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osteopontin plays a pivotal role in in increasing severity of respiratory syncytial virus infection

Viviana Sampayo-Escobar
University of South Florida, viviana@mail.usf.edu

Osteopontin Plays a Pivotal Role in Increasing Severity of Respiratory Syncytial Virus Infection

by

Viviana Sampayo-Escobar

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Department of Molecular Medicine
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University of South Florida

Major Professor: Shyam S Mohapatra, Ph.D.
Nanjundan Meera, Ph.D.
Srinivas Bharadwaj, Ph.D.
Kennet Ugen, Ph.D.
Andreas Seyfang, Ph.D.

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DEDICATION

First and foremost, I would like to dedicate this dissertation to God for giving me strength throughout this program. I also dedicate this work to my husband, Adrian Avila who has encouraged me all the way during this long process, and to my precious daughter, Antonella Avila-Sampayo for bringing relief and joy to my life.

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ABSTRACT

The molecular mechanisms underlying susceptibility to severe respiratory syncytial virus (RSV) infection remain poorly understood. Herein, we report on the role of osteopontin (OPN) in regulation of RSV infection in human epithelial cells and how interleukin-1 beta (IL-1β), a cytokine secreted soon after RSV infection, when persistently expressed can induce OPN expression leading to increased viral infection. We first compared OPN expression in two human epithelial cell lines: HEK-293 and HEp-2. In contrast to HEp-2, HEK-293 expresses low levels of pro-caspase-1 resulting in decreased IL-1β expression in response to RSV infection. We found a correlation between low IL-1β levels and a delay in induction of OPN expression in RSV-infected HEK-293 cells compared to HEp-2. This phenomenon could partially explain the high susceptibility of HEp-2 cells to RSV infection versus the moderate susceptibility of HEK-293 cells. Also, HEK-293 cells expressing low levels of pro-caspase-1 exhibit decreased IL-1β expression and delayed OPN expression in response to RSV infection. HEK-293 cells incubated with human rIL-1β showed a dose-dependent increase in OPN expression upon RSV infection. Also, incubation with rOPN increased RSV viral load. Moreover, HEp-2 cells or mice infected with a mucogenic RSV strain RSV-L19F showed elevated levels of OPN in contrast to mice infected with the laboratory RSV strain rA2. This correlated with elevated levels of OPN following infection with RSV-L19F compared to rA2. Together, these results demonstrate that increased OPN expression is regulated in part by IL-1β, and the

interplay between IL-1β and OPN signaling has a pivotal role in the spread of RSV infection.

INTRODUCTION

Objective

The objective of this dissertation is to explore the role of OPN in the pathogenesis and inflammatory response to RSV and to evaluate OPN as a regulator of severe RSV infection.

Motivation

The motivation for this work is to elucidate the role of OPN in RSV infection and its contribution to the outcome of the disease. The work presented herein will reveal the mechanism by which OPN contributes to RSV infection. Our approach allows us to evaluate the role of IL-1β in the regulation of OPN expression during RSV infection, as well as OPN's role during the inflammatory response thus influencing the onset of infection and its severity.

Scope

The scope of this project is to have a better understanding of the inflammatory response to RSV infection. Severe RSV infection is associated with increased viral replication and uncontrolled inflammation. This uncontrolled inflammation is a major contributor to the disease pathology presented by patients with severe RSV infection. In the future, these findings would help to develop new drug targets for treatments and/or

prophylaxis of RSV infection because they may lead to development of pharmacological strategies to regulate Il-1β and OPN expression during the infection, which could minimize the inflammatory response and control the replication of the virus.

Overview

Chapter 1 introduces background information on RSV infection, the susceptible population and available treatments, as well as the biology of the virus and molecular mechanisms developed by the virus to disrupt the immune response. Chapter 2 introduces a detailed description of all molecular biology techniques used and experimental designs. Chapters 3-5 will cover three areas: 1) how OPN expression is affected by RSV infection and disease; 2) the role of RSV-induced IL-1β expression during up-regulation of OPN; and 3) the correlation between OPN and severe RSV infection. Each chapter will include relevant background information and explanation of the results and discussion. Chapter 6 will summarize all the findings and contributions of this study. A correlation of all the results is included to assist readers in making quick approximations of the data presented and to demonstrate the usefulness of the model.

CHAPTER 1

RESPIRATORY SYNCYTIAL VIRUS - LITERATURE REVIEW

1.1 RSV Clinical Relevance

Respiratory syncytial virus (RSV) infection is one of the first pathogens encountered by the infant immune system. It is estimated that RSV infects most people by two years of age, and it may re-infect individuals throughout life since infection does not lead to persistent or long-lasting immunity [1-3]. Severe RSV infection is common in infants and individuals above 65 years of age because their immune system is either immature or gradually diminished due to age advancement; thus their capacity to efficiently respond to an infection is impaired [4, 5]. The global incidence of RSV infection is estimated to be 34 million cases per year in children younger than five years, and results in more than 90,000 hospitalizations a year in the United States [6-8]; the overall incidence of RSV disease in elderly adults in the U.S. is ~3-4% with an estimated 10,000 deaths per year [5, 9, 10]. Most primary infections are identified by cold-like symptoms, coughing, fever, shortness of breath, runny nose and lethargy; however, some RSV infections will lead to severe obstruction of airways, alveolar epithelium damage, and edema resulting in pneumonia and/or bronchiolitis that may require hospitalization. Death may ensue if complications persist [10-12]. Despite the significant impact RSV has on current health care systems and decades of intense research in the field, no vaccine or effective treatment is available and the infection is typically managed solely by supportive

care. Currently, Palivizumab is the only prophylactic drug approved by the Food and Drug Administration (FDA) for use in the United States. It is based on neutralizing antibodies targeting the fusion glycoprotein of RSV; however, Palivizumab is administered by multiple intramuscular (IM) injections before and during the RSV season which makes it an expensive prophylactic treatment only approved for high-risk infants [13-15].

Remarkably, viral infections trigger 80-85% of asthma exacerbations in children, and around 45% in adults [16-18]. Moreover, viral infections stimulate immune cells to produce inflammatory cytokines, which increase mucus production in the airways resulting in airway obstruction. Viral stimulation of pro-inflammatory cytokines also causes airway hyperresponsiveness that has been associated with irreversible pulmonary damage in children with cystic fibrosis [19-22]. RSV infection is associated with wheezing and asthma exacerbations [16, 20, 23, 24]. The nature of this association is not fully understood, but the fact that RSV infection induces a Th2-like cytokine profile may explain the risk for the development of asthma after the infection [20, 25-27].

1.2 Biology and Structure

RSV is a negative-sense, non-segmented, single-stranded RNA virus with a 15.2 kb genome that belongs to *Paramyxoviridae* family, order *Mononegavirales* [28, 29]. The negative-sense genome contains 10 viral genes that encode 11 viral proteins. Each gene is transcribed into a messenger RNA (mRNA) and codes for a single viral protein, except the M2 mRNA which is known to have two overlapping open reading frames that lead to the expression of two different proteins M2-1 and M2-2 [29, 30]. Briefly, once RSV enters

the host airway epithelial cells via direct fusion of the viral envelope on lipid raft domains within the plasma membrane [31]. The RSV-F and RSV-G proteins mediate the fusion and attachment process by interacting with different cellular receptors such as CX3 chemokine receptor 1 (CX3CR1), toll like receptor-4 (TLR-4), annexin II, nucleolin, CD44 receptor, among others [32-34]. Although the exact function of the previously mentioned receptors remains inconclusive. It is known that the activation of cell signaling pathways contribute to successful entry of the virus and subsequent production of viral filaments [33]. Following the fusion event comes RSV internalization process that leads to release of the genomic material and the RSV-L RNA dependent RNA polymerase (RdRp) to the cytoplasm where transcription and replication take place; hence the polymerase generates polyadenylated and capped mRNAs that are translated to non-structural and structural proteins [35, 36]. Finally, the anti-genome is the template used by the RdRp to produce adequate genomic negative-sense RNA which is encapsidated with the viral nucleoprotein and assembled with all the other viral proteins before the virus is released from the infected cell. The budding process allows progeny viral particles to retain the host cell plasma membrane as their envelope (Fig. 1).

RSV genes are transcribed in the following order. First are the non-structural proteins 1 and 2 (NS1/NS2) which, although not found in the virion, are accessory proteins present in the infected cells that have been involved in disruption of the antiviral immune response by inhibiting the type I interferon (IFN) signaling pathway at different points, thus facilitating viral replication [37-39]. Those proteins are followed by the nucleocapsid protein (N) which is critical for viral transcriptional activity and encapsidates both the genomic RNA and the antigenome. The phosphoprotein (P) is an important co-factor in

RNA synthesis that gets phosphorylated in order to create a functional polymerase complex [40, 41]. Likewise, the matrix protein (M) is essential during viral assembly, release, and production of infectious viral particles [42]. The next three glycoproteins are heavily glycosylated proteins found anchored to the surface of the viral envelope: a small hydrophobic protein (SH) whose function is not fully elucidated but is suggested to play an immumodulatory role [43]; the attachment glycoprotein (G) which, while not required for *in vitro* infection, seems important for optimal *in vivo* viral growth and facilitates viral adsorption to the host cells allowing the attachment of the virus to the cell [44]; and the fusion protein (F) which mediates the penetration of the virus to the cell by allowing the fusion of the viral envelope and the host cell membrane. F protein also allows the passage of RSV from cell to cell and the fusion of neighboring infected cell plasma membranes producing giant multinucleated cells known as syncytia; the formation of which is characteristic of RSV infection [45, 46]. M2-1 is a transcription elongation factor that allows transcription through the intergenic regions and increases the expression of genes found at the distal region; M2-2 regulates viral transcription by changing the polymerase complex (N, P and L proteins) from a transcriptional to a replicative mode which is critical for a proper propagation of the virus [30, 47, 48]. Lastly, the large protein (L) is the major subunit of the RNA-dependent RNA polymerase [49].

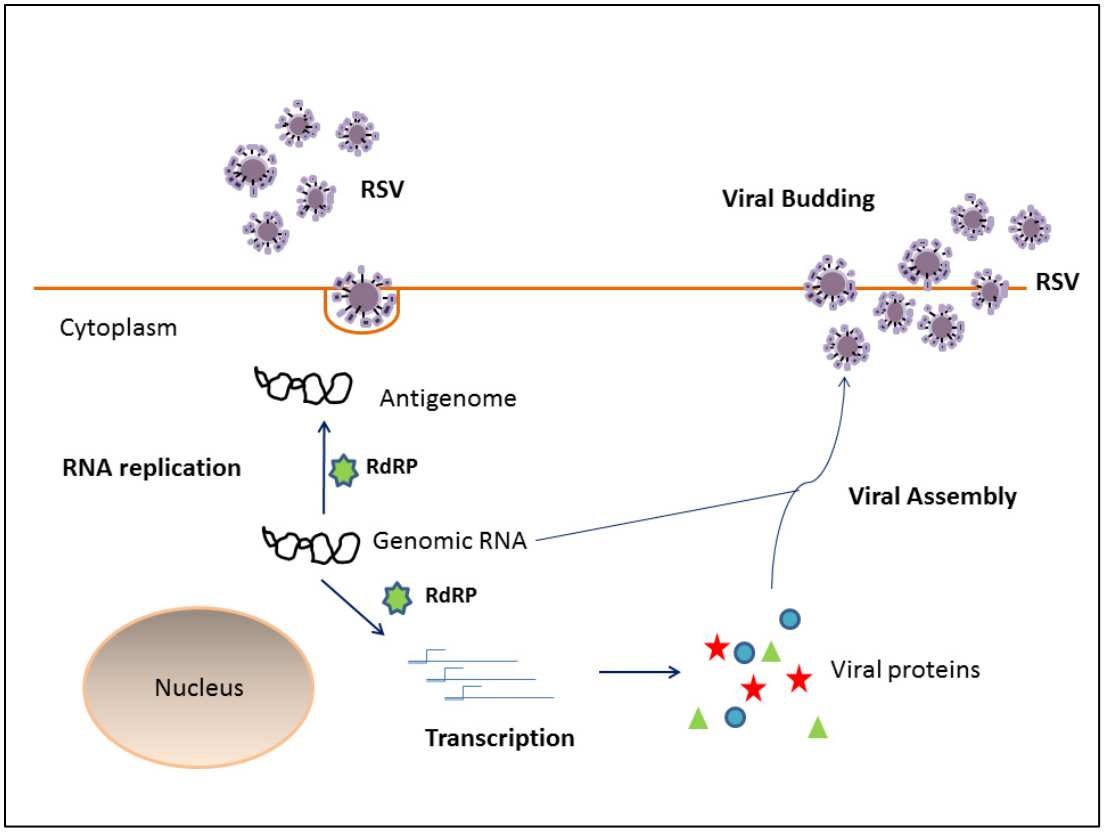

Figure 1. RSV replication cycle. RSV-F and RSV-G glycoproteins mediate fusion and attachment of the virus to the airway epithelial cell. The virus enters the cell and releases its encapsidated genomic material and the RSV RNA-dependent RNA polymerase (RdRp) into the cytoplasm where RNA replication takes place and the RdRp enzyme uses the genome as a template to generate mRNA that codify the 11 viral proteins. Lastly, the genomic RNA is encapsidated and assembled with the other viral proteins to produce progeny viral particles that bud from the host cells while taking the plasma membrane as their envelope.

1.3 Antigenic Subgroups

Although there exists only one serotype of RSV, it consists of two major antigenic subgroups (A and B). These subgroups have been identified through gene sequencing and cross-neutralization reactions with monoclonal antibodies [50]. Although disparities have been reported in all the structural proteins, the major differences between subgroups A and B are found in the G and F surface proteins, with respectively 53% and 90% amino acid sequence identity [50-52]. The two strains alternate during yearly epidemics thus leading to their re-circulation, which may account for frequent re-infections during the same or next RSV season [53]. Additionally, some studies have suggested that RSV A is more prevalent and associated with greater severity of disease compared to RSV B [49, 51, 54]. On the contrary, others found that strain differences do not play a significant role in the onset and severity of RSV disease [49, 55].

