a) IFN I abx mice also had significantly higher levels of catalase mRNA. Significance was determined using student's *t*-test (a) and one way ANOVA (b). ** $p < 0.01$.

FIGURES CHAPTER 3.

Figure 1.

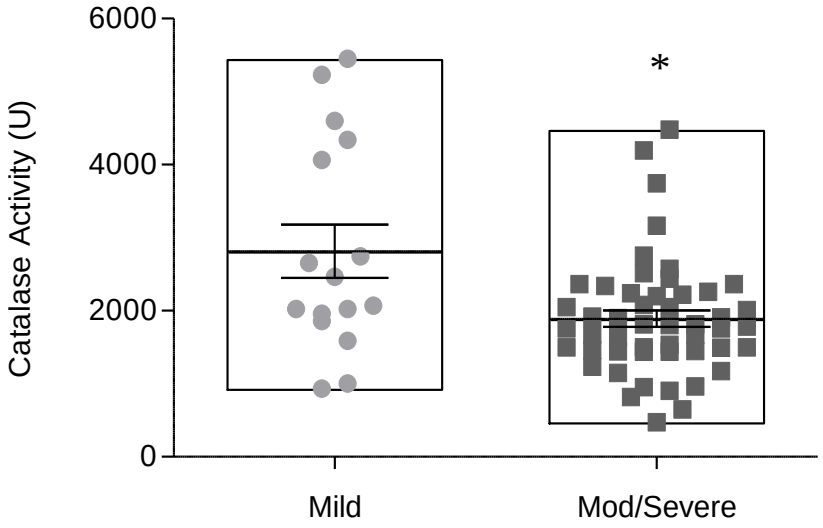


Figure 1. Catalase activity correlates negatively with RSV disease severity in patient NPS samples. NPS samples were obtained from patients with a flush of PBS under suction. Patients were grouped into mild and moderate/severe groups based on hospitalization status, oxygen requirements and PICU admission. Catalase activity was determined with protein/volume normalized samples. Patients in the mild group had significantly higher catalase activity compared to moderate/severe groups. Significance was determined using student's *t*-test, with unequal variances and $p<0.05$.

Figure 2.

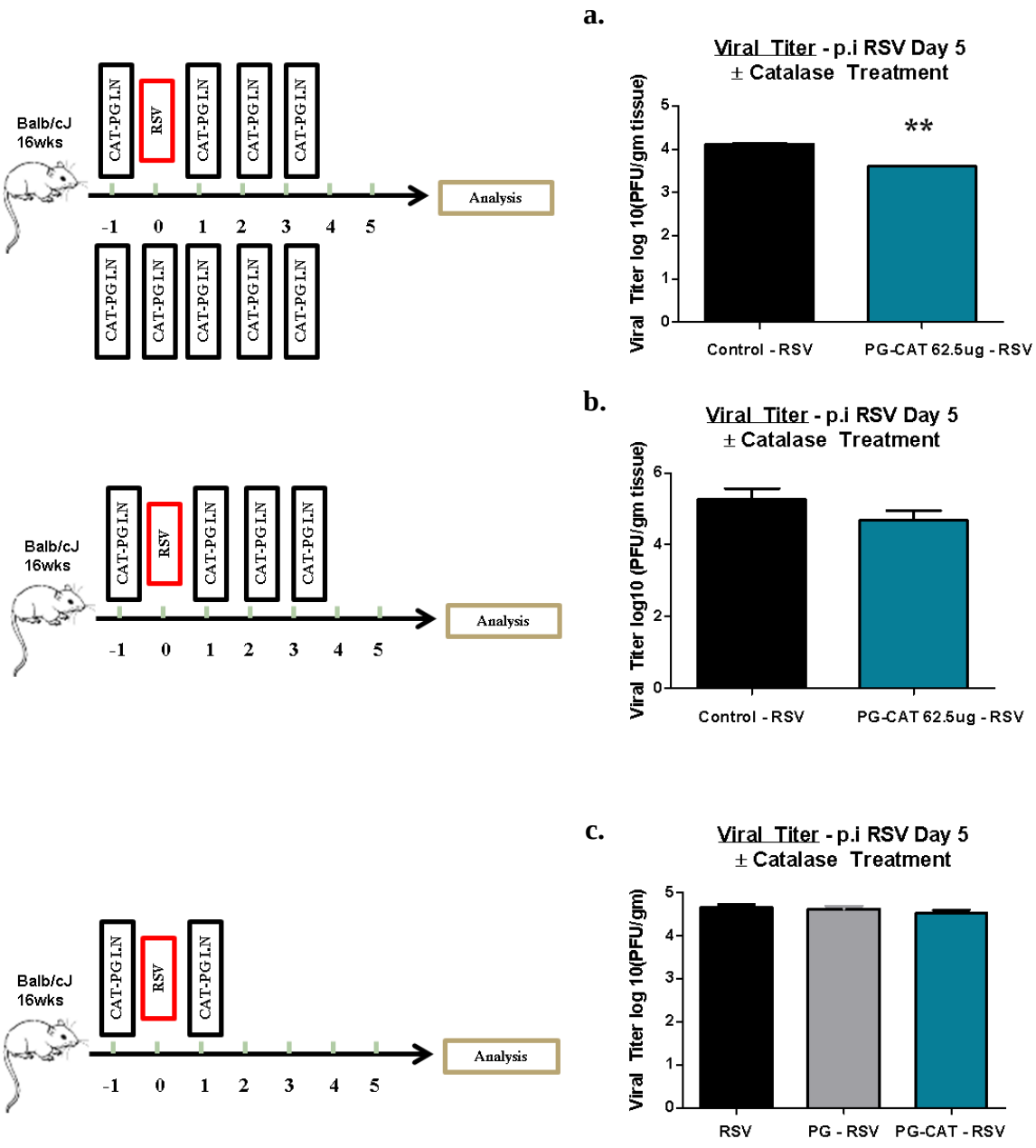


Figure 2. Optimization of catalase treatment protocol and effects on RSV viral titer. Several models of PG-CAT treatment were practiced prior to utilization of one optimal method. (a) 14-16 week old BALB/c mice were treated twice daily with 62.5 µg of PG-CAT intranasaly (i.n.) and sacrificed at D5 p.i with RSV. Viral titers were determined using plaque assays with lung tissue lysates. PG-CAT treatment resulted in ~0.6log10 reduction in viral titer. (b) Mice were treated with only one dose of treatment daily upto D3 p.i with RSV and viral titers were similarly reduced ~0.6log10. (c) Mice were treated

with a single dose D(-1) and D1 with respect to RSV infection, no effects were seen on RSV viral titer. Statistical significance was determined using student's *t*-test (a-b) and one-way ANOVA (c). ** $p<0.01$.

Figure 3.

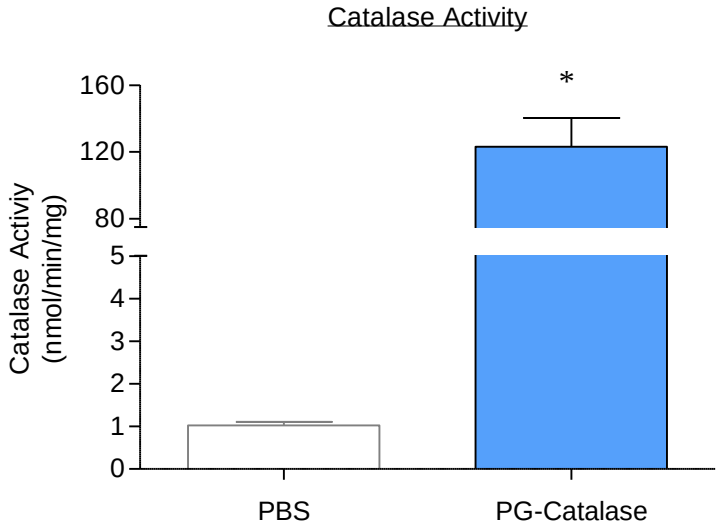


Figure 3. PG-CAT treatment results in enhanced and sustained catalase activity in bronchoalveolar lavage fluid (BALF) – 14-16wk old BALB/c mice were treated with 62.5 μg of PG-CAT and sacrificed at D1 post-treatment. Lungs were flushed with 1mL of cold PBS and BALF was centrifuged to remove cellular fractions. Catalase activity assay was performed and PG-CAT treatment resulted in significantly enhanced catalase activity even 24h post treatment. Statistical significance was determined using student's *t*-test. * $p<0.05$.

Figure 4.

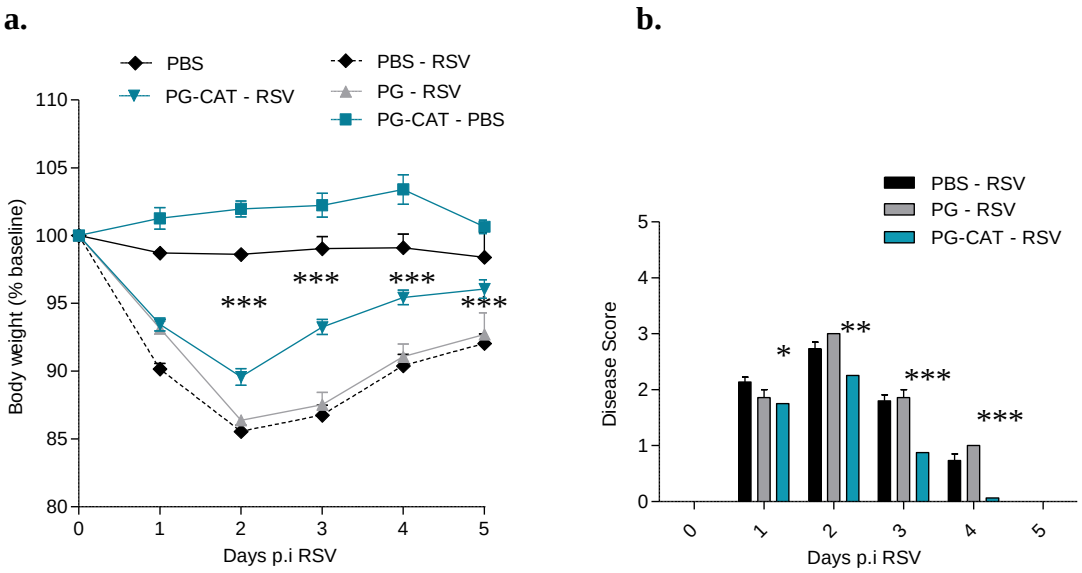


Figure 4. Exogenous catalase treatment improves RSV clinical disease parameters. 14-16 wk BALB/c mice were treated with PG-CAT or controls (PBS or PG only) and infected with $5x10^6$ PFU/mouse of RSV. (a) Mice were monitored daily for body weight loss and (b) clinical disease score (based on ruffled fur, hunched back etc.). PG-CAT treatment resulted in a significant improvement in both parameters. Significance was determine using repeated measures ANOVA and *** represent comparison to both infected controls. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

Figure 5.

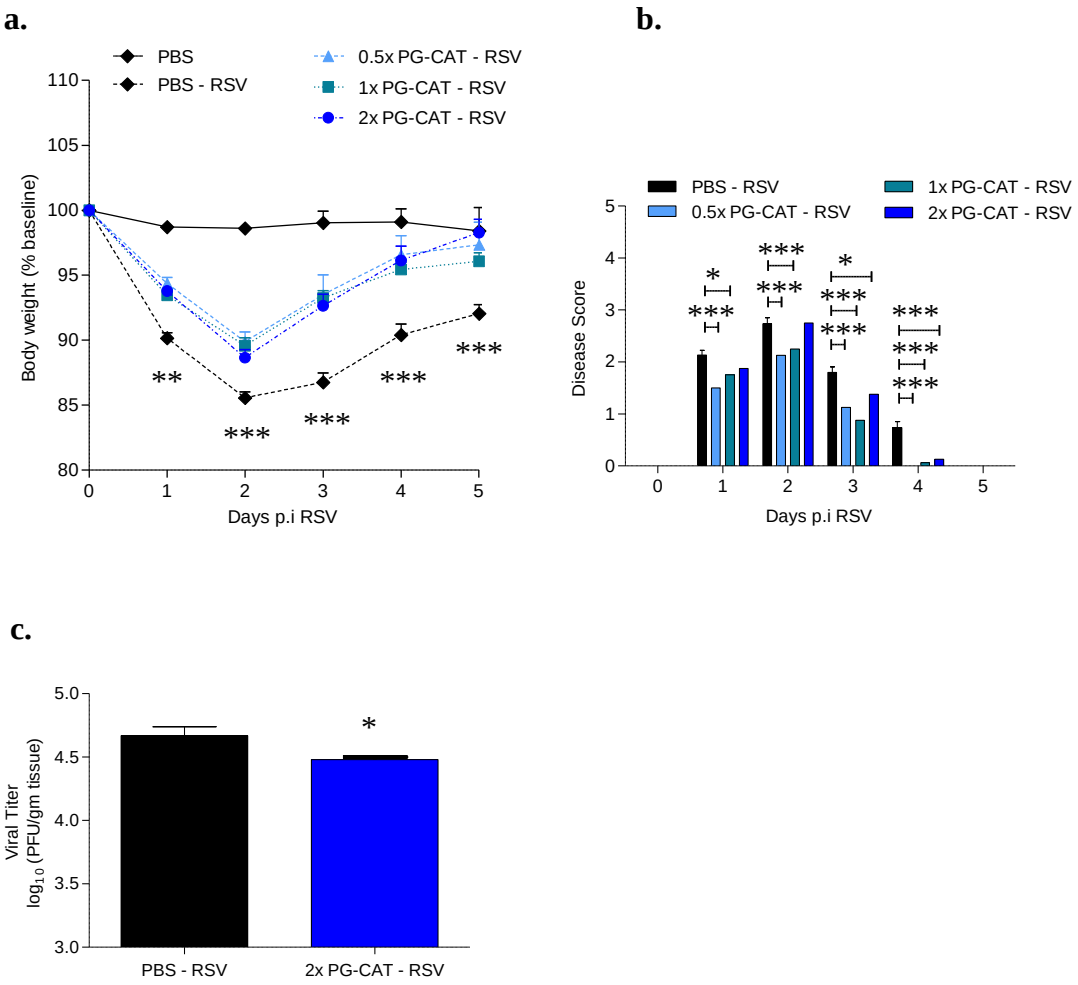


Figure 5. Different dosages of exogenous catalase provide similar improvements in RSV clinical disease parameters. 14-16 wk BALB/c mice were treated with PG-CAT (1x – 62.5 µg, 0.5x – 31.25 µg, and 2x – 125 µg) or controls (PBS or PG only) and infected with $5x10^6$ PFU/mouse of RSV. (a) Mice were monitored daily for body weight loss and (b) clinical disease score. All PG-CAT treatments resulted in a significant improvement in both parameters. (c) 2x treatment showed a significant improvement in levels of RSV viral titer by CPE (plaque assay). No changes seen with 0.5x and 1x. Significance was determine using repeated measures ANOVA (a-b) and student's *t*-test (c), and *** represent comparison of all treatments vs RSV. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

Figure 6.

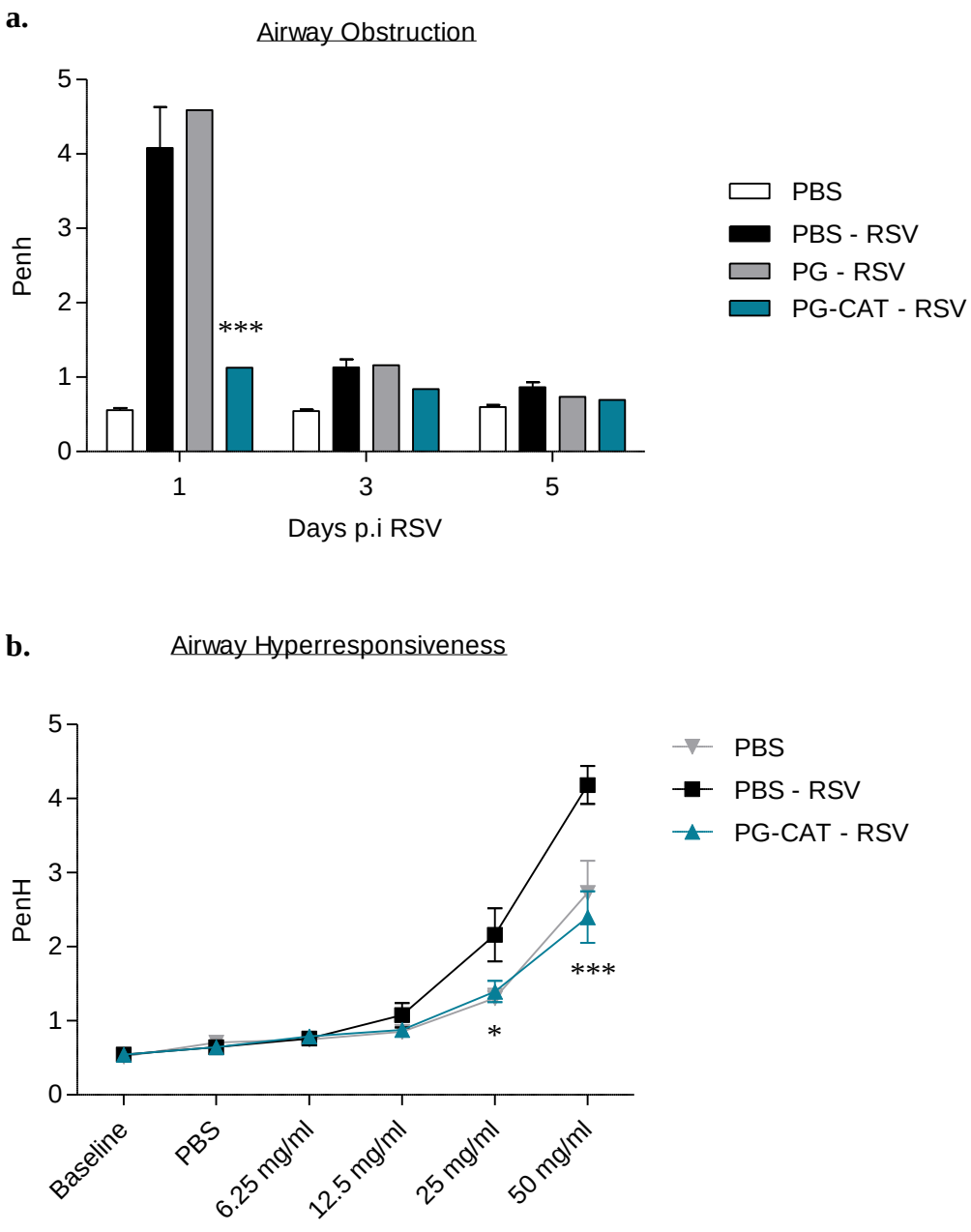


Figure 6. PG-CAT treatment results in significant improvements in airway function parameters. 14-16 wk BALB/c mice were treated with PG-CAT or controls (PBS or PG only) and infected with $5x10^6$ PFU/mouse of RSV. Airway function parameters were determined using the full body plethysmography apparatus: BUXCO. (a) Baseline airway obstruction was determined at D1, D3 and D5 p.i with RSV. PG-CAT treatment resulted in significant improvement at D1 p.i. (b) Airway hyperresponsiveness (AHR) was determined using increasing dosages of methacholine at D8 p.i with RSV. PG-CAT treatment resulted in a significant improvement in AHR as well. Significance was

determine using repeated measures ANOVA and *** represent comparison to both infected controls. * $p<0.05$, and *** $p<0.001$.

Figure 7.