1.4 Immune Response to RSV

Host innate and cellular immunity mediates the clinical outcome and resolution of microbial infections, but it also controls the propensity for severe disease and re-infection [56]. Airway epithelial cells, immune cells and tissues express pattern-recognition receptors (PRRs) capable of recognizing pathogen-associated molecular patterns (PAMPS), thus activating the innate immune response, causing the release of pro-inflammatory cytokines leading to pathogen clearance, but also mediating disease pathology [57]. During viral infections the cascade of signaling events is initiated mainly by retinoic acid-inducible gene (RIG)-like receptors (RLRs), which include RIG-I, MDA5

and LGP2; toll-like receptors (TLRs); and (NOD)-like receptors (NLRs) that recognize viral genomic material such as single-stranded RNA (ssRNA), double stranded RNA (dsRNA) and DNA [57, 58]. Upon RSV infection, ssRNA and dsRNA are detected in the endosomal compartment by TLR-3 and TLR-7, and in the cytosol by MDA5, RIG-I and NLR receptors [59]. The stimulation of these signaling pathways leads to activation of transcription factors IRF-3, IRF-7 and NF-kB that result in production of type I interferon (IFNα,β) and pro-inflammatory cytokines [58].

The IFN response pathway is considered the first line of defense against viral infection; hence a proper production of type I IFN (IFNα and IFNβ) is essential during the innate immune response to control the infection [60]. IFNα/β are secreted and bind to cell surface receptors (IFNAR) that induce the transcription of interferon-stimulated genes (ISG) to elicit antiviral, antiproliferative and immunomodulatory activities [61]. Interestingly, RSV has the capability to subvert the host immune response, particularly the IFN signaling pathway. Expression of the nonstructural RSV proteins, NS1 and NS2, has been associated with poor induction of the type I IFN response. Infections with RSV lacking either the NS1 or NS2 gene have shown enhanced IFN induction and reduced replication efficiency [38, 62]. NS1 and NS2 cause proteosomal degradation of host STAT2, and likewise a decrease in NF-κB activation [63, 64]. Particularly, NS1 protein binds to the mitochondrial antiviral signal protein (MAVS) and NS2 interacts with RIG-I but not MAVS [38, 63]. Therefore, evidence demonstrates that the RSV NS1 and NS2 proteins together antagonize IFN induction [38].

In addition to type I IFN production, epithelial cells respond to RSV infection by expressing inflammatory cytokines resulting in tissue remodeling, increased mucus

production, and airway obstruction that worsens the symptoms and results in exacerbation of allergy and asthma. Other cytokines produced during RSV infection include interleukin-6 (IL-6), IL-8, IL-1β, IL-10, IL-13, TNFα, RANTES (CXCL5), IL-17, macrophage inflammatory protein 1α (MIP-1α), GM-CSF, IL-1α, IL-25, and IL-33 [62, 65]. In an effort to elucidate the genes that are associated with RSV pathology and age-related weakening of the immune system our lab previously analyzed 84 different antiviral genes and established the association between those genes, age and infection. Five genes out of 84 were associated with both advanced age and RSV infection, RIG-I, IFNAR1, TLR-8, IL-1β, and Osteopontin (OPN) [4]. Our previous results showed that aged mice express pro-inflammatory cytokines even prior to infection which may explain the susceptibility of these animals to a severe form of disease caused by RSV infection and their difficulty to clear the infection efficiently compared to the young group [4].

As with other viral infections, RSV infection triggers recruitment and activation of immune cells: natural killer cells (NK), macrophages, monocytes, granulocytes, T lymphocytes and dendritic cells (DC) [66]. Although these cells can induce a direct effector immune response that controls the local infection, they also serve as a bridge to initiate a proper acquired immune response [67]. In mouse models of RSV infection, neutralizing antibodies control the spread of the infection from upper to lower respiratory tract [68]. In humans, RSV induces Immunoglobulin A (IgA) secretion in the upper respiratory tract which, although a short-lived immune response, controls the infection and its progression to the lower respiratory tract [69]. Although passive immunity is transitory during RSV infection, neutralizing antibodies against RSV have been reported in serum from full term newborns; thus primary infections of infants usually does not lead

to a severe form of infection since maternal antibodies protect them of a lower tract respiratory infection [70-72]. Later in life neutralizing antibodies are developed during natural infections, with higher titers in adults compared to infants infected, which could be explained by the latter group's immature immune system [73].

Unfortunately, RSV induces a short immune response that does not protect from future infections. Additionally, the uncontrolled inflammation increases the severity of the disease. Further studies are required to establish the role of the inflammatory response and its association with disease progression so that effective treatments can be developed.

1.5 Current Treatments

RSV was first discovered in 1956 in a laboratory chimpanzee with respiratory tract infection. It was soon established as a leading viral pathogen able to cause serious respiratory infection in the infant population. Despite years of intense research and several attempts to identify new and effective treatments to minimize clinical hospitalizations and complications due to RSV infection, there is no vaccine available for RSV infection and the standard treatment is basic supportive care with limited pharmacologic treatment in extreme cases [74].

Ribavirin is the only antiviral drug approved by the U.S. Food and Drug Administration (FDA) for the treatment of RSV. It acts by inhibiting the transcription and replication of a variety of viruses, thus it is not exclusively used to treat RSV infection [75, 76]. In fact, ribavirin is not as effective for RSV as it is for other viruses and it is only used

as a treatment of severely ill children and immunocompromised patients infected with RSV in order to reduce the disease progression from upper to lower respiratory tract infection thus reducing mortality [77, 78].

Another well-known approach is the administration of a prophylactic treatment consisting of a humanized monoclonal antibody against the F protein of RSV (Palivizumab) made of 95% human and 5% mouse antibody sequence, thus helping to reduce pulmonary viral replication [79, 80]. Palivizumab is currently administered to children at high risk of RSV disease, for instance, premature infants, infants born with congenital heart disease, neuromuscular disorders, and immunocompromised children among others. It is administered monthly during the RSV season by intramuscular route in a dose of 15 mg per kg; a maximum of 5 consecutive doses has been recommended by the American academy of pediatrics to ensure immune protection; moreover a 55% reduction in hospitalization by RSV has been reported and attributed to treatment with palivizumab [80, 81]. Despite the benefits of a prophylactic treatment in infants at risk of severe RSV disease, the use of palivizumab is limited due to the high cost of the treatment [80].

RSV vaccine development has been challenging and remains a priority since RSV infection does not lead to sustained or long lasting immunity. The first unsuccessful attempt to develop an RSV vaccine consisted of a formalin-inactivated vaccine; recipients had an increased rate of infection and hospitalizations due to the infection compared to the control group; additionally, the death of two participants of the clinical trial was attributed to the vaccine [82]. Further studies proposed that the formalin-inactivated virus was not able to stimulate PRRs; consequently, it did not trigger an appropriate innate

immune response but the activation of other arms of the immune system that led to strong priming of T cells towards a Th2 lineage, resulting in the increased expression of Il-4 and Il-5 both associated to enhanced inflammatory infiltrate and pulmonary eosinophilia [83, 84]. Although inducing a robust immune response is the aim of vaccination, formalin-inactivated RSV vaccine is a good example of how vaccines can be harmful when uncontrolled stimulation of the immune response is induced [85].

1.6 Concluding Remarks

Despite more than six decades of research aiming to develop effective treatments and vaccine candidates for RSV infection, the treatments available remain supportive and there is still a lack of cost-effective prophylactic treatment or effective vaccine candidates. Thus, severe RSV infections remain a constant risk for infant, elderly and individuals who are immunocompromised. A proper understanding of the immune response to the infection is essential since it is known that severe RSV infection is not only driven by an increase in viral replication but also by an imbalanced immune response that leads to exaggerated inflammation [86].

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CHAPTER 2

GENERAL METHODS

This section presents the general molecular biology techniques used during the course of the study. Two different approaches will be presented, *in vivo* and *in vitro* models of RSV infection; both are broadly used in the field to study RSV infection and its effect on the immune response and the pathology of disease.

2.1 Mice

Osteopontin-deficient knockout mice (OPN KO) (strain B6.Cg-Spp1tm1Blh/J) and wild type (WT) C57BL/6 were purchased from Jackson Laboratory. All animal work was approved by and performed in accordance with the policies of the University of South Florida Institutional Animal Use and Care Committee. Mice were acclimated for 7 days prior to the start of experiments. Mice were provided with standard rodent chow and water ad libitum.

2.2 Cell Culture

Human epithelial type 2, HEp-2, (CCL 23; American Type Culture Collection, Rockville, MD) and human embryonic kidney 293 cells, HEK-293, (ATCC CRL-1573)

were maintained in Dulbecco´s modified Eagle´s medium (DMEM, HyClone) supplemented with 5% fetal bovine serum (FBS, HyClone) and 1% penicillin-streptomycin (GIBCO). Cells were incubated at 37°C in a humidified incubator with 5% CO2/95%.

2.3 Virus Purification, Infection and Plaque Assay

Three different sources of RSV were used: rgRSV-A2 which expresses green fluorescent protein, the mucogenic rA2-L19F (RSV-L19F) recombinant variant of the A2 strain with the fusion protein replaced with that of the line 19 RSV, and a version of the line 19 that expresses a red fluorescent protein (RSV-KL19F). The virus was propagated by infecting 60% confluent HEp-2 cells with a multiplicity of infection (MOI) of 0.1 plaque forming units (pfu) per cell for two hours at 37°C with gentle rocking every 15 minutes, after which the medium was replaced by fresh DMEM containing 5% FBS. Cells and media were collected when 70-80% of the cells showed cytopathological effects. RSV was pelleted through a layer of 30% glycerol (0.22 µm-filtered) in 0.1 M MgSO4 and 50 mM HEPES, pH 7.5. The viral particles were pelleted by centrifuging at 11,600 rpm in an SW28 rotor for 3 h at 4°C. Supernatants were carefully aspirated without disturbing the viral pellets and the viral pellets were resuspended in pre-cooled, 0.22 µm-filtered 50 mM HEPES, pH 7.5, 0.1 M MgSO4, 150 mM NaCl. The resuspended pellet was aliquotted and stored at −80°C until use. UV-inactivation of RSV was performed by irradiating aliquots of virus with 1200 mJ of UV for 20 mins using a Stratalinker.

For all the experiments, a monolayer of HEp-2 or HEK-293 cells at 80% confluence was infected with 0.1, 1 or 10 MOI (as indicated in the figure legends). Cells were

incubated with the viral inoculum in Opti-MEM containing 2% FBS (Life Technologies) for 2 hours at 37°C. After this, the infectious medium was replaced by fresh DMEM with 5% FBS. At 24, 48 or 72 hours post infection (hpi) the cell supernatants and pellets were collected for viral plaque assay, RNA or protein analysis.

WT or OPN KO mice were intranasally infected with 1 x 10^6 plaque forming units (pfu)/mouse of RSV-L19F in a BSL2, HEPA-filtered, ventilated hood. Mice were euthanized 1, 3 or 5 days post-infection (dpi). Lungs were collected for RNA or protein extraction.

To determine the viral titers, HEp-2 cells were seeded in 24 well plates to 80% confluence and infected in duplicates with serial dilutions of cell supernatants during 2 hours at 37°C. Infectious media was removed and cells were overlaid with 1 ml of 0.8% methylcellulose in DMEM supplemented with 5% FBS. 5 dpi, the cell monolayers were fixed overnight with 1 ml of 80% cold methanol. Next day, cells were rinsed with phosphate-buffered saline (PBS, Hyclone) and incubated with primary monoclonal antibody against RSV fusion protein (AbD Serotec). Plaques were visualized using an anti-mouse IgG horseradish peroxidase antibody (HRP)(Sigma) and developed with 4 CN peroxidase substrate (KPL); the dark purple spots were counted and each spot represented one plaque-forming unit (PFU).

2.4 Quantitative RT-PCR

Total RNA was isolated from cells using TRIzol (Life Technologies). Samples were treated with recombinant DNase I (Life Technologies) to remove any contaminating DNA.

1 µg of RNA was reverse transcribed using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific) as described in manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed on the BioRad CFX384™ Real-time PCR Detection system using DyNamo Color Flash SYBR master mix (Thermo Fisher Scientific). The sequences of all primers used in this study are as follows (forward and reverse): RSV-N, 5'- CAT CTA GCA AAT ACA CCA TCC A-3′ and 5′-TTC TGC ACA TCA TAA TTA GGA GTA TCA A-3′; human IL-1β (PrimeTime® -qPCR primers-IDT), 5'- GAA CAA GTC ATC CTC ATT GCC-3' and 5'-CAG CCA ATC TTC ATT GCT CAA G-3'; human OPN, 5'-TGG CCG AGG TGA TAG TGT G-3' and 5'- CGG GGA TGG CCT TGT ATG-3'; human IFN-β, 5'-CAA CTT GCT TGG ATT CCT ACA AAG-3' and 5'- TGC CAC AGG AGC TTC TGA CA-3' . All samples were run in four replicates and the data were analyzed using normalized gene expression (ΔΔCt). Expression of all genes was normalized to control hypoxanthine-guanine phosphoribosyltransferase (HPRT): mouse HPRT, 5′-GCT GAC CTG CTG GAT TAC ATT AA-3′ and 5′-TGA TCA TTA CAG TAG CTC TTC AGT CTG A-3′; human HPRT, 5'- AGG AAA GCA AAG TCT GCA TTG TT-3' and 5'- GGC TTT GTA TTT TGC TTT TCC A-3'.