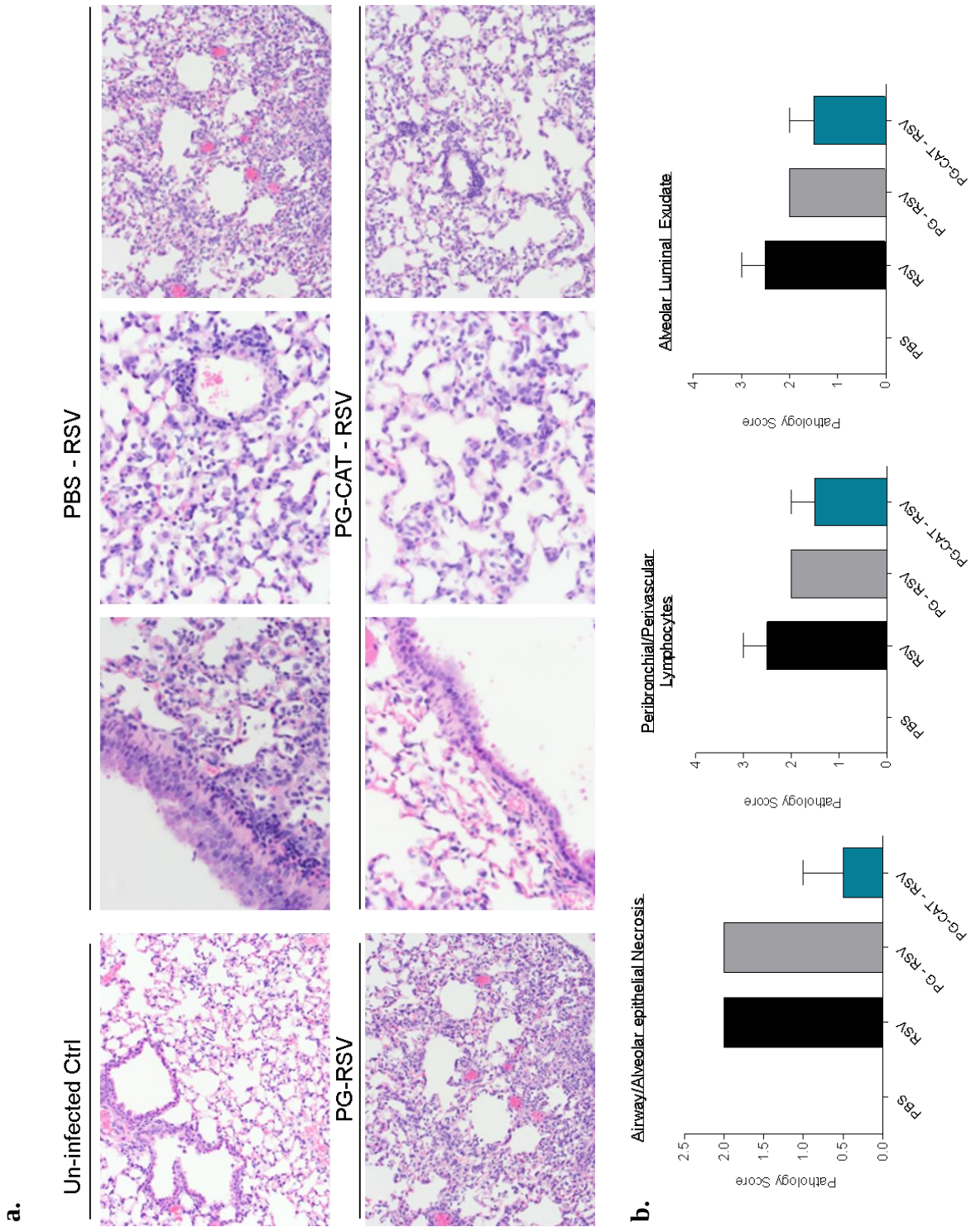
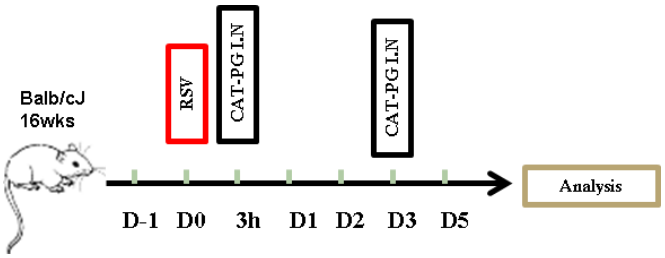
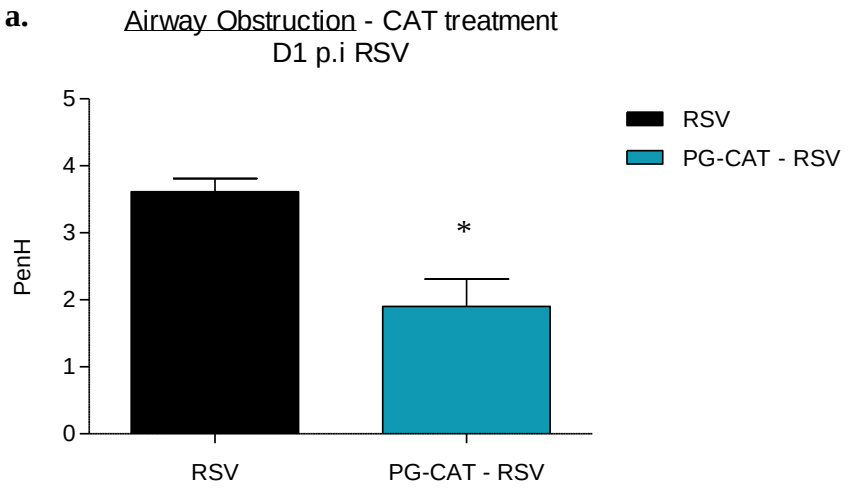


Figure 7. Exogenous catalase treatment improves pulmonary pathology caused by RSV infection . 14-16 wk BALB/c mice were treated with PG-CAT or controls (PBS or PG only) and infected with $5x10^6$ PFU/mouse of RSV. Mice were sacrificed at D3 p.i and

lungs were flushed with 10% NB formalin prior to paraffin embedding and sectioning. Slides were stained with H&E stain and visualized for pathological changes. (a) PG-CAT treatment resulted in a significant decrease in airway epithelial damage, pneumocyte hypertrophy, interstitial pneumonia and overall airway structure damage compared to infected controls (PBS – RSV and PG – RSV). (b) Slides were scored by a blinded trained pathologist and scored for severity. PG-CAT treatment resulted in a reduction in airway epithelial necrosis, lymphocyte infiltrate and alveolar luminal exudate.

Figure 8.





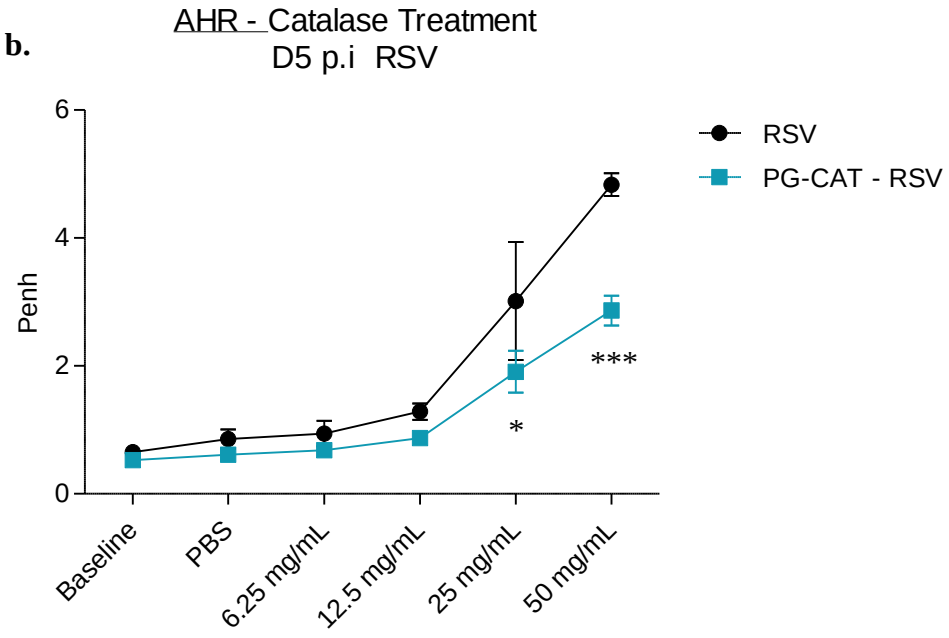


Figure 8. Exogenous catalase treatment given p.i with RSV still results in airway function improvements. 14-16 wk old BALB/c mice were infected with $5x10^6$ PFU/mouse of

RSV, prior to PG-CAT treatment at 3h and D3 p.i. Airway obstruction was measured at D1 p.i and PG-CAT treatment resulted in a significant reduction in airway obstruction, measured as described before. (b) AHR performed at D5 p.i with RSV also showed that PG-CAT treatment significantly reduced hyperreactivity in mice. Significance was determined using student's *t*-test (a) and repeated measures ANOVA (b). * $p<0.05$ and *** $p<0.001$.

Figure 9.

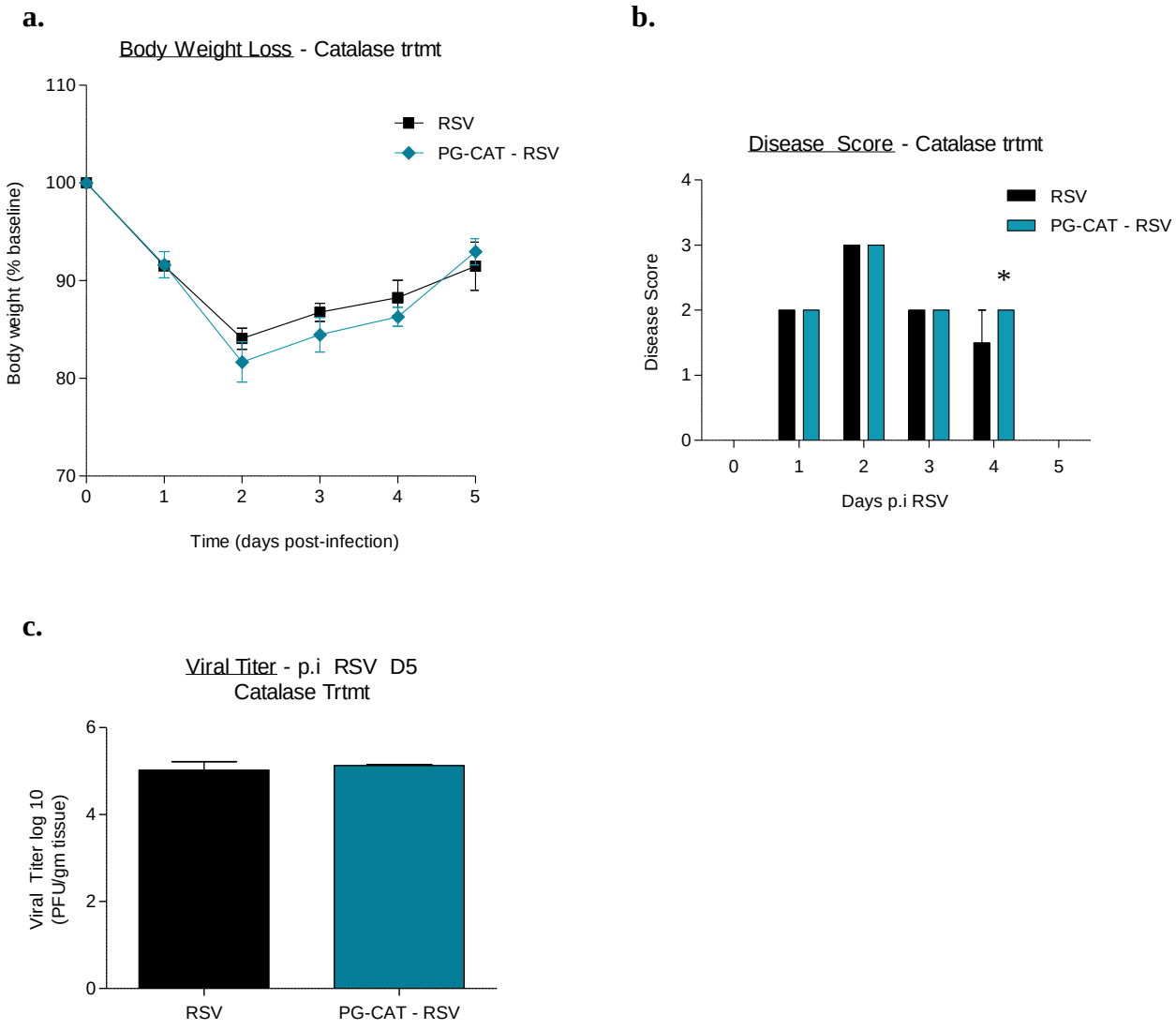


Figure 9. Exogenous catalase treatment p.i with RSV does not alter clinical disease parameters. 14-16 wk old BALB/c mice were infected with $5x10^6$ PFU/mouse of RSV, prior to PG-CAT treatment at 3h and D3 p.i. (a) Mice were observed daily for body weight loss and (b) clinical disease. No changes were observed in either parameters. (c) Mice were sacrificed D5 p.i and lungs were harvested for viral titer by plaque assay. PG-CAT treatment did not result in significant changes in viral titer. Significance was determined using repeated measures ANOVA (a-b) and student's *t*-test (c). * $p<0.05$.

Figure 10.

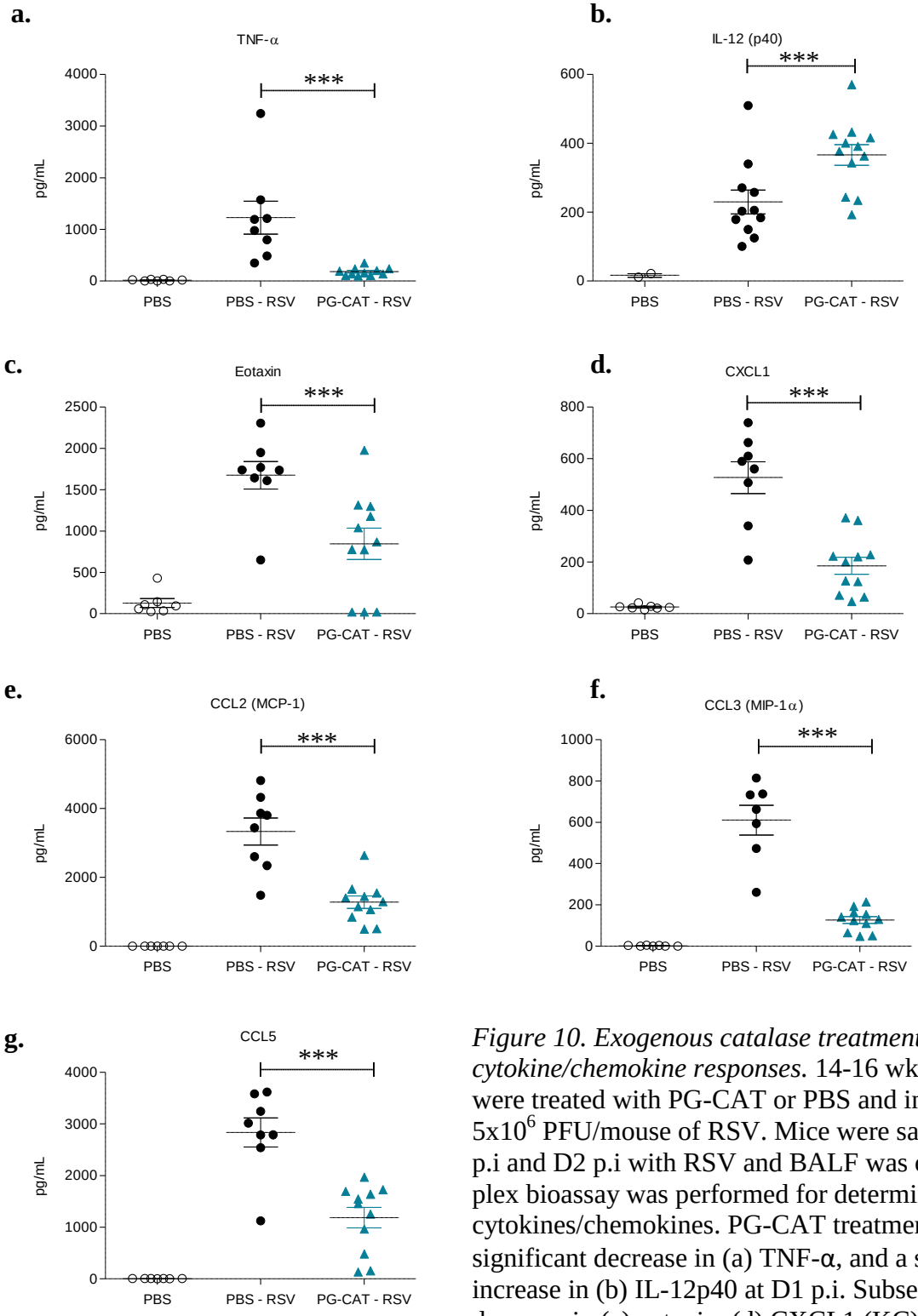


Figure 10. Exogenous catalase treatment modulates the cytokine/chemokine responses. 14-16 wk BALB/c mice were treated with PG-CAT or PBS and infected with $5x10^6$ PFU/mouse of RSV. Mice were sacrificed at D1 p.i and D2 p.i with RSV and BALF was obtained. A 23-plex bioassay was performed for determination of key cytokines/chemokines. PG-CAT treatment results in a significant decrease in (a) TNF-α, and a significant increase in (b) IL-12p40 at D1 p.i. Subsequently a decrease in (c) eotaxin, (d) CXCL1 (KC), (e) CCL2 (MCP-1), (f) CCL3 (MIP-1α) and (g) CCL5 (RANTES) was also observed. Significance was determined using one-way ANOVA. *** $p<0.001$.

Figure 11.

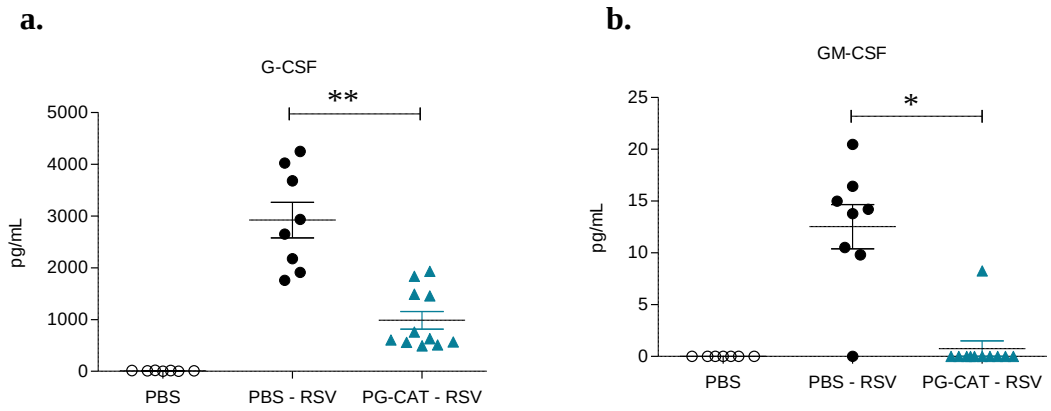


Figure 11. Exogenous catalase treatment significantly reduces growth factors. 14-16 wk BALB/c mice were treated with PG-CAT or PBS and infected with $5x10^6$ PFU/mouse of RSV. Mice were sacrificed at D2 p.i with RSV and BALF was obtained. A 23-plex bioassay was performed for determination of key growth factors. PG-CAT treatment resulted in a significant reduction in (a) G-CSF and (b) GM-CSF levels. Significance was determined using one-way ANOVA.