2.5 Western Immunoassay

Cells were plated in 6-well culture plates the day before treatment/infection and harvested in lysis buffer containing 1% NP-40, 150 mM NaCl, 50 mM Tris-HCl pH 8.0 and a protease inhibitor cocktail (Thermo Fisher Scientific). After removal of cellular debris by centrifugation, total protein concentration was measured at 660nm using protein assay

reagent (Thermo Fisher Scientific) and 25 µg of total protein were separated in a precast 12% mini-PROTEAN TGX gel (Bio-Rad) and transferred to a nitrocellulose membrane (Bio-Rad). The membrane was incubated with a rabbit polyclonal antibody to human OPN (Abcam, ab181440) and a mouse monoclonal antibody to β-actin (Sigma). Proteins were detected by incubating with a secondary anti-mouse IgG-HRP and/or anti-rabbit IgG-HRP, followed by the ECL reagent kit (Pierce). Images were captured using a ChemiDoc XRS+ imaging system (Bio-Rad).

2.6 Immunofluorescence Microscopy

HEp-2 cells were plated in 8-well chamber slides the day before infection. Cells were mock infected or infected with 1 MOI of RSV strain rA2-L19F. Cells were fixed with cold 4% paraformaldehyde 24 or 48 hpi and stained with a goat polyclonal antibody against RSV (Millipore, ab1128) and a rabbit polyclonal antibody to OPN (ABCAM), followed by indirect immunofluorescence using secondary anti-goat IgG Alexa Fluor-555- and anti-rabbit IgG Alexa Fluor-488-conjugated antibodies. Slides were mounted with 4′, 6-diamidino-2-phenylindole (DAPI) containing anti-fade mounting media (Southern Biotech). A minimum of 10 images at 200X magnification were collected per slide with a DP72 digital camera on a BX51 Olympus fluorescence microscope on the appropriate fluorescence emission filter channels.

2.7 Enzyme-Linked Immunosorbent Assay (ELISA) in Lung Homogenates

Snap frozen lungs were homogenized in cold lysis buffer containing 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 10% Glycerol, 5 mM EDTA and a protease inhibitor cocktail. Tissue debris was pelleted by centrifugation at 4°C for 10 min at 300×g and protein concentration in the supernatants was measured at 660 nm with protein assay reagent (Thermo Fisher Scientific). Mouse osteopontin ELISA kit (RayBiotech) and mouse IL1-β ELISA kit (BioLegend) were used per manufacturer's instructions.

2.8 Flow Cytometry Assay for CD44 and RSV Infected Cells

HEp2 or HEK-293 cells were seeded on 6-well plates at an appropriate cell density to reach 70 to 80% confluence. On the day of the experiment, each set of cell cultures was infected with RSV-KL19F for 2 hours. 24 hpi, cells were washed with PBS, detached with accutase (Life Technologies) and stained with DAPI to determine cell viability, FITC mouse anti-human CD44 antibody (BD Bioscience) or isotype control antibody. The percentage of cells expressing GFP (CD44 +) or RFP (RSV +) in each cell culture was determined by flow cytometry (BD FACSCanto II). Data was analyzed using BD FACS diva software.

2.9 Recombinant IL-1β and OPN Treatment

HEK-293 cells were infected with 1 MOI of rA2-L19F as described previously. Afterwards, the infectious media was replaced by fresh DMEM 5% FBS containing

different concentrations (0 - 1 and 10 ng/ml) of human rIL-1β (PeproTech). Similarly, HEK-293 were treated four hours before infection with different concentrations (0 - 1 – 10 - 50 - 100 and 200 ng/ml) of human rOPN (PeproTech) diluted in fresh DMEM 5% FBS. Cells were infected with rA2-L19F at 1 MOI, and two hours after infection the media were replaced by fresh DMEM 5% FBS containing rOPN. Protein and supernatants were collected for viral titering and western immunoassay.

2.10 Caspase I Inhibitor (Ac-YVAD-CHO) Treatment

HEp2 cells were treated with 10 μM of caspase I inhibitor (Sigma-Aldrich) or DMSO (vehicle control) two hours preceding the infection. Afterwards, cells were infected with 1 MOI of RSV-L19F, and two hours later the infectious media was replaced by fresh media containing 10 μM caspase I inhibitor or DMSO. Protein and RNA were isolated 24 hpi.

2.11 CD44 Receptor-Neutralization and RSV Infection

HEp-2 cells were seeded at 24-well plate the day before infection. On the infection day, cells were pre-treated at room temperature during 20 minutes with 10 μg broad spectrum rat-anti human CD44 antibody (clone A020) or normal rat IgG (control) (Millipore). After the pre-treatment, cells were infected with RSV-KL19F for two hours at 37°C and infectious media was replaced with fresh growth media. After 24 hours, the

percentage of RSV positive cells was determined by flow cytometry (BD FACS Canto II). Data was analyzed using BD FACS diva software.

2.12 Statistical Analysis

All the experiments were performed in triplicate and repeated at least twice. Statistical significance for each experiment was determined using Analysis of variance (ANOVA), $p<0.05$. Calculations were performed and graphs produced using Prism 6.0 software (Graphpad Software, San Diego, CA, USA). Graphs of results show the mean and error bars depict the standard error of the mean, +/- SEM.

CHAPTER 3

OPN EXPRESSION DURING RSV INFECTION IS REGULATED BY IL-1β

3.1 Introduction

As described in chapter 1, Respiratory Syncytial Virus (RSV) induces the expression of cytokines and chemokines in the cells that it infects. This intensifies the inflammatory process and contributes to progression of the RSV infection from a mild upper respiratory infection to a severe lower respiratory infection that could lead to death when uncontrolled [1]. Understanding the diversity of cytokine function during infection can be a complicated process not only because different cell types secrete cytokines at different times during the infection, but also because the function of cytokines can vary depending on whether they act in an autocrine, paracrine or endocrine manner, thus contributing to their functional pleitrophy [2, 3].

At present the mechanisms that contribute to severe RSV infection and viral pathogenesis remain unclear. Our lab has aimed to elucidate factors associated with RSV disease progression. Among those factors, Osteopontin (OPN) and Interleukin-1 beta (IL-1β) were associated with progression of RSV disease [4]. This chapter aims to elucidate the role of IL-1β during RSV infection and how it plays a major role in amplifying the inflammatory response by regulating OPN expression and the progression of the infection.

3.1.1 Inflammasome Activation

The innate immune response involves recognition of Pathogen Associated Molecular Patterns (PAMPs) and Damage Associated Molecular Patterns (DAMPs) by numerous pattern recognition receptors (PRR). The activation of these receptors leads to inflammation that aims to fight the infection or control harmful effects of chemicals on the cell [5]. The inflammasome pathway is a component of the innate immune response that gets activated upon pathogen recognition and signals of danger to the cells; the final products of inflammasome activation are Interleukin-1 beta (IL-1β) and Interleukin-18 (IL-18), which are known to amplify the inflammatory response and also modulate cell death [5, 6]. Five different receptors have been associated with inflammasome activation: nucleotide-binding oligomerization domain (NOD), leucine-rich repeat (LRR)-containing protein, NLR family members (NLRP1, NLRP3 and NLRC4), the proteins absent in melanoma 2 (AIM2) and pyrin [7]. Out of those, NLRP3, also known as NALP3, is the most well defined inflammasome pathway [8].

Generation of IL-1β requires assembly of the multi-protein inflammasome complex. There are two signals required for effective inflammasome activation. i) The priming signal ensures enough expression of the IL-1β precursor (pro-IL-1β) and an inflammasome receptor, such as NLRP3. The first signal is controlled by recognition of the pathogenic insult through PRR, which leads to activation of NF-κB and subsequent expression of pro-IL-1β and genes related to the inflammasome complex [9]. The canonical inflammasome complex NLRP3 is composed of the NLRP3 sensor, the adaptor protein (ASC) and pro-caspase 1 [9, 10]. ii) The second signal involves activation of NLRP3 complex that leads to the processing of pro-caspase 1 to its active form, caspase-

1. This step is critical since caspase-1 is required for the proteolytic cleavage of the inactive cytoplasmic precursors pro-IL-1β [11]. The proposed mechanisms of caspase-1 activation include pore formation and potassium efflux, lysosomal rupture that leads to release of cathepsin B, and the generation of mitochondrial reactive oxygen species (ROS) [10, 12, 13]. Briefly, after a pathogen is recognized by PRR and the levels of pro-IL-1β and NLRP3 are elevated, the monomers of NLRP3 undergo oligorimerization and the pyrin domain (PYD) of ASC protein allows their interaction [14]. Subsequently, the complex NLRP3-ASC allows the recruitment of pro-caspase-1 to the complex through a Caspase Recruitment Domain (CARD); hence pro-caspase-1 gets cleaved and generates an enzymatically active heterodimer of 20 kDa (p20) and 10 kDa (p10) subunits [14, 15]. The activation of caspase-1 leads to further processing of pro-IL-1β and pro-IL-18 that results in maturation and secretion of IL-1β and IL-18 [15-18].

The genetic material (RNA or DNA) of several viral infections is recognized by PRR within the cells, which in turn activates the inflammasome pathway and contributes to the antiviral response [19]. NLRP3 activation is seen in influenza virus, Encephalomyocarditis Virus (EMCV), hepatitis C virus, Respiratory Syncytial Virus (RSV), Sendai Virus, and Dengue Virus infections, among others [19-23]. Among these, influenza virus infection is known to induce a robust IL-1β expression via activation and recruitment of NLRP3, ASC and caspase-1 [24, 25]. The activation of the first signal during influenza infection is granted by the recognition of the viral RNA by TLR-7; following TLR-7 activation the influenza M2 protein induces proton flux which activates the second signal that leads to inflammasome complex assembly and activation [24, 26, 27]. Similarly, RSV infection induces IL-1β expression via activation of TLR-2 and TLR-4 that leads to pro-IL-1β and

NLRP3 expression (first signal), and a potassium and ROS efflux that triggers inflammasome assembly (second signal) [28, 29].

3.1.2 Inflammasome Regulation

Inflammasome activation is essential to clear pathogens and damaged cells. After it has carried out its function inflammasome activity is typically downregulated since exaggerated inflammasome activation is associated with multiple diseases where uncontrolled inflammation disrupts the host balance and causes harm to the cells and tissues [30].

IL-1β is a strong pro-inflammatory cytokine that exerts its biological function by binding to its receptor, IL-1 receptor type I (IL-1R1). This binding results in the activation of intracellular signaling pathways which result in initiation of a cascade of inflammatory mediators [31, 32]. In contrast, a naturally occurring antagonist protein, IL-1 receptor antagonist (IL-1Ra), is essential to maintain equilibrium during the inflammatory response. It binds to IL-1R1 with higher affinity than IL-1β and inhibits the recruitment of accessory proteins required for activation of pro inflammatory pathways [33]. Of note, the interaction of IL-1β and IL-1R1 has been associated to the pathophysiology of some forms of pulmonary inflammation and airway remodeling (Fig. 2) [33, 34].

As mentioned, regulation of inflammasome activation is fundamental for the maintenance of homeostatic biological functions; after inflammasome activation has exerted its function and the harmful stimulus is no longer an issue for the cell, the expression of Interferon (IFN) may down-regulate NLRP3 activity. For instance, IFN-γ induces the expression of inducible nitric oxide synthase (iNOS) which produces nitric

oxide (NO) that hinders NLRP3 activity, whereas type I-IFNs disrupt the expression of pro-IL-1β and pro-IL-18 and inhibit their cleavage via generation of NO and enhanced secretion of IL-10 that decrease the expression of the pro-cytokine forms [35-38]. In addition, the activation of NLRP3 is also controlled by miR-223 that silences NLRP3 mRNA expression and leads to decreased protein levels, thus decreasing the activation of the pathway [39, 40].

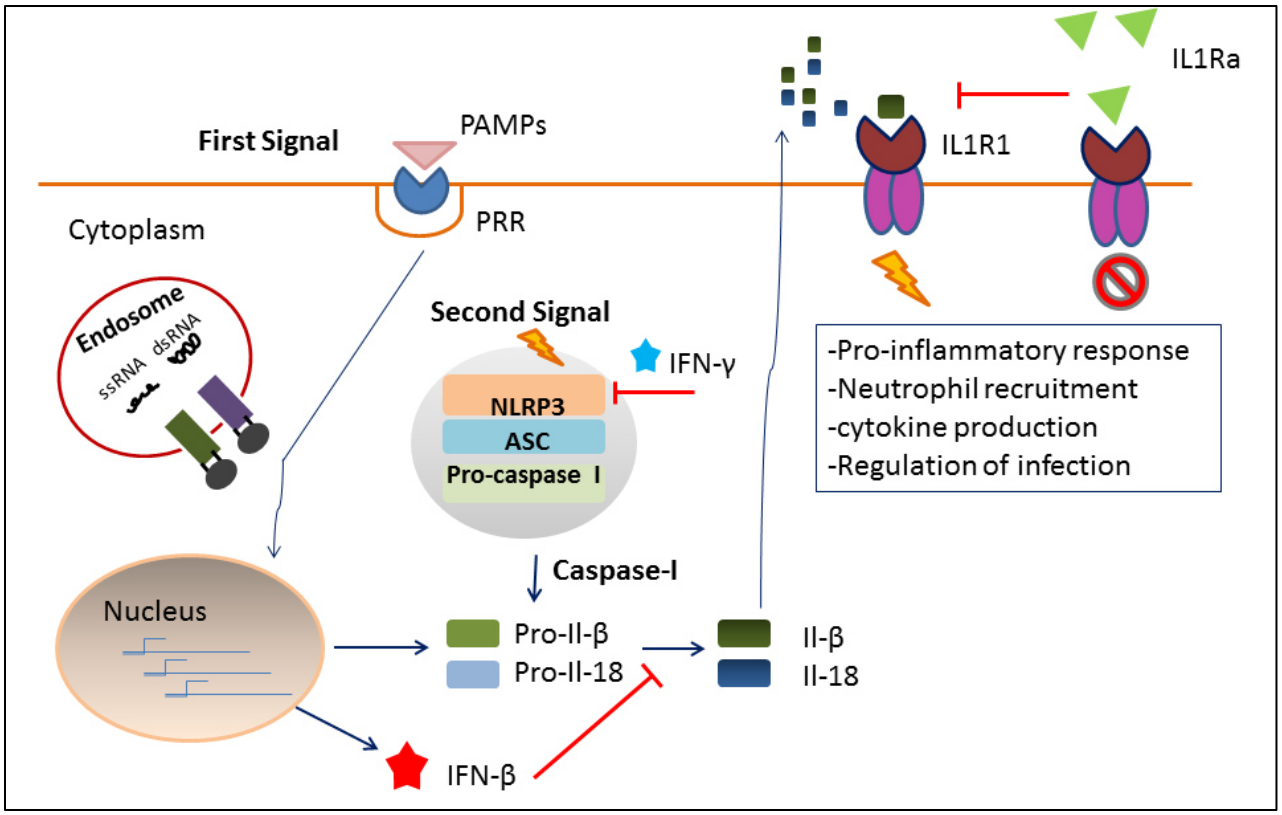

Figure 2. Activation and regulation of the Inflammasome Pathway. The recognition of Pathogen Associated Molecular Patterns (PAMPs) by several Pathogen Recognition Receptors (PRR) results in activation of the inflammasome pathway. Two signals are required: a priming signal that results in the stimulation of PRR which leads to expression of inflammasome components such as NLRP3, pro-Il-1β and pro-IL-18 (first signal); and the activation signal that involves oligomerization of NLRP3 and recruitment of the adaptor protein ASC and pro-caspase 1, thus leading to caspase-1 activation and the processing and secretion of the inflammatory cytokines, IL-1β and IL-18, which allows the interaction of the soluble cytokine with its receptor IL-1R1 so it can exert its function (second signal). Regulatory mechanisms aim to maintain the balance of the inflammatory response; hence secretion of type I IFN and IFN-γ results in down-regulation of NLRP3 activity that leads to inhibition of the inflammasome pathway. In addition, IL-1Ra expression has higher affinity to the receptor and its binding to IL-1R1 blocks the biological function of IL-1β.