Figure 12

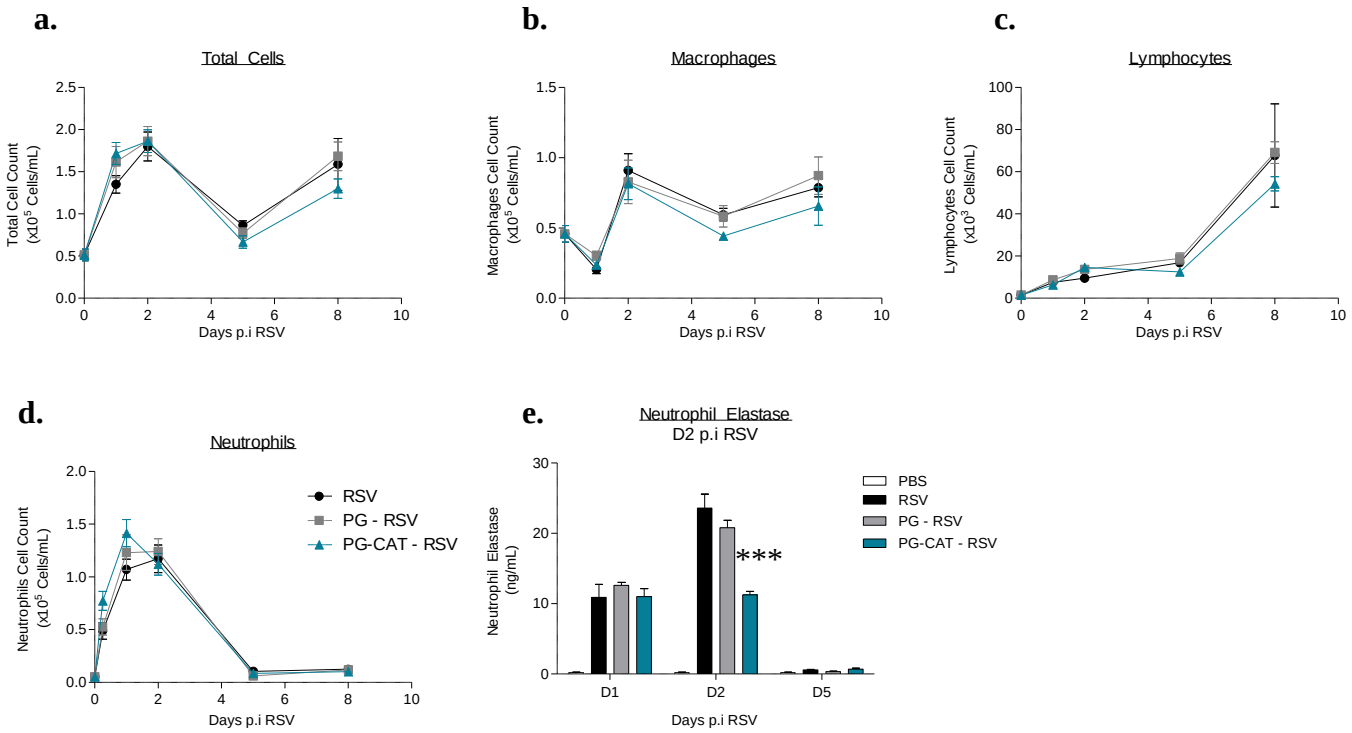


Figure 12. Exogenous catalase treatment does not alter immune cell infiltration but reduces neutrophil activity. 14-16 wk BALB/c mice were treated with PG-CAT, PG only, or PBS and infected with $5x10^6$ PFU/mouse of RSV. Mice were sacrificed at D1, D2, D5, and D8 p.i with RSV and BALF was obtained for differential cell counts. No changes were observed in levels of (a) total cells, (b) macrophages, (c) lymphocytes, or (d) neutrophils. (e) BALF obtained at D1, D2, and D5 p.i was used to determine levels of neutrophil elastase. PG-CAT treatment resulted in a significant reduction in neutrophil elastase levels at D2 p.i. Significance was determined using repeated measures ANOVA.

Figure 13.

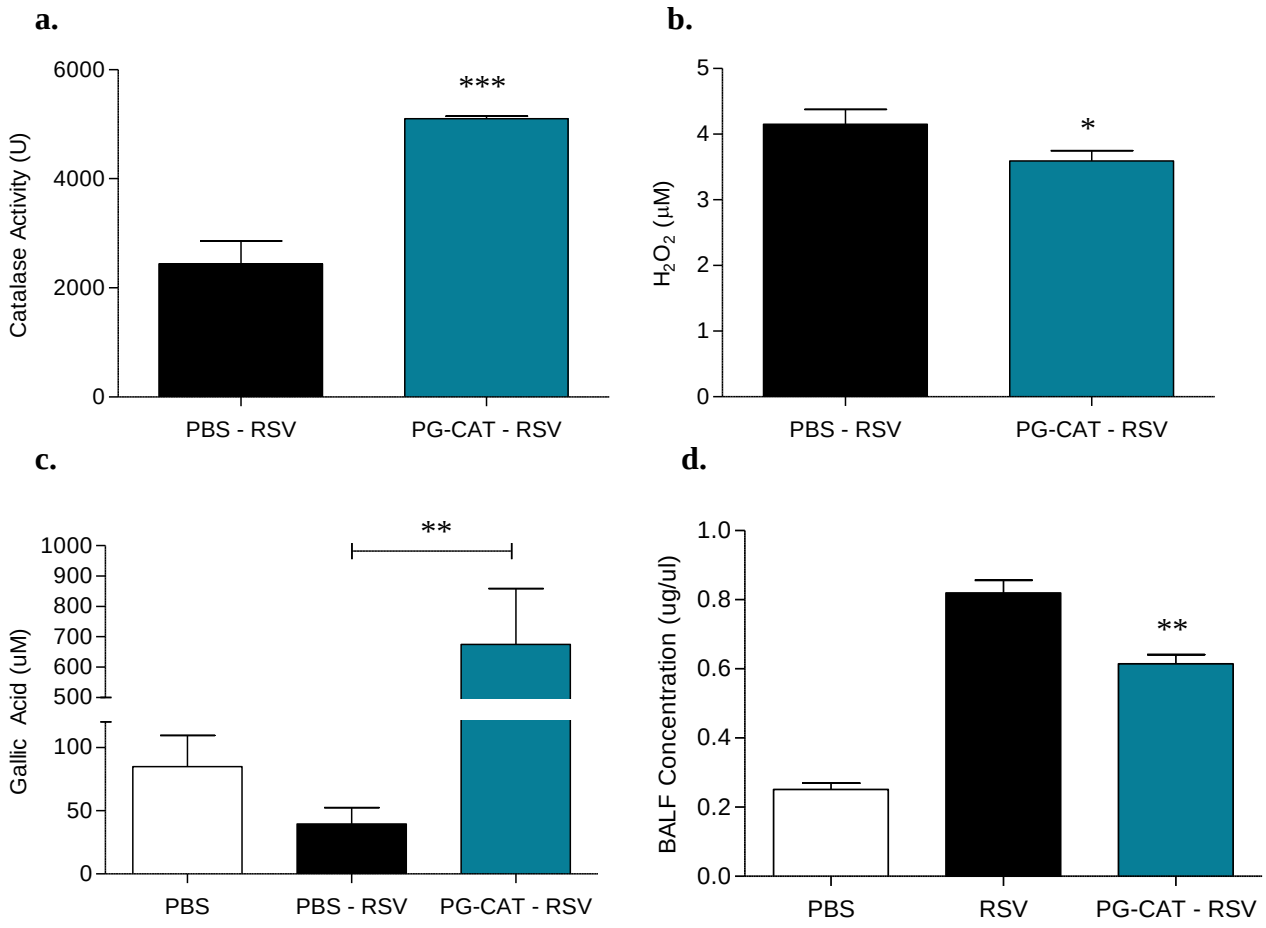


Figure 13. Role of reactive oxygen species in exogenous catalase treatment mediated improvements in RSV disease parameters. 14-16 wk BALB/c mice were treated with PG-CAT, or PBS and infected with $5x10^6$ PFU/mouse of RSV. BALF was obtained at D1 and D2 p.i with RSV. (a) Catalase activity was determined at D2 p.i and PG-CAT treatment still maintained a significant increase in catalase activity at this time. (b) A reduction in overall H_2O_2 levels were also observed at D1 p.i with RSV. (c) A hydroxyl radical antioxidant capacity (HORAC) assay was also performed to directly determine the antioxidant capacity of PG-CAT treatment, and treatment resulted in a significant enhancement in HORAC levels. (d) Total protein measured as a parameter of vascular permeability and epithelial barrier damage was also reduced at D2 p.i. Overall these results show that PG-CAT treatment successfully results in enhanced antioxidant tone, resulting in improvement in airway barrier function. Significance was determined using student's *t*-test (a-b) and one-way ANOVA (c-d).

Figure 14.

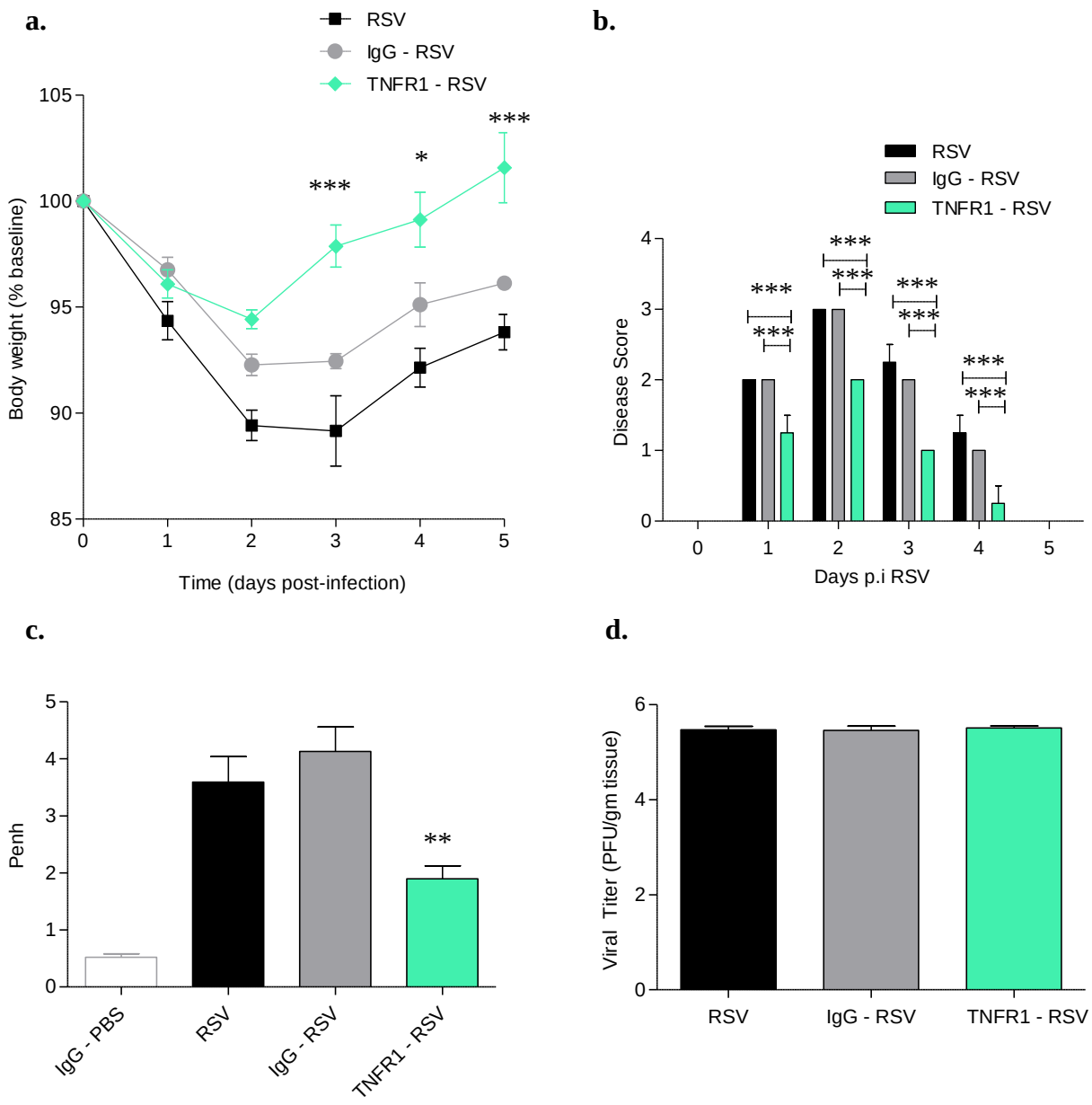


Figure 14. Role of TNF-α in exogenous catalase mediated improvements in RSV disease parameters. 14 wk BALB/c mice were treated with 80 µg of TNFR1 antibody intranasally and infected with $5x10^6$ PFU/mouse of RSV D1 post treatment. Control mice were treated with either PBS or IgG. (a) Mice were observed for body weight loss and (b) disease score. TNFR1 antibody treatment resulted in a significant reduction in both disease parameters. (c) airway obstruction obtained at D1 p.i with RSV was also reduced with TNFR1 treatment. (d) No changes were observed in viral titer. Significance was determined using repeated measures ANOVA (a-b) and one –way ANOVA (c-d). Significances are represented as a combination of comparison to RSV and IgG – RSV. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

Figure 15.