3.1.3 IL-1β Expression during Infections

Although the exact role of IL-1β during viral infection is not fully elucidated, recent studies reported a dual function of IL-1β during influenza infection where infection of IL-1R1 knockout mice led to diminished inflammation but also decreased survival. The previous findings were explained by impairment in the recruitment of neutrophils and failure to recruit and activate CD4+ and CD8+ T cells [31, 41, 42]. Additionally, another study showed that lung fibroblast cells infected with influenza virus exhibited a reduction of pro-inflammatory cytokines such as IL-6, IL-8 and TNF-α when they were pre-treated with IL-1Ra [43]. In agreement, IL-1Ra treatment used in patients with active chronic bacterial infections such as *Sreptococcus pneumoniae* and *Streptococcus aureus* reduces inflammation without exacerbating the infection [32]. Consequently, a controlled expression of IL-1β seems to be essential for a proper antimicrobial response; however, chronic production of IL-1β is detrimental as it increases the production of CXCL1, MIP-2 and matrix metalloproteases that are associated with chronic infections and tissue damage [34]. These findings demonstrate that persistent expression of IL-1β and other cytokines, although necessary to control viral replication early during the infection, also correlate with increased replication of the virus and severity of the disease [44].

To validate the role of IL-1β as a regulator of viral replication, a recombinant murine leukemia virus strain expressing IL-1β (FMLV-IL-1β) was used to infect murine fibroblasts or BALB/c mice; *in vivo* and *in vitro* FMLV-IL-1β infections caused increased permissiveness to the infection that resulted in higher viral replication as compared to their FMLV counterpart. Although the mechanism is not fully understood, the authors speculated that IL-1β enhances the susceptibility of the cells to the infection and/or up-

regulates viral transcription in the infected cells by inducing activation and proliferation of neighboring cells, which in turn increases their chance of infection [45].

Although IL-1β expression mediates viral clearance early during the infection, late expression of this cytokine has been associated with increased lung pathology characterized by increase mucus production, edema, cellular infiltration and a “cytokine storm” due to the capacity of IL-1β to orchestrate and amplify the inflammatory process that leads to severe infection [46]. Previous studies have shown that infection of NLRP3 KO mice with influenza virus results in exacerbation of the disease and difficulty clearing the infection; the previous results showed that NLRP3 activation and an appropriate IL-1β expression is essential for the resolution of the infection and overall survival of influenza infected mice [47, 48]. Additionally, when compared to WT mice infected with NLRP3, KO mice exhibited decreased neutrophil and monocyte recruitment and reduced cytokine production which correlated with their impaired antiviral response and susceptibility to the infection [48].

On the other hand, a proper control of the infection is accomplished when activation of NLRP3 is reduced later during the infection, proving that prolonged IL-1β expression is tightly associated with persistent and severe infection [49, 50]. Likewise, our previous studies in a murine model that compared the progression of RSV infection in aged vs. young mice showed that aged mice express higher levels of IL-1β and OPN prior to infection compared to their younger counterparts, and this pro-inflammatory state that comes with aging impairs the antiviral response once those mice are exposed to RSV infection [4, 51]. Current evidence has shown that RSV induced cytokines control the onset of the infection, pathogenesis and evolution of the disease by disrupting the balance

between elimination of the virus and inflammation; likewise, uncontrolled inflammation is the base of many diseases. Consequently, this chapter aims to elucidate the regulatory role of IL-1β orchestrating the pro-inflammatory state during RSV infection by modulating the levels of OPN expression. We hypothesize that persistent IL-1β expression causes increased OPN levels that in turn negatively regulate the antiviral response, which leads to increased RSV infection.

3.2 Results

3.2.1 RSV Infection Up-regulates IL-1β and OPN mRNA Expression in Epithelial Cells

Our previous study in a murine model of infection showed that higher basal levels of IL-1β and OPN in aged mice are associated with impaired antiviral response that leads to increased susceptibility to RSV infection. *In vitro*, to evaluate the correlation of IL-1β and OPN expression with susceptibility to RSV infection, we examined RSV infection in commonly used and highly susceptible HEp-2 cells and HEK-293 cells. HEK-293 cells were selected because they express low levels of endogenous pro-caspase1, which is essential for IL-1β expression; therefore, they constitute a good model to compare viral infection and OPN expression in the absence of IL-1β [28]. We found a significant increase in expression (reported as fold expression compared to mock-infected cells) of RSV-N and IL-1β transcripts 24, 48 and 72 hpi in RSV-L19F-infected HEp-2 cells compared to RSV-L19F-infected HEK-293 cells (Fig. 3A and B). We compared OPN expression levels obtained by qPCR performed on RNA extracted from HEp-2 and HEK-293 cells infected with 1 MOI of RSV-L19F. In a similar pattern to that of IL-1β, OPN

expression was significantly increased 48 hpi in RSV-L19F-infected HEp-2 cells. In contrast, HEK-293 cells showed decreased RSV infection, lack of IL-1β expression, and delayed OPN mRNA expression that was up-regulated 72 hpi (Fig. 3C). These results suggest that increased RSV infection up-regulates both OPN and IL-1β expression. Also, lack of IL-1β expression in HEK-293 cells correlates with resistance to RSV infection and delays OPN expression.

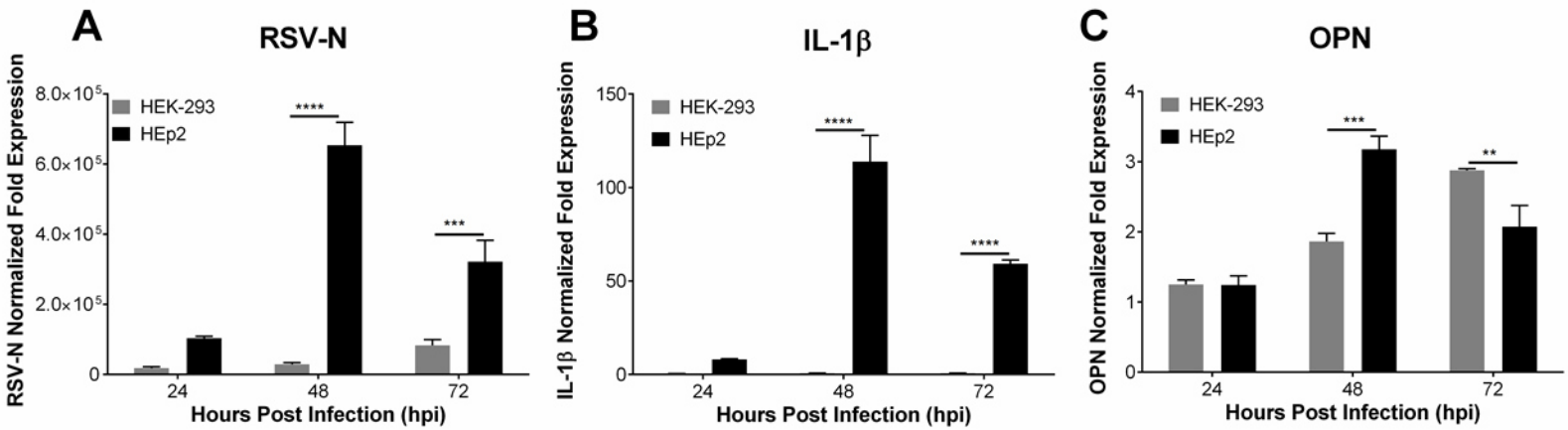

Figure 3. IL-1β and OPN mRNA expression in RSV infected cells. HEp-2 and HEK-293 cells were mock infected or infected with 1 MOI of RSV-L19F. RNA was isolated at 24, 48 and 72 hpi (A-C) Expression levels of RSV-N, IL-1β and OPN were determined by qPCR. Results are presented as fold-change in expression of RSV-N, IL-1β or OPN mRNA normalized to the control (HPRT). qPCR data are represented as means ±SEM. Experiments were performed in triplicate. ** $p < 0.01$, *** $p < 0.001$**** $p < 0.0001$.

3.2.2 RSV Infection-Induced IL-1β Regulates OPN Expression.

While the regulation of OPN expression is not fully understood, it is known that the expression of OPN is regulated by different stimuli, depending on the cells and tissues where it is expressed [52-54]. To examine the role of IL-1β in the induction of OPN expression and to confirm that the production of OPN is not intrinsically impaired in HEK-293 cells, cells were infected with 1 MOI of RSV-L19F, then the infectious medium was replaced with growth medium containing 1 or 10 ng/ml of human rIL-1β. Incubation of HEK-293 cells with human rIL-1β lead to increased OPN expression 24 hours after treatment. In addition, the RSV-L19F-infected HEK-293 cells treated with rIL-1β showed higher levels of OPN expression compared to uninfected cells treated only with rIL-1β (Fig. 4A). While rIL-1β increased the expression of OPN after mock or RSV-L19F infection, it also contributed to an increase in viral yield in RSV-infected cells (Fig. 4B).

Since HEK-293 and HEp-2 cells are likely to have other genetic differences which may influence OPN expression or susceptibility to RSV infection, we treated HEp-2 cells with a caspase-1 inhibitor (Ac-YVAD-CHO) in order to mimic the reduced IL-1β expression seen in HEK-293 and establish that IL-1β is responsible for the observed differences in RSV infection and OPN expression between the two cell lines. Cells were treated with the inhibitor and subsequently infected with RSV-L19F (1 MOI). Our results showed that cells treated with caspase-I inhibitor showed decreased IL-1β mRNA levels (Fig. 4C). RSV-N mRNA levels and OPN protein expression were significantly reduced in those cells treated with the inhibitor as compared to control group (Fig. 4D and E). These results demonstrate the regulatory effect of IL-1β and OPN expression in the progression of RSV infection.

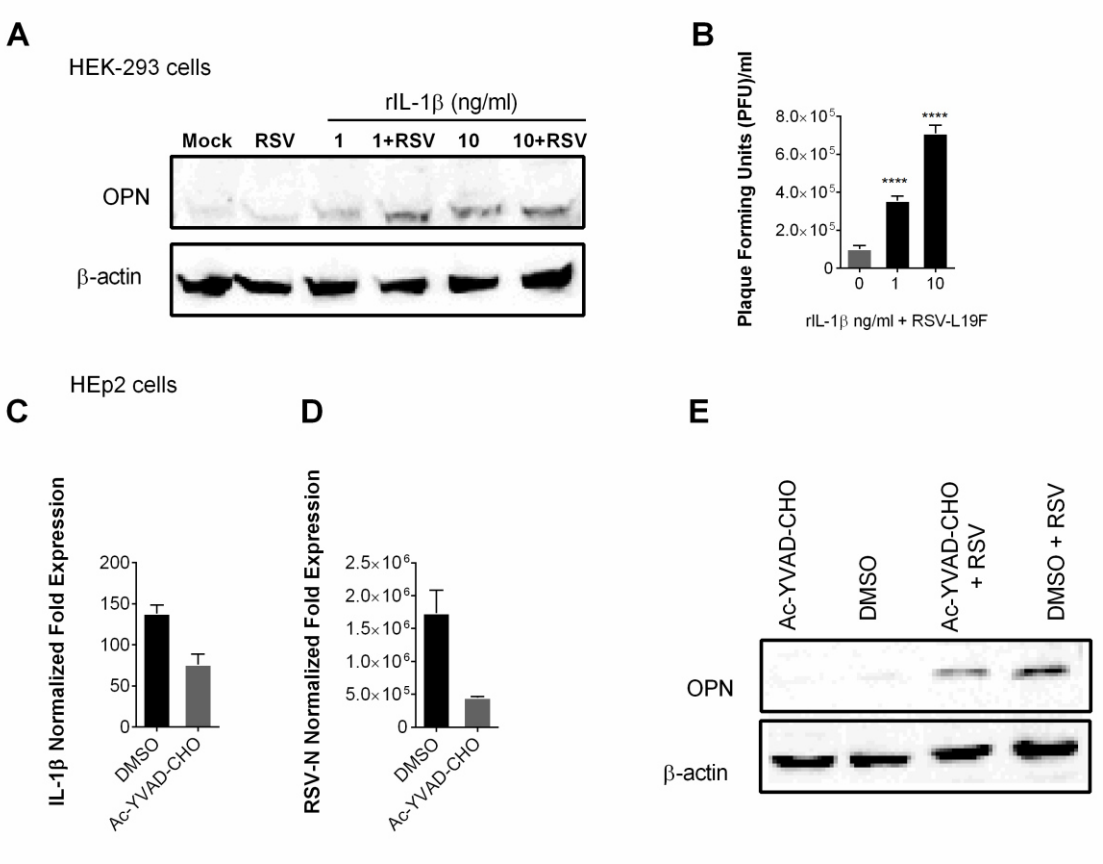

Figure 4. RSV induced IL-1 β regulates OPN expression during RSV infection. HEK-293 cells were mock-infected or infected with 1 MOI of RSV-L19F. 2 hours after the infection cells were treated with increasing concentrations of human rIL-1β (0, 1 or 10 ng/ml). (A) Protein was harvested 24 hpi for western blots. 25μg of protein lysate was loaded in each lane. OPN is seen at 55 kDa and β-actin (loading control) at 42 kDa. (B) Plaque viral titers were obtained from the supernatants collected 24 hpi. (C-E) HEp-2 cells were pre-treated two hours prior RSV-L19F infection with 10 μM of Ac-YVAD-CHO (caspase-I) inhibitor. Cells were infected with 1 MOI of RSV-L19F and infectious media was replaced with fresh media containing 10 μM of Ac-YVAD-CHO. RNA and protein were collected 24 hpi. (C and D) Expression levels of IL-1β and RSV-N were determined by qPCR. Results are presented as fold-change in expression of IL-1β or RSV-N mRNA normalized to the control (HPRT). (E) 25μg of protein lysate was loaded in each lane. OPN is seen at 55 kDa and β-actin (loading control) at 42 kDa. qPCR data are represented as means ±SEM. Experiments were performed in triplicate. **** $p < 0.0001$.

3.2.3 rOPN increases RSV Titers in a Dose-Dependent Manner

To better understand the effect of increased OPN expression on viral yield during RSV infection and to validate that OPN expression is sufficient for the increase in viral titer seen in HEK-293 cells after treatment with rIL-1β. We used varying concentrations of human rOPN (0 - 1 - 10 - 50 - 100 and 200 ng/ml) to pretreat HEK-293 cells four hours before infection with 0.1 MOI of RSV-L19F. After infection, the medium was replaced with fresh growth medium containing rOPN at the same concentrations. 24 hours after infection, there was a significant dose-dependent increase in viral titers in cells treated with rOPN, further confirming that OPN promotes RSV infection (Fig. 5A). Additionally, to establish which step of the infection is benefited from OPN treatment, an intermediate OPN concentration (100 ng/ml) known to increase RSV infection was selected to treat the cells at three different time points: 1) four hours before the infection, 2) during the infection, or 3) after the infection. Our results show a significant increase in viral titers of cells treated with human rOPN during or after the infection (Fig. 5B). As an additional test of rOPN effect during the infection, HEp-2 and HEK-293 cells were infected with 0.1 MOI of the clinical strain Line 19 that expresses a red fluorescent protein-RFP (RSV-KL19F) in the presence of rOPN. We evaluated the number of RSV infected cells using flow cytometry. We found that treatment with rOPN significantly increased the percentage of infected HEp-2 and HEK-293 cells by 23% and 20% respectively (Fig. 5C). These findings suggest that OPN could facilitate the entry of the virus into the cells and increase cell permissiveness to RSV infection.

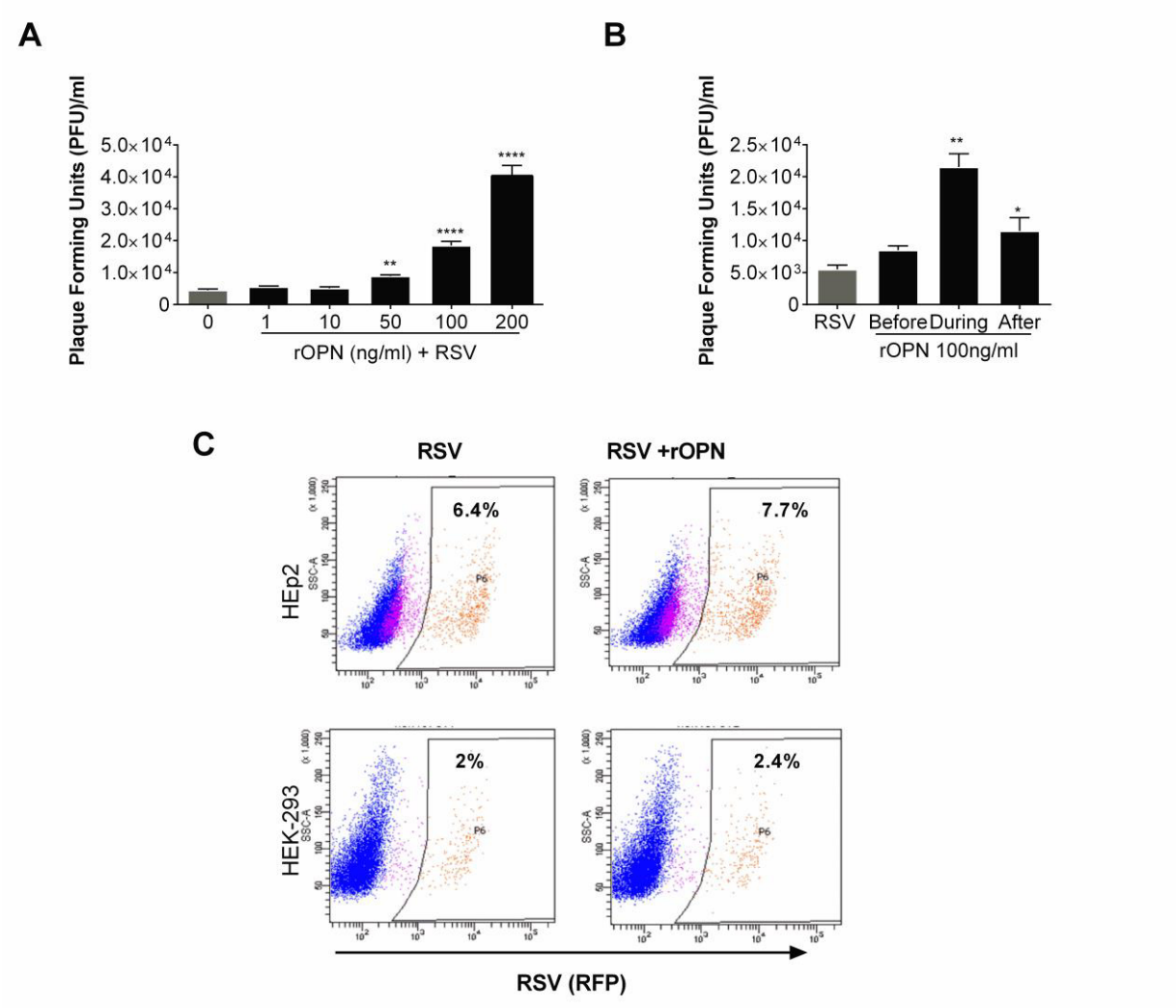

Figure 5. Human rOPN increases RSV titers in a dose-dependent manner. HEK-293 cells were treated with increasing concentrations of human recombinant OPN (rOPN) (0 to 200 ng/ml) four hours before infection. Cells were infected with 0.1 MOI of RSV-L19F and infectious media was replaced with fresh media containing the appropriate concentration of rOPN. (A) Supernatants were collected 24 hpi and viral titers were determined by plaque assay. (B) HEK-293 cells were treated with 100 ng/ml at different time points: before, during or immediately after the infection. (B) Supernatants were collected 24 hpi and viral titers were determined by plaque assay. Result of a representative experiment is shown. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. (C). Hep-2 or HEK-293 cells were infected with 0.1 MOI of RSV-KL19F or RSV-KL19F in the presence of 100 ng/ml of rOPN. After 24 hours, the infected HEp-2 or HEK-293 cells were gated for RFP-expression (RSV + cells). Result of a representative experiment is shown. Experiments were performed in triplicate.

3.3 Discussion

A major finding of our study is that IL-1β is involved in regulating OPN levels during RSV infection. Our results suggest that OPN plays an important role in RSV infection and propagation *in vivo* and *in vitro*, and IL-1β amplifies the inflammatory response by inducing OPN expression, resulting in increased viral loads. Our results show that although RSV infection can induce significant OPN expression in the absence of IL-1β, in the presence of IL-1β it can enhance and accelerate the infection process.

The induction of OPN is controlled by a variety of cytokines, growth factors and hormones [52, 55, 56]. A previous study showed that IL-1β dramatically increased OPN expression during pulmonary fibrosis through the activation of ERK1/2 but not by JNK pathway [57, 58]. Likewise, IL-1β is one of the factors released at sites of injury that contributes to enhanced OPN expression [59]. RSV infection induces a substantial increase of IL-1β, a pro-inflammatory cytokine known to induce the expression of a plethora of downstream pro-inflammatory cytokines including OPN, thus resulting in magnification of the inflammatory process [28, 29, 60, 61]. We have explored the association between IL-1β and OPN up-regulation during RSV infection and our data shows that OPN and IL-1β expression leads to increased viral infection. We also show a delay in OPN mRNA expression levels in infected HEK-293 cells compared to HEp-2 cells, suggesting that the impaired production of IL-1β in HEK-293 cells partially contributes to the reduced expression of OPN in the infected HEK-293. The increased early OPN expression in HEK-293 cells treated with rIL-1β proves that the production of OPN is not intrinsically impaired in HEK-293 cells but instead is dependent on IL-1β signaling. The correlation between increased OPN protein levels and increased RSV viral

titers in HEK-293 cells treated with rIL-1β further confirms the role of these two pro-inflammatory cytokines in the regulation of RSV infection. Similarly, to rule out other differences between HEK-293 and HEp-2 cells, we inhibited caspase-I expression in HEp-2 cells and showed that this inhibition leads to a decrease in IL-1β expression that also results in decreased OPN expression and decreased viral infection.

We have consistently observed an increase in OPN expression in cells and mice infected with RSV. In order to evaluate the feedback effect of high OPN levels on RSV infection we treated HEK-293 with human rOPN during RSV infection. Our results showed that HEK-293 cells treated with human rOPN displayed a dose-dependent increase in viral titers, suggesting that OPN has a regulatory effect on RSV infection. Moreover, the results of studies on timing of OPN action show the prominent effect of OPN during the infection process itself. Cells treated with rOPN during and immediately after the infection yielded higher viral titers, while cells pre-treated with OPN before the infection did not show a significant difference in viral titers when compared to RSV infected cells without rOPN treatment. This suggests that OPN mediates increased entry and/or assembly of the virus leading to a significant increase in plaque viral titers.

Further, cells treated with rOPN became infected with RSV at a significantly higher rate than untreated cells.

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CHAPTER 4
OPN EXPRESSION DURING RSV INFECTION

4.1 Introduction

Osteopontin is a multifunctional secretory protein also known as secreted phosphoprotein 1 (SPP 1) and early T-lymphocyte activation-1 (Eta-1) [1]. Although it was first identified in osteoclasts and is highly expressed in bone, OPN is also secreted by a variety of cells and tissues including macrophages, smooth muscle cells, epithelial and endothelial cells [1-3]. OPN has anti- and pro-inflammatory activity depending on cell context; hence it has been associated with multiple physiological and pathological conditions.

The duality of OPN function can be explained by the complexity of its structure, which has six translated exons that can undergo alternative splicing and multiple post-translational modifications. This results in a number of OPN forms with different functions: OPN-a, the full length protein; OPN-b, which lacks exon 5; and OPN-c, which lacks exon 4 (Fig. 6) [1, 4]. Although the function of this alternative splicing has not been characterized in many diseases, it has been reported that excision of exon 5 is essential to maintain OPN physiological functions; hence OPN-b expression is associated with anti-apoptotic activity. Lack of exon 4 in OPN-c results in increased solubility that makes OPN

more accessible to its integrin receptors, thus enhancing signaling that leads to cell migration and cancer metastasis [5-8]. The full-length OPN is composed of 300 amino acids and is expressed as a 33 to 75 KDa protein depending on the specific post-translational modifications [9-11].

The induction of OPN is controlled by a variety of cytokines, growth factors, and hormones [12]. Examples of growth factors involved with OPN regulation are transforming growth factor β, epidermal growth factor and fibroblast growth factor [9, 12]. Additionally, the glucocorticosteroids dexamethasone, progesterone and other hormones can regulate its expression [9]. OPN expression is also regulated by mediators of acute inflammation such as IL-1β, tumor necrosis factor α (TNF-α), IL-2, IL-3, NO and IFN-β among others [13-16]. Up-regulation of OPN has been shown after activation of phosphoinositide 3-kinase pathways (PI3K), extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK) [1].

OPN contains a highly conserved Arg-Gly-Asp (RGD) integrin binding domain that allows its interaction with αvβ1, αvβ3, αvβ5, αvβ6, α8β1, α5β1 integrins and the CD44 hyaluronic acid receptor [8, 11]. The association of OPN with these receptors also explains the diversity of OPN function and its association with multiple signaling pathways [11].