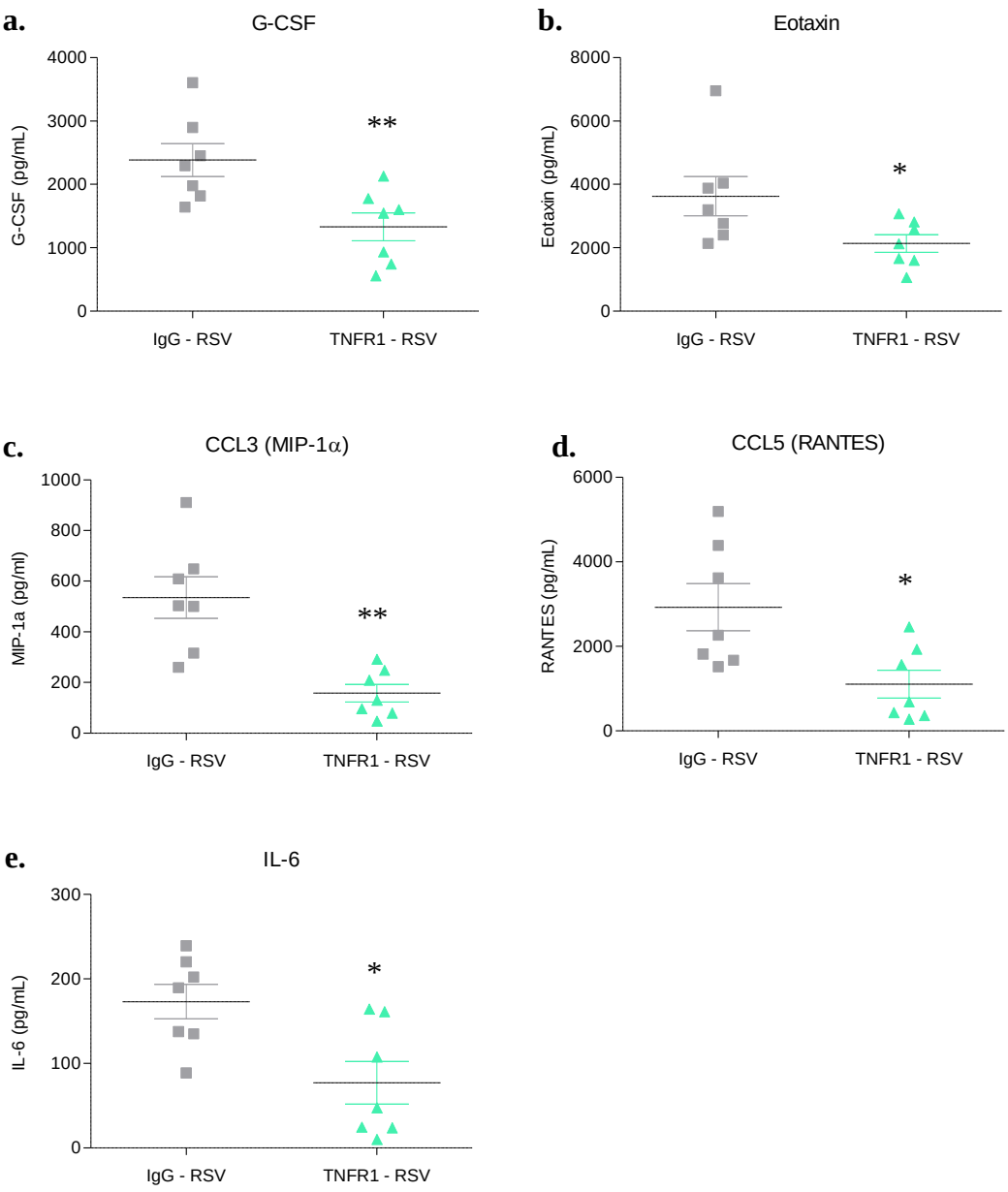


Figure 15. TNFR1 blockade results in a similar reduction in cytokines, chemokines, and growth factors. 14 wk BALB/c mice were treated with 80 µg of TNFR1 antibody intranasally and infected with $5x10^6$ PFU/mouse of RSV D1 post treatment. Control mice were treated with IgG. BALF was obtained from mice at D2 p.i and a 23-plex bioassay was performed. Levels of (a) G-CSF, (b) Eotaxin, (c) CCL3 (MIP-1α), (d) CCL5 (RANTES), and (e) IL-6 were determined. TNFR1 blockade resulted in a significant decrease in all of the above. Significance was determined using student's *t*-test. * $p<0.05$ and ** $p<0.01$.

TABLES

Table 1.

GENE TARGET	PRIMERS
Catalase	GAACGAGGAGGAGAGGAAACG CGGGCCCCATAGTCAGG
Mx1	CCCTGCTACCTTTCAAAATAACTCTC CTCCCAATATTCGTCTGCAC
PML	CTGCTATCGTCCAACTCGTG AAAAGATCCTCCTGCCCAAC

Table 1. *Primers utilized for mRNA and qRT-PCR analysis*

Table 2.

RACE	CLINICAL SEVERITY	CC	CT/TT	P-VALUE
African American (n = 32)	Mild	16 (94%)	1 (6%)	NS
	Mod/severe	13 (87%)	2 (13%)	NS
	Severe only	5 (100%)	0 (0%)	0.000
Caucasian (n = 67)	Mild	20 (61%)	13 (39%)	NS
	Mod/severe	30 (88%)	4 (12%)	4.0e-5
	Severe only	7 (100%)	0 (0%)	0.000
Hispanic (n = 37)	Mild	12 (86%)	2 (14%)	NS
	Mod/severe	21 (91%)	2 (9%)	0.03
	Severe only	3 (100%)	0 (0%)	0.000

Table 2. *Presence of catalase polymorphism (CT/TT – rs1001179) is a correlate of protection from RSV disease severity.* Nasopharyngeal samples were obtained from patients, and DNA was extracted for DNA sequencing. Patients were grouped in mild, moderate, and severe categories based on clinical parameters. Presence of catalase polymorphism (CT – heterozygous or TT – homozygous) was analyzed with disease severity. Patients with either CT/TT had lower representation in moderate/severe groups, and were not represented at all in the severe category. Statistics were performed with exact test goodness of fit test via a binomial test.