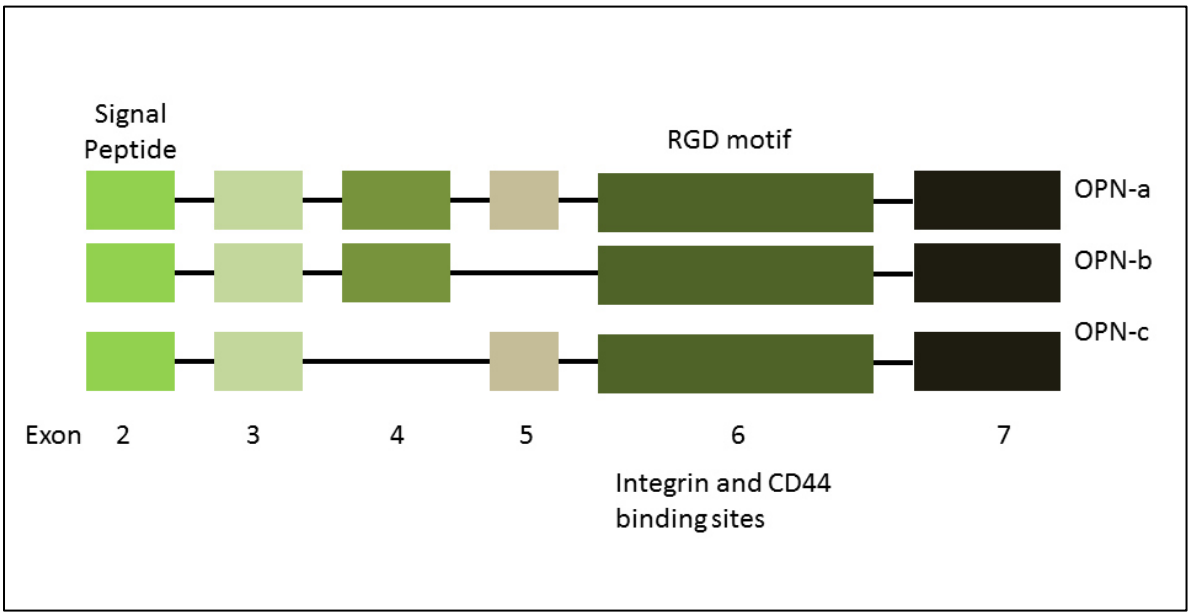

Figure 6. Splice variants of Osteopontin. The OPN gene has six translated exons which produce the full length protein (OPN-a 33kDa) when all are transcribed. Deletion of exons 5 and 4 leads to OPN-b and OPN-c expression respectively. Exon 2 contains the signal peptide required for OPN secretion, whereas exon 6 has a tripeptide Arg-Gly-Asp (RGD) domain that is recognized by integrins and CD44 receptor.

4.1.1 Role of OPN Expression and Regulation of the Immune Response

OPN modulates the immune response by facilitating neutrophil and macrophage migration to the site of injury. It also promotes dendritic cell maturation and migration to the lymph nodes where they can present antigens to naïve T cells, thus serving as a bridge between the innate and the cell-mediated immune response [9]. Likewise, OPN has been associated with Th17-related pathologies like human T-lymphotropic virus 1 infection, acute coronary syndrome, arthritis, and *Trypanosoma cruzi* infection, where endogenous levels of OPN seem to positively regulate the Th17 cytokine response that promotes inflammation [17-19].

Recent studies have shown that OPN expression was up-regulated in serum and bronchial tissue samples from mild to moderate asthmatics, and increased levels of OPN correlated with the severity of the disease [20-22]. Similarly, OPN levels are up-regulated in serum and tissue samples from chronic rhinosinusitis and allergic patients [16, 20]. Xanthau et al. further explored the mechanism by which OPN is involved in asthma and allergic diseases by showing that during primary sensitization, OPN blocks the migration of plasmacytoid dendritic cells to draining lymph nodes, resulting in an increased inflammatory response and exacerbated allergic symptoms. However, during secondary antigenic challenge OPN up-regulation resulted in inhibition of conventional dendritic cells recruitment, which consequently reduced the Th2 response and associated airway hyperresponsiveness [23]; thus, the role of OPN in respiratory disease remains inconclusive.

OPN function has been the focus of research in several microbial infections; it has been credited with antimicrobial functions against *Klebsiella pneumoniae* and *Mycobacterium tuberculosis* [24, 25]. OPN deficiency leads to severe endodontic infections [26]. Some researchers found a significant increase in OPN protein in the lungs of WT mice after influenza infection; however, there was no viral load or lung pathology difference between infected WT and OPN KO mice. A second study showed that OPN KO mice exhibited severe pathology and decreased survival after influenza infection [27, 28]. Although the authors did not explain the difference in the results, the discrepancies could be attributed to the difference in the inoculum used to infect the mice. Similar to influenza, RSV induces a substantial increase of IL-1β, which is a critical cytokine able to amplify the pro-inflammatory response that follows infection [29, 30]. RSV infection induces the expression of several cytokines including IL-1β, IL-6 and chemokines such as IL-8 and TNF-α that contribute to inflammation and the pathology of the infection [31-33]. However, whether the inflammation contributes to increased viral load and spread of infection is unclear.

In an effort to dissect the molecular basis of severity of RSV infection, previously we conducted a microarray analysis and identified several genes that are influenced by aging and RSV infection. These studies led to identification of five up-regulated genes including osteopontin (OPN), which hitherto had not been studied in RSV infection [34]. Thus, the main goal of this chapter is to establish the effect of OPN during RSV infection; our hypothesis is that OPN increases cell permissiveness to RSV infection and

accelerates the infection process, which leads to increased viral titers in epithelial cells expressing OPN.

4.2 Results

4.2.1 RSV Replication is not required for Increased OPN Expression

To assess baseline levels of OPN and subsequent changes in expression upon RSV infection, HEp2 cells were infected with RSV-L19F at 1 MOI (1 PFU/cell) and the levels of OPN were confirmed by immunostaining of mock- or RSV-L19F-infected HEp2 cells; results showed elevated OPN expression in the virus-infected cells compared to mock-infected cells. There were comparatively fewer OPN-positive cells in the mock-infected than RSV-L19F-infected cells 48 hpi (Fig. 7A).

To examine the role of RSV replication during OPN induction and to determine whether RSV proteins alone could initiate OPN induction, HEp2 cells were infected with increasing doses (0.1, 1 and 10 MOI) of UV-inactivated RSV-L19F, native RSV-L19F or mock treatment. To evaluate the productivity of the infection, supernatants were used for RSV titration by plaque assay. As expected, the number of PFU increased in a dose-dependent manner from 0.1 to 10 MOI in RSV-L19F infected cells. There was no productive infection in the UV-attenuated virus (Fig. 7B). Protein was isolated 48 hpi for Western blot analyses. OPN expression was proportional to the MOI dose of the RSV—higher in 10 MOI-infected cells compared to 0.1 or 1 MOI-infected cells (Fig. 7C). Furthermore, OPN expression was higher in RSV-L19F-infected cells than in cells

infected with the UV inactivated virus. However, at 10 MOI of UV-inactivated RSV there was some up-regulation in the expression of OPN, suggesting that RSV proteins or nucleic acids present in the inactivated form of RSV continued to contribute to OPN induction and expression.

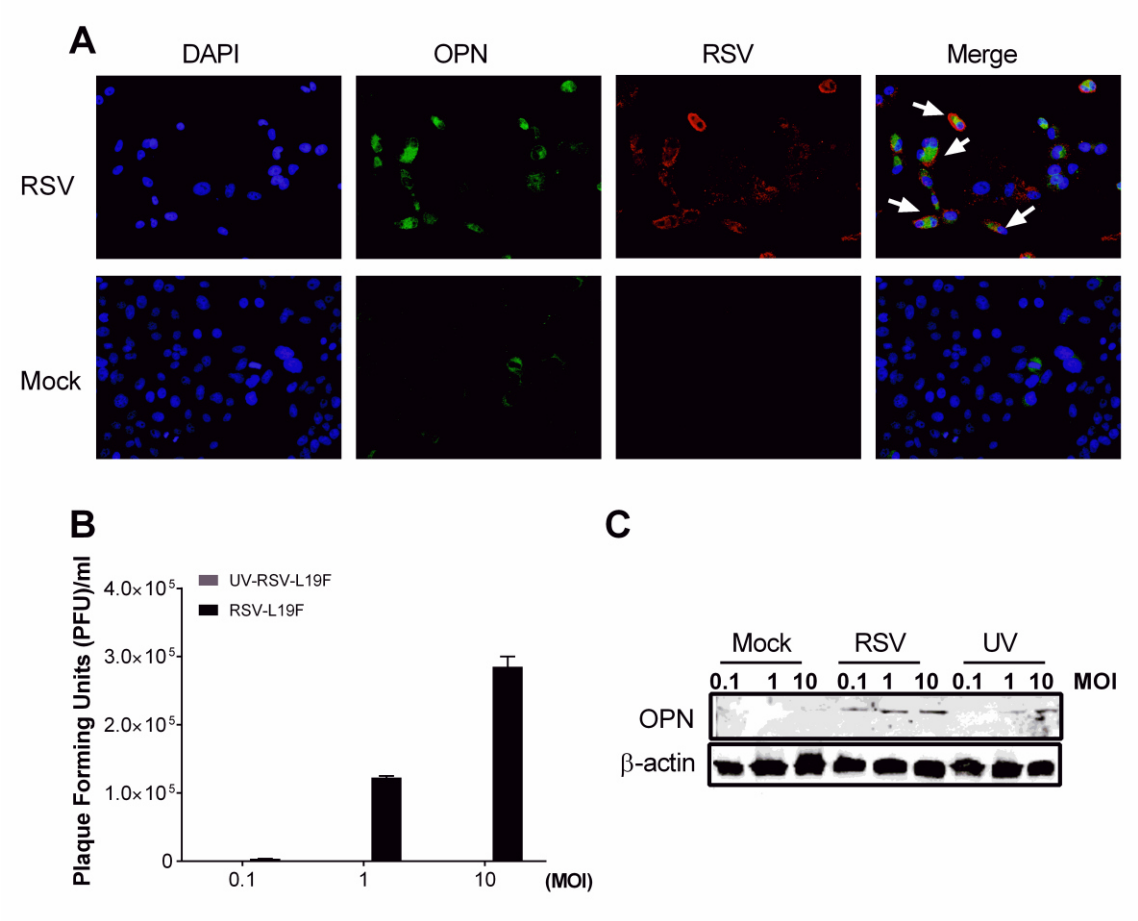

Figure 7. RSV infection induces OPN expression in a dose dependent manner. HEp2 cells were mock-infected or infected with RSV-L19F (1 MOI). (A) Images of Hep2 cells stained with polyclonal antibody against RSV and OPN at 48 hpi. Representative images of RSV-positive (red) cells, OPN positives (green) cells, and DAPI (blue) nuclear staining at 200X magnification. (B) RSV titer by plaque assay of supernatants for increasing doses (0.1, 1 and 10 MOI) of UV-inactivated and native RSV-L19F at 48 hpi. (C) OPN protein (55 kDa) expression in HEp2 cells at 48 hpi after mock, RSV and UV-inactivated RSV treatment in the indicated doses analyzed by western blots. β-actin (loading control) at 42 kDa was probed as the loading control.

4.2.2 CD44 Expression Mediates RSV Infection in Epithelial Cells

CD44 is known to be a receptor for OPN. To evaluate the expression of CD44 in mock or RSV infected cells, HEp-2 and HEK-293 cells were infected with 0.5 MOI of RSV-KL19F. 24 hours after infection, cells were stained with a FITC-CD44 antibody and gated for CD44 or isotype control (green) and RSV (red) expression. We found higher cell surface expression of CD44 in the HEp-2 cell line (96.7%) than in HEK-293 (73.7%). We also found a significant difference in the number of RSV positive cells, confirming that HEp-2 is more permissive to RSV infection (Fig. 8). These results suggest that the earlier OPN expression in HEp-2 cells and the concomitant CD44 expression could be key mediators for OPN signaling during RSV infection that potentiates the infection of HEp-2 cells.

To test whether CD44 expression is required for RSV infection, the CD44 receptor was neutralized with a broad-spectrum rat anti-human CD44 antibody. Briefly, HEp-2 cells were pre-incubated for 20 minutes at room temperature and then infected with RSV-KL19F (0.5 MOI) for two hours, after which the infectious media was replaced with fresh growth media. 24 hpi we assessed the number of RSV positive cells (RFP +) by flow cytometry. We found ~52.4% of the cells were RSV positive in the cells pre-treated with CD44 antibody compared to 70.5% RSV positive cells in the control group. Our results showed a decrease in RSV positive cells following treatment with anti-CD44 antibody prior to infection compared to normal rat IgG control (Fig. 9). Together, these data suggest that CD44 facilitates the RSV infection process.

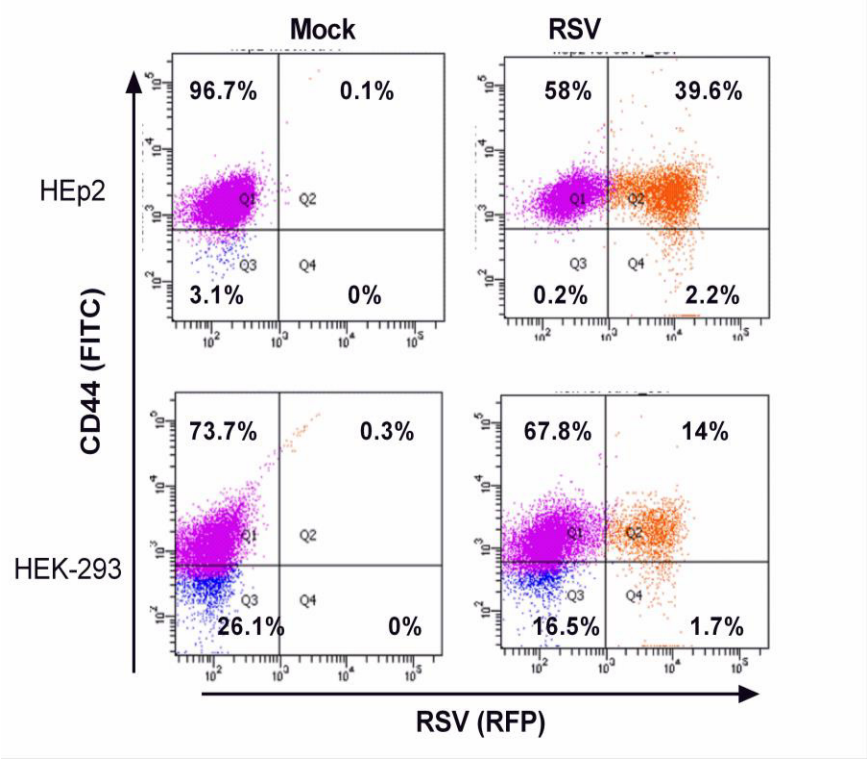

Figure 8. CD44 expression mediates RSV infection. HEp-2 or HEK-293 cells were infected with 0.5 MOI of RSV-KL19F (red). After 24 h, the cells were stained with FITC mouse anti-human CD44 antibody (green) and also gated for RSV expression (red). (Top panel) Flow cytometry analysis of HEp-2 cells infected with RSV-KL19F. (Bottom panel) Flow cytometry analysis of HEK-293 cells infected with RSV-KL19F. Result of a representative experiment is shown. Experiments were performed in triplicate.

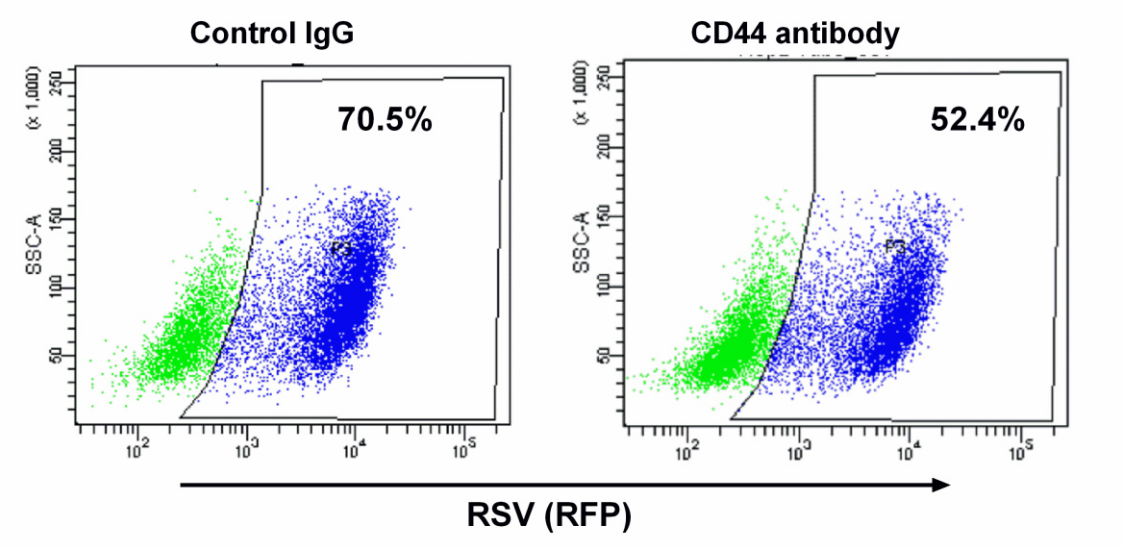

Figure 9. Neutralization of CD44 receptor reduces the number of RSV positive cells. Hep-2 cells were pre-treated with 10 µg of broad spectrum rat-anti human CD44 antibody (clone A020) or normal rat IgG (control). After the pre-treatment, cells were infected with RSV-KL19F (0.5 MOI) and infectious media was replaced with fresh growth media. After 24 hours, the percentage of RSV positive cells was determined by flow cytometry. Result of a representative experiment is shown. Experiments were performed in triplicate.

4.3 Discussion

To our knowledge our lab is the first one to determine the importance of OPN for RSV infection *in vitro* and *in vivo*. Our data has shown the association between increased OPN levels and increased RSV replication [34].

To establish the mechanism for OPN up-regulation and also to verify that OPN was expressed in human epithelial cells infected with RSV, we infected HEp-2 cells with RSV-L19F and verified through immunostaining and qPCR that RSV triggers OPN expression. Also, we found up-regulation of OPN after infection with UV irradiated-RSV-L19F, while the UV inactivated virus led to lower expression of OPN protein levels compared to replicating RSV-L19F. Therefore, loading more antigenic proteins and nucleic acids initiates OPN induction, suggesting replication is not absolutely required for the expression of OPN.

Also, we found that HEp-2 highly expressing the OPN receptor CD44 showed a higher percent of infected cells compared to HEK-293 cells whose expression of CD44 is lower. Although the basis of OPN regulation of RSV entry and/or assembly remains unclear, one possibility is that OPN modulates the fusion process itself, thus aiding virus entry. This idea is supported by the observation that the OPN receptor (CD44) co-localizes with RSV F protein and results in viral filament formation and syncytia formation which benefit the infectious process [35]. Additionally, the decrease we observed in RSV-positive cells after CD44 receptor neutralization evidenced that any manipulation of components of the lipid rafts which disrupts CD44 signaling could negatively affect the

infection process; thus our results suggest that the interaction of proteins at the lipid rafts, like OPN and its receptor (CD44), could favor the infection process.

In support of our findings, OPN has been associated with lung disease progression [36, 37]. During lung infection, OPN expression was associated with impairment of the innate immune response and decreased antibacterial function of effector antimicrobial peptides [38], which resulted in increased susceptibility to infection by *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* [38]. Another study showed lower *S. pneumonia* bacteria loads in the lungs of OPN KO mice as compared to WT infected mice, suggesting that OPN enhanced the primary infection of the respiratory tract by increasing the viability of the bacteria [39].

Overall increased OPN levels during bacterial infections have been reported and associated to increase susceptibility to infections; however, the role of OPN during viral infection remains inconclusive. During viral lung infection, higher levels of OPN in the serum of patients infected with influenza correlated with increased severity of the infection and pulmonary edema which can be explained by increased pro-inflammatory cytokine production [40]. Additionally, OPN up-regulation has been proposed as a key cytokine modulating chronic hepatitis C infection (HCV); and increased OPN expression was found in cells that are more susceptible to HCV infection while neutralization of OPN expression correlates with decreased viral replication [36]. Similarly, Dengue infection is another example of OPN up-regulation during exacerbation of a viral infection [41].

With regard to lung pathology, OPN is not only associated with infectious diseases; several studies also associated OPN expression and lung cancer progression [42-44].

OPN has been postulated to increase metastasis and anti-apoptotic activity of the cancer cells, which results in increased metastatic phenotype in lung cancer [44, 45].

4.4 References

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CHAPTER 5

SEVERE RESPIRATORY SYNCYTIAL VIRUS INFECTION AND OSTEOPONTIN EXPRESSION

5.1 Introduction

Respiratory syncytial virus (RSV) is one of the first pathogens encountered by infant immune systems. The infection is regularly characterized by cold like symptoms. However, complications arising from RSV infection can cause acute lower respiratory infection (ALRI) leading to bronchiolitis and pneumonia in up to 30% of the infected infants [1]. In the United States alone, RSV causes up to 90,000 hospitalizations and 1.7 million visits to the doctor office [2]. RSV infection represents a healthcare burden as treatment of its related disease costs up to $400 million each year [2, 3], which is not surprising if we consider that by the age of two years old all infants are expected to suffer from the infection at least once [4].

RSV is known to cause re-infection throughout life because the immune response to the infection does not lead to long lasting immunity. Therefore, assessing the risk factors associated with severe RSV infection is important to protect individuals that are more susceptible to complications [5, 6]. Among these risk factors are pre-term infants, children with malignancies, congenital diseases, acquired or congenital immune deficiency, cystic fibrosis, Down syndrome, and other severe respiratory diseases [7].

RSV is a major producer of inflammation of the small airways, also known as bronchiolitis. This is one of the leading causes of infant hospitalization and admission to

the intensive care unit (ICU) [8-11]. A recent study evaluated the association between bronchiolitis and viral infections in young children (≤ 5 years). The authors found that RSV was detected by RT-PCR in 73% of the samples whereas Rhinovirus (RV) was found in 30% of them and the combination RSV and RV in 41% of all the samples [12].

Severe RSV infection has been recognized as a risk factor for the development of pneumonia in the elderly (> 65 years) [13]. A retrospective study done in adults in the United States evidenced that RSV is associated with higher numbers of severe infections, associated hospitalizations and increased mortality than Influenza infection, particularly in older adults that were immunocompromised [14].

RSV infection is associated with a set of pathological changes of the respiratory epithelium. These include necrosis of the tissue, goblet cell hypermetaplasia, severe inflammation and a plug of mucus that leads to airway obstruction and edema, which is a hallmark of severe respiratory disease [15-17]. Both the viral load and the host immune response control the pathological changes following RSV infection [18]. *In vivo*, several studies have aimed to elucidate the pulmonary changes associated to RSV infection. Recent studies have used murine models to mimic RSV disease. The authors used two different viral strains, A2 and line 19 [15, 19]. The A2 strain is a common strain of human RSV available from the American Type Culture Collection (ATCC).The line 19 strain is derived from a clinical isolate and is associated with severe RSV disease and mucus production [20, 21]. In comparison, BALB/c mice infected with line 19 shown significant increase in gob5, MUC5AC and IL-13 protein expression that correlate with mucus production and airway obstruction compared to native A2-infected mice [20, 21]. In addition, the mucogenic line 19 is associated with increased viral loads which can explain

the increase in mucus production since more viral factors are being expressed and are thought to contribute to the inflammatory process [15].

In humans, RSV infects ciliated airway epithelial cells leading to necrosis of epithelial cells and tissue damage that results in accumulation of cell debris, macrophages, neutrophils and mucus production that leads to airway obstruction [22]. In addition, bronchoalveolar lavage (BAL) secretions associated with severe RSV infection are rich in neutrophils, pro-inflammatory cytokines and chemokines such as Interleukin-6 (IL-6), IL-10, IL-5, IL-4, IL-13, tumor necrosis factor-α (TNFα), and MCP-1 among others [23, 24]. Therefore, a robust inflammatory response is associated with the clinical outcome and disease severity in humans. However, other host genetic factors and specific features associated with the virus itself also have an influence in the severity of the disease [25].

The current understanding of RSV disease in humans is incomplete. Therefore, modeling RSV disease *in vivo* remains a good approach to increase our knowledge of viral pathogenesis and progression of the disease. RSV infection has been studied in chimpanzees, cattle, cotton rats, guinea pig, sheep and mice [24, 26]. Ideally, a good animal model of RSV infection should mimic pathological characteristics of the human infection. Infection by the line 19 antigenic strain of the virus has been associated with similar effects to the ones seen in human infection including airway hyperreactivity, increased mucus production and similar cytokine production [27].

Although RSV was discovered more than five decades ago and despite the ongoing research and clinical trials, there is no treatment or vaccine available. Immunoprophylaxis remains the key to prevent severe RSV infection in high-risk

population [28, 29]. OPN is up-regulated in other inflammatory lung diseases, such as asthma, and RSV has been implicated in asthma exacerbation. Therefore, we reasoned that OPN might be involved in regulating susceptibility to severe RSV infection. To test this hypothesis we investigated the role of OPN in RSV infection.

In this chapter we compare human epithelial cells and mice infected with RSV-L19F, a strain that produces severe infection associated with increased viral loads, to rgRSV-A2 which induces mild infection and moderated viral loads. This allows us to establish if OPN expression is correlated with increased spread of the virus between cells and increased cell permissiveness to RSV infection.

5.2 Results

5.2.1 OPN Expression is a Marker of High RSV Loads in HEp-2 Cells (*in vitro)*

To investigate the role of OPN in determining the severity of RSV infection, HEp-2 cells were infected with rgRSV-A2 or RSV-L19F. The latter is known to induce severe RSV infection and higher viral loads in a mouse model [15]. *In vitro*, infection with rgRSV-A2 and RSV-L19F resulted in similar plaque viral titers and RSV-N gene expression at 24 hpi (Fig. 10A and B). However, at 48 hpi RSV-L19F-infected cells had a significantly increased number of viral plaques and a similar increase in RSV-N mRNA expression when compared to cells infected with rgRSV-A2 (Fig. 10A and B). Following the same pattern, we found no significant difference in the gene expression of IL-1β at 24 hpi with the two RSV strains, but we noted a significant up-regulation of IL-1β mRNA expression 48 hpi with RSV-L19F compared to rgRSV-A2 (Fig. 10C). Remarkably, IFN-β mRNA

levels were significantly elevated 24 hpi in rgRSV-A2-infected cells compared to those infected with RSV-L19F. This may in part account for the difference in viral titers and RSV-N transcripts observed in cells infected with the different viral strains (Fig. 10D). Western blot analysis revealed an increase in OPN protein expression at 24 hpi that remained high at 48 hpi in RSV-L19F-infected cells. Cells infected with rgRSV-A2 showed less OPN protein expression at 24 hpi and the expression started to return to baseline levels by 48 hpi (Fig. 10E).