ILLUSTRATIONS

Illustration 1. Role of type I interferon in RSV disease pathogenesis

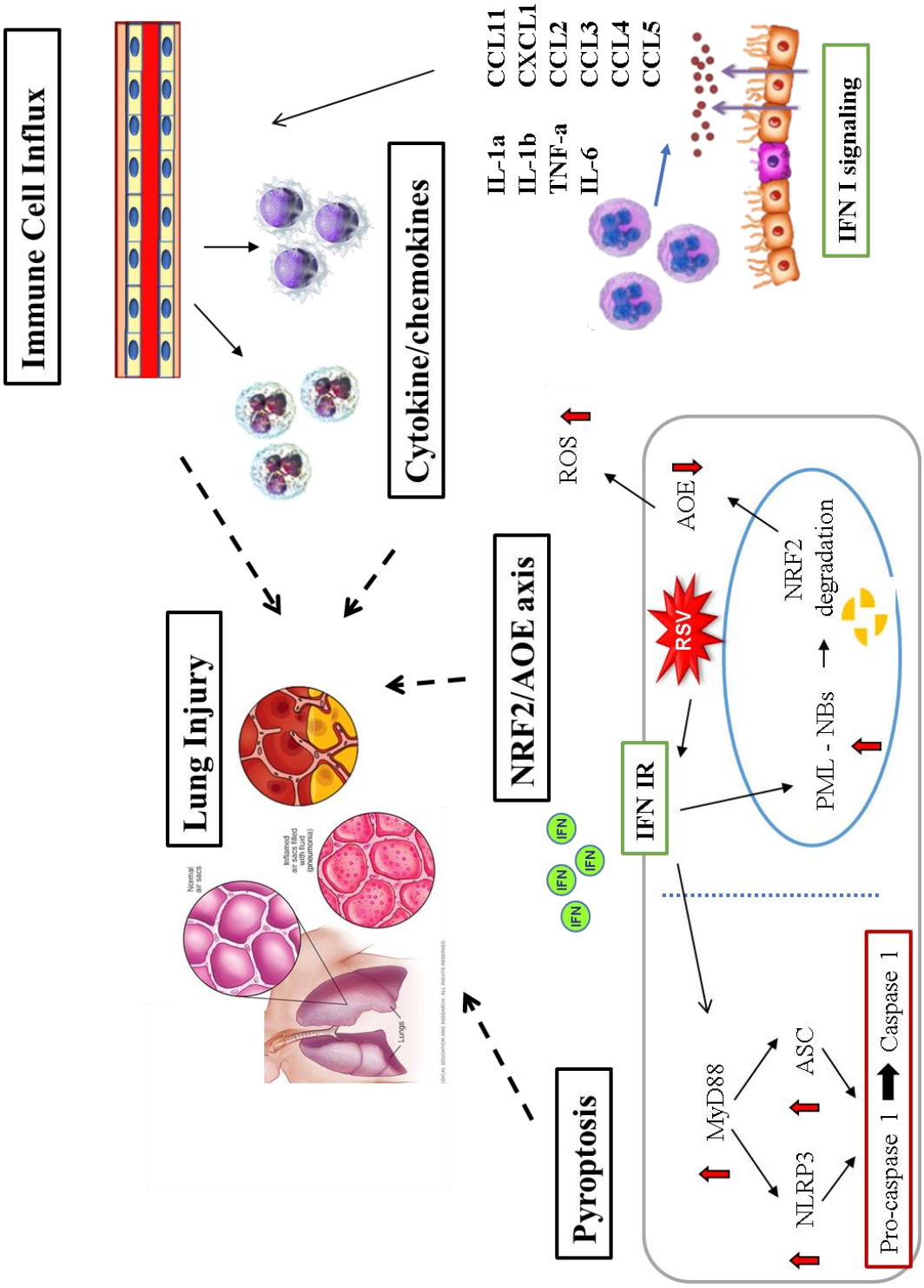
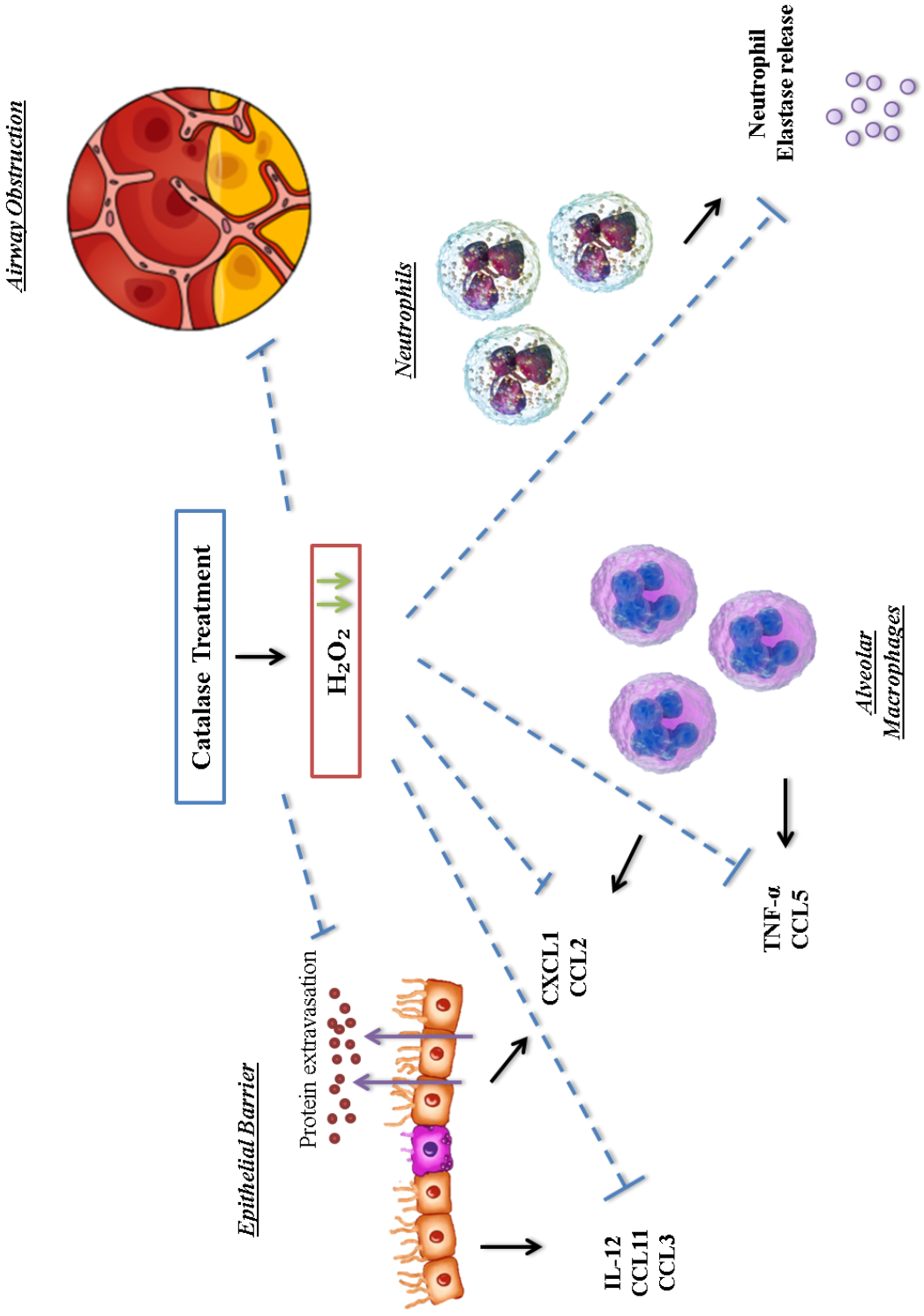


Illustration 2. PG-catalase treatment mediated protection in RSV infection



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Curriculum Vita

NAME: Maria Ansar

PRESENT POSITION AND ADDRESS:

- MD/PhD Student – May 2013 – May 2021
- Graduate Student in Department of Microbiology and Immunology – August 2015 – May 2019
- Current Address: 2828 61st Street Apt # 1004, Galveston, Texas. 77551.
- Cell Phone #: 240-449-7429

BIOGRAPHICAL:

- Birthdate: April 20, 1990
- Birthplace: Islamabad, Pakistan
- Citizenship: U.S Citizen
- Business Address: 2.314 Research Building 6, University of Texas Medical Branch, Galveston, Texas, 77555.

EDUCATION:

INSTITUTION AND LOCATION	**DEGREE**	**START DATE MM/YYYY**	**END DATE *(or expected end date)* MM/YYYY**	**FIELD OF STUDY**
University of Texas Medical Branch, Galveston, TX.	Ph.D.	08/2015	05/2019	Microbiology and Immunology
University of Texas Medical Branch, Galveston, TX.	M.D	08/2013	05/2021	Medicine
University of Maryland, College Park, MD.	BSc.	08/2008	05/2012	Cell Biology and Molecular Genetics
University of Maryland, College Park, MD.	BSc.	08/2008	05/2012	Biochemistry

1. CURRENT RESEARCH ACTIVITIES:

Currently, I work in Dr. Roberto P. Garofalo's lab in the Pediatrics Allergy and Immunology Department. My project focuses on the role of antioxidants and interferon

signaling in modulating disease progression during Respiratory Syncytial Virus (RSV) infections in pediatric populations. RSV virus mainly infects children < 2 years of age, and is a cause of significant morbidity and mortality in this age group. Currently, there are no effective therapeutics or vaccines available for RSV infection, despite > 33.1 million infections globally each year. It is well known that RSV infections lead to extensive inflammatory damage and upregulation of inflammatory transcription factors such as NF-κB, which further leads to upregulation of tumor necrosis factor-α, IL-1, and IL-6. Recent work in the Garofalo lab has additionally shown that there is also downregulation of anti-oxidant transcription factors (i.e. NRF-2), as well as antioxidant enzymes such as catalase, superoxide dismutase (SOD) etc. My current project involves delivery of exogenous catalase, SOD, and other antioxidant enzymes in an attempt to restore antioxidant capabilities in the RSV mouse model. We are currently testing direct intranasal delivery of pegylated-Catalase as a potential therapeutic for RSV infection. Additionally, I also take part in some of the lab's clinical research activities which involve collecting nasopharyngeal secretion samples from patients for this project and others, in order to determine antioxidant levels and additional parameters in human subjects. It is our hypothesis that restoring the antioxidant potential can ameliorate some of the inflammatory damage done by RSV infection and ameliorate disease parameters such as airway hyperresponsiveness and pathology. Additionally, I am also studying the underlying mechanisms that lead to RSV related disease as well as NRF-2 degradation. Our preliminary results suggest a possible role of interferon signaling and downstream generation of promyelocytic protein (PML) – nuclear bodies that could lead to the degradation of NRF-2. Our aim is to determine if inhibition of these pathways could also provide further targets for therapeutics and enhance current knowledge regarding RSV pathogenesis.

The goal of all of these studies is to discover ways to decrease disease severity, and morbidity in the mouse model of RSV, so that it can be utilized for patients in the future. Currently, there is no vaccine for RSV and treatments remain non-specific and sub-sufficient. Therefore, this work carries the potential to be a significant contribution in finding better therapeutics for RSV. Both of these projects have been funded through support described below:

a. GRANT SUPPORT:

i. Institute of Translational Sciences at the University of Texas Medical Branch – NRSA TL1 Training Core (TL1TR001440) National Center for Advancing Clinical Sciences, NIH. December 2015 – December 2017

ii. Graduate School of Biomedical Sciences at University of Texas Medical Branch – McLaughlin Fellowship. December 2017 – August 2019

2. MEDICAL EDUCATION RESEARCH

a. At UTMB I have worked with Dr. Huda Sarraj (Jun 2017 – Oct 2018), in the Institute of Translational Sciences. I worked with a small group of students from diverse departments, focusing on studying interprofessional relationships among graduate and medical students. UTMB has an extensive interprofessional education (IPE) network, and all participants including myself have had several different IPE experiences. Each of us individually wrote autoethnographic accounts of our experiences, discussed narratives in a groups setting, and analyzed our narratives for common themes highlighting strengths, weaknesses and future directions for the IPE program. The data was analyzed through ongoing group meetings and discussions. Our manuscript has been published in the Journal of Interprofessional Practice and Education, and I am a second author as described below. We have also presented our research at the Innovations in Health Science Education Meeting:

i. Ansar, M.; Dimet, A.; Levine CB.; Miller A,; Moon J.; Rice, C.; Schaeffer, A.; Andersson, J.; Ekpo-Otu, S.; McGrath, E.; and Sarraj, H. (2018). An Autoethnographical Account: Attitudes of medical and graduate students in the biomedical sciences before and after interprofessional experiences. Innovations in Health Science Education Meeting, Austin, TX.
    1. Selected for oral presentation

ii. Levine, CB.; Ansar, M.; Dimet, A.; Miller A,; Moon J.; Rice, C.; Schaeffer, A.; Andersson, J.; Ekpo-Otu, S.; McGrath, E.; and Sarraj, H. (2019). Student evaluation of interprofessional experiences between medical and graduate biomedical students, *Journal of Interprofessional Practice and Education*, 9(1), 1-12.

3. PAST RESEARCH PROJECTS

a. **Undergraduate Research (University of Maryland)** – I worked in Dr. Silvia Muro's lab (2010-2013) focusing on manipulating endocytic pathways to allow more efficient nanocarrier mediated drug delivery. The goal of my work was to improve nanocarrier mediated drug delivery in lung vasculature. The main targets were lysosomes in endothelial cells in order to improve lysosomal enzyme replacement therapy for diseases such as Niemann Pick and Fabry disease. My specific project consisted of determining whether endocytic pathways could be manipulated to target drugs to one receptor, while allowing endocytosis to occur through a non-related pathway. For this purpose nanocarriers were targeted to Mannose-6-Phosphate receptor and sphingomyelinases were utilized to allow internalization via Cellular Adhesion Molecule (CAM)-mediated endocytic pathway. Using this approach we were able to have internalization of 1-4.5 micron sized carriers via the M6P receptor, which is a clathrin mediated endocytic receptor and therefore was limited to carriers of <250 nm size. My work was published as a first author paper (see publications for details), and was presented as part of my honors thesis for which I received High Honors.

b. Clinical Research (Johns Hopkins University) – After completion of my undergraduate degree, I took part in two clinical research projects in the following year.

i. I worked with Dr. Peter Mogayzel (2012) on a statistical project encompassing the pediatric Cystic Fibrosis (CF) population at the CF clinic at Johns Hopkins Hospital. I acquired data from medical charts to determine the impact of types of infections, frequency and length of hospital stay, medications administered and other factors had on a positive or negative impact on patient pulmonary function testing. My work was meant to highlight the most significant factors for determining aggressive treatment vs. early discharge. The assembled data was then referred to a trained statistician for conclusions. The work provided me invaluable experience in patient care and medical treatment of Cystic Fibrosis.

ii. I also worked with Dr. Beth Laube at Johns Hopkins University from 2012 -2013. We worked on determining the effect of *Pseudomonas aeruginosa* infections on mucociliary clearance in Cystic Fibrosis patients. My contribution was to observe the consent process, physical exams and assist in administration of the tests. We used gamma radiation to follow Tc-99 clearance in patients with and without P. *aeruginosa* infections. At completion of my involvement the project was still ongoing, however early analysis suggested diminished mucociliary clearance with established *Pseudomonas aeruginosa* infections.

4. WORK HISTORY

INSTITUTION AND LOCATION	Position	START DATE MM/YYYY	END DATE *(or expected end date)* MM/YYYY
University of Maryland, College Park, MD.	Research Assistant	05/2012	05/2013
University of Maryland, College Park, MD.	Library Assistant	08/2009	04/2010

5. MEMBERSHIP IN SCIENTIFIC SOCIETIES/PROFESSIONAL ORGANIZATIONS:

1. **2018 – 2019 –** Immunology Journal Club co-sponsor
2. **2017 – 2018 –** American Association of Immunologists
3. **2017 – current –** American Medical Association
4. **2017 – current –** Immunology Journal Club
5. **2016 – current –** Microbiology and Immunology Student Organization
6. **2016-2017 -** National Student Research Forum – co-Director
7. **2014-2015 -** Pediatric Student Association
8. **2013 – current -** Muslim Medical Association
9. **2013- current -** Global Health Program
    a. **Hands and Feet Mission Trip –** Dec – Jan (2018 – 2019)
10. **2013- 2016 -** MD/PhD Mentor Program (Big Sib Little Sib)

6. COMMUNITY OUTREACH

1. **2018 –** Careers in Healthcare & Biomedical Science – Clear Lake High School
2. **2016 – current -** St. Vincent's Intern Program – *additional volunteering every month*
3. **2014 – current –** United to Serve

7. HONORS:

1. **2019** –Blocker Scholar Fellowship in Biomedical Research
2. **2018** – University Federal Credit Union Graduate School Scholarship
3. **2018** – American Association of Immunologists Travel Award – oral presentation
4. **2018** – Excellence in Infectious Disease and Immunology Research Award
    a. *National Student Research Forum – oral presentation*
5. **2018** – Blue Ribbon Award – Translational Science meeting – poster presentation
6. **2017** - Michael Tacheeni Scott Endowed Scholarship
7. **2017 – 2019** – McLaughlin Fellowship Award, UTMB
8. **2016-2018 -** Clinical & Translational Science NRSA TL1 Fellowship Award, UTMB
9. **2012** - Phi Lambda Upsilon, UMCP
10. **2012** - Office of Multi Ethnic's Students Excellence Award, UMCP

11. **2012** - Dr. P. Arne. Hanson Memorial Award, UMCP
12. **2011-2012** - Howard Hughes Medical Institute Research Fellowship, UMCP
13. **2010-2012** - Cellular Biology and Molecular Genetics Departmental Honors,
    a. Recipient of High Honors for Research, UMCP
14. **2009-2011** - John J. Leidy Scholarship, UMCP
15. **2008-2012** - University of Maryland Honors Program, UMCP

8. PUBLICATIONS:

a. ARTICLES IN PEER-REVIEWED JOURNALS:

i. **Ansar, M**.; Chambliss, J.M.; Kelley, J.P.; Ivanciuc, T.; Spratt, H.; Garofalo, R.P.; and Casola, A. (2019) A polymorphism in the catalase gene promoter confers protection against severe RSV bronchiolitis, *Free Radical Biology and Medicine.* (submitted)

ii. Komaravelli, N.; **Ansar M**.; Garofalo, R.P.; and Casola, A. (2017). Respiratory syncytial virus induces NRF2 degradation through a PML-RNF4 dependent pathway, *Free Radical Biology and Medicine*, 113, 494-590.

iii. Ivanciuc, T.; Sbrana, E.; **Ansar, M.**; Bazhanov, N.; Szabo C.; Casola, A.; and Garofalo, RP. (2016) Hydrogen sulfide is an antiviral and anti-inflammatory endogenous gasotransmitter in airways, *American Journal of Respiratory Cell and Molecular Biology*, **55,** 684-696.

iv. **Ansar, M**.; Serrano, D.; Bhowmick, T.; Papademetriou, I.; Muro, S. Biological functionalization of drug delivery carriers to bypass size restrictions of receptor-mediated endocytosis independently from receptor targeting, *ACS Nano*, 2013, 7 (12), pp 10597–10611. PMCID: PMC3901850

b. REVIEWS:

i. Bazhanov, N.; **Ansar, M**.; Ivanciuc, T.; Garofalo, R.P.; Casola, A. (2017). Hydrogen sulfide: a novel player in airway development, pathophysiology of respiratory diseases and antiviral defenses. *American Journal of Respiratory Cell and Molecular Biology*. PMID: 28481637

c. ABSTRACTS:

i. **Ansar, M.**; Ivanciuc, T.; Casola, A.; and Garofalo, R.P. (2019) Intranasal administration of catalase ameliorates respiratory syncytial virus induced airway disease via modulation of innate immune responses, American Thoracic Society Meeting, Dallas, TX.

ii. **Ansar, M**.; Morris, D.; Ivanciuc, T.; Casola, A.; and Garofalo, RP. (2019) TNF-α blockade reduces clinical severity of respiratory syncytial virus infection without altering viral growth, McLaughlin Colloquium on Infection and Immunity, Galveston, TX.

iii. **Ansar, M**.; Ivanciuc, T.; Casola, A.; and Garofalo, RP. (2018). Protective effect of antibody-mediated interferon blockade during respiratory syncytial virus infection, Immunotherapy for Infectious Diseases Conference, Galveston, TX.

iv. **Ansar, M**.; Komaravelli, N.; Ivanciuc, T.; Casola, A.; and Garofalo, RP. (2018) Detrimental Role of type I interferon signaling in respiratory syncytial virus infection, American Association of Immunologists Annual Meeting, Austin, TX.

v. **Ansar, M**.; Chambliss, JM.; Komaravelli, N.; Ivanciuc, T.; Casola, A.; and Garofalo, RP. (2018) Role of antioxidant enzyme catalase in respiratory syncytial virus infection, Translational Science, Washington D.C., MD.

vi. **Ansar, M**.; Ivanciuc, T.; Casola, A.; and Garofalo, RP. (2018) Antioxidant enzyme supplementation: a potential therapeutic approach for respiratory syncytial virus infection, 59th National Student Research Forum, University of Texas Medical Branch, Galveston, TX.

vii. **Ansar, M**. (2018). Catalase Administration as a potential treatment for respiratory syncytial virus infection, T1-T4 in 3 minutes competition – Institute for Translational Sciences, University of Texas Medical Branch, Galveston, TX.

viii. **Ansar, M**.; Komaravelli, N.; Casola, A.; and Garofalo, R.P. (2017) Interferon related regulation of innate antioxidant response in respiratory syncytial virus infection. American Thoracic Society colloquium, Washington D.C, MD.

ix. **Ansar, M**.; Komaravelli, N.; Casola, A.; and Garofalo, R.P. (2017). Interferon-mediated modulation of innate immune responses. Abstract for poster presentation. McLaughlin Colloquium on Infection and Immunity, University of Texas Medical Branch, TX

x. **Ansar, M.;** Ivanciuc, T.; Casola, A.; and Garofalo, RP. (2016). Interferons modulate innate inflammatory responses and exacerbate Respiratory Syncytial Virus infection. Abstract for poster presentation. Texas Regional Immunology Conference, MD Anderson, Houston, TX.

xi. **Ansar, M.**; Ivanciuc, T.; Casola, A.; and Garofalo, R.P. (2016). Effect of Antioxidant Enzyme Superoxide Dismutase 1 Upregulation on Respiratory Syncytial Virus Infection. Abstract for Poster Presentation. 4th Annual

Clinical and Translational Research Forum, University of Texas Medical Branch, Galveston, TX.

xii. **Ansar, M.**; Bhowmick, T.; Papademetriou, I.; Serrano, D. and Muro, S. (2013). Functionalization of Drug Delivery Carriers to Bypass Size Restrictions of Receptor-Mediated Endocytosis and Enhance Intracellular Delivery. Abstract for Poster Presentation, International Translational Nanomedicine Conference, Northeastern University, Boston, MA.

xiii. **Ansar, M.**; Serrano, D. and Muro, S. (2011). Sphingomyelinase Facilitates Endocytosis of Micrometer-Sized Materials, Such as Drug Carriers. Abstract for poster presentation, Bioscience Day, University of Maryland, College Park, MD.