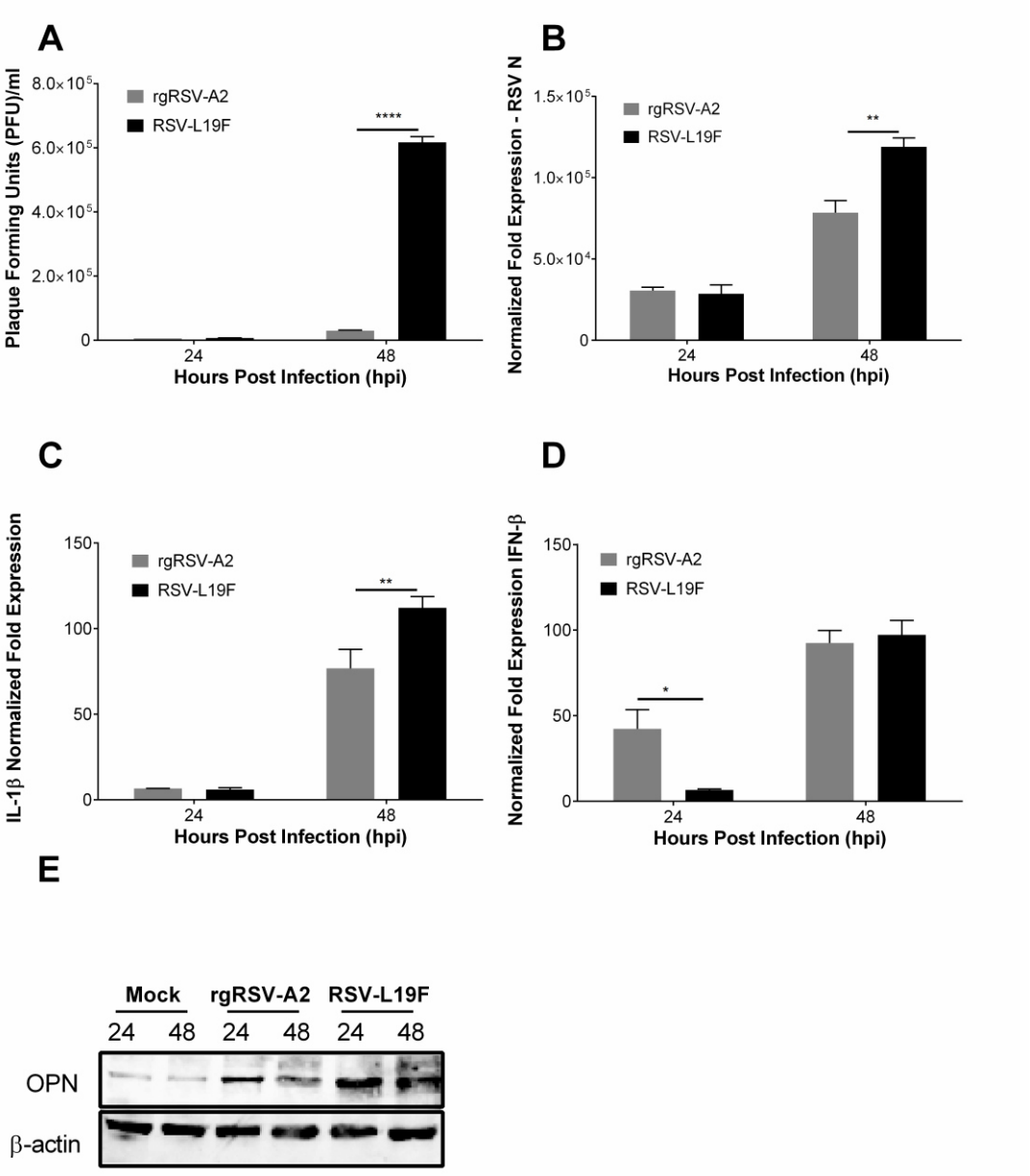

Figure 10. OPN is a marker of high RSV loads in HEp-2 cells. HEp-2 cells were mock-infected or infected with 1 MOI of RSV-L19F or rgRSV-A2. RNA, supernatants and protein were isolated 24 and 48 hpi. (A) Plaque titers were obtained from supernatants of HEp-2 cells infected with RSV-L19F or rgRSV-A2. (B - D) Expression of RSV-N, IL-1β and IFN-β were determined by qPCR. Results are presented as fold-change in expression of RSV-N, IL-1β or IFN-β mRNA normalized to the control (HPRT). (E) Western blot analysis of OPN expression. 25µg of protein lysate was loaded in each lane. OPN is seen at 55 kDa and β-actin (loading control) at 42 kDa. Experiments were performed in triplicate. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

5.2.2 RSV Replication is diminished in Mice Lacking OPN (OPN KO).

To determine the time points where IL-1β and OPN expression are induced and their effect during RSV infection, we infected OPN KO and WT mice intranasally with the mucogenic virus, RSV-L19F. Mice were euthanized 1, 3 and 5 dpi. As we reported before [21], RSV infection resolves much faster in OPN KO mice than WT. This was evidenced by a significant decrease in RSV-N transcripts at 5 dpi in the lungs of OPN KO mice as measured by qRT-PCR. There were no significant differences in RSV-N amplification 1 or 3 dpi, but the infection did not progress to 5 dpi in OPN KO mice as it did in WT mice (Fig. 11A). Secretion of IL-1β was also measured upon infection since it was suspected to play a role in controlling OPN expression during RSV infection. We found a significant increase in IL-1β expression 1 dpi in both OPN KO and WT mice infected with RSV-L19F, yet there was no significant difference between the two strains of mice at 1 dpi. Nonetheless, at 5 dpi RSV infected WT mice had increased levels of IL-1β while in the OPN KO mice IL-1β levels returned to baseline (Fig. 11B). The expression of OPN in RSV-L19F-infected WT mice was also determined and compared to mock-infected WT. OPN protein levels were measured by ELISA of lung homogenates. We observed a significant increase in OPN protein levels at 3 and 5 dpi in WT mice infected with L19F (Fig. 11C). As expected, the levels of OPN were below the detection limit in RSV-L19F or mock-infected OPN KO mice (Fig. 11C).

5.2.3 OPN Expression is a Marker of high RSV Loads (*in vivo)*

Furthermore, we infected WT mice in increased doses of RSV-L19F (1 or 3 x 10^6) and found a significant increase in OPN protein levels that correlated with the increased

viral concentration used to infect those mice (Fig. 12A). We also evaluated the effect of mild or severe RSV infection *in vivo* and how it influenced OPN expression. WT mice were infected with mock, RSV-L19F or rgRSV-A2. Similar to the results found *in vitro*, WT mice infected with RSV-L19F exhibited higher OPN protein levels compared to those infected with rgRSV-A2 (Fig. 12B). These results suggest that increases in OPN expression levels are closely associated with RSV viral loads.

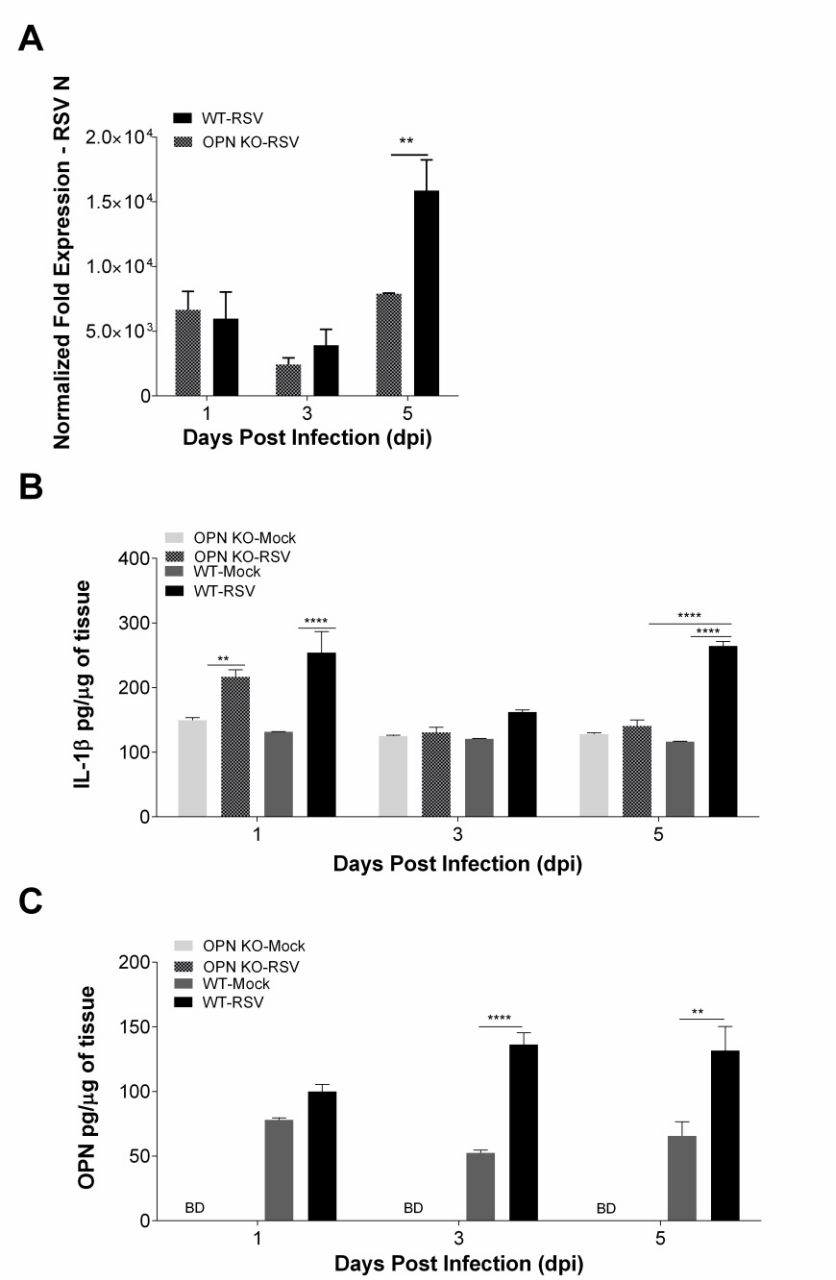

Figure 11. RSV replication is diminished in mice lacking OPN (OPN KO). C57BL/6 and OPN KO mice were mock-infected or infected with 3 x 10^6 RSV-L19F. Lungs were collected 1, 3 and 5 dpi for protein and RNA extraction. (A) RSV-N mRNA expression levels were determined by qPCR and results represented as fold-change in expression of RSV-N mRNA normalized to the control (HPRT). (B and C) Levels of OPN and IL-1β in lung homogenates were determined by ELISA. BD: Below detection limits. Lung homogenates were obtained from individual mice and not pooled (n>4 per group). ** $p < 0.01$, **** $p < 0.0001$.

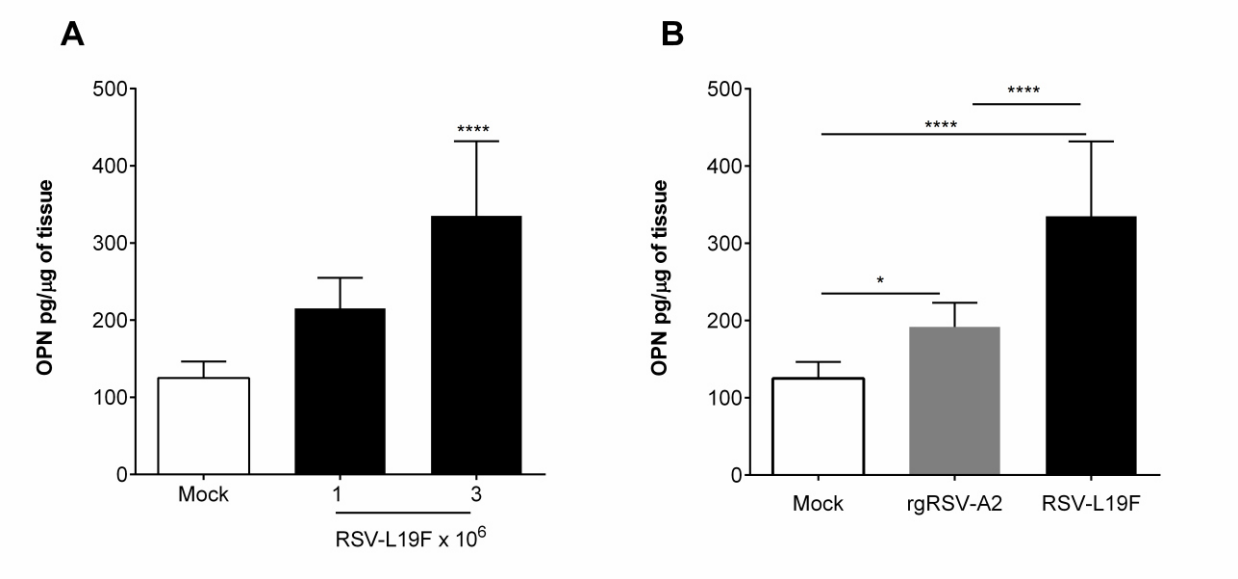

Figure 12. OPN expression is a marker of high RSV loads *in vivo*. (A) C57BL/6 mice were mock-infected or infected with 1 or 3 x 10^6 RSV-L19F. (B) C57BL/6 mice were mock-infected or infected with 3 x 10^6 RSV-L19F or rgRSV-A2. (A-B) Lungs were collected 3 dpi for protein isolation and levels of OPN in lung homogenates were determined by ELISA. Lung homogenates were obtained from individual mice and not pooled (n>4 per group). * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

5.3 Discussion

A comparative analysis of virus infection, IL-1β, OPN and IFN-β expression by two RSV strains (lab isolate rA2 versus the mucogenic strain RSV-L19F) showed that OPN expression was significantly higher in RSV-L19F compared to rA2. Also, our *in vivo* studies in a mouse model of RSV infection showed that IL-1β expression preceded OPN expression in WT mice after RSV infection. Importantly, we found a significant difference in infection at 5 dpi, where WT infected mice exhibited increases in RSV-N gene amplification compared to OPN KO mice. This data is consistent with a previous study done by our lab where we found decreased viral titers from lung homogenates and fewer RSV-positive lung cells in the OPN KO mice infected with RSV compared to WT mice [21]. Further, these results are consistent with reports of increased levels of OPN positively correlated with severity of lung inflammation [30-34]. OPN up-regulation in serum and tissue samples from chronic rhinosinusitis and allergic patients also correlated with severity of disease [30, 35, 36]. In agreement with these studies, a recent paper found a positive correlation between increased levels of OPN in human serum and disease severity in influenza patients [37]. Our *in vitro* findings suggest that IFN-β reduces the viral infection resulting in decreased IL-1 β and OPN expression and it is consistent with previous reports where RSV-A2 infection resulted in significantly higher expression of IFN-α than RSV-L19F in human epithelial cells and BALB/c mice [15, 38]. Additional studies have reported decreased OPN levels in serum samples from multiple sclerosis patients treated with IFN-β, supporting this INF-β dependent mechanism of OPN regulation [39-41]. Also, an inverse relationship between IFN-β and OPN expression reported in recent data indicates that the fusion protein of the virus could directly modulate

type I IFN expression and that the increase in viral loads after RSV-L19F infection lead to higher expression levels of viral non-structural proteins (NS1 and NS2). These are known to disrupt type I IFN response and therefore may act to increase OPN [15, 42-44]. Together, these pathophysiological differences between non-severe and severe RSV strains suggest that OPN could be used as a marker of severe RSV infection.

Proper control of the inflammatory response is required not only for effective viral clearance but also for prevention of subsequent complications caused by imbalanced immune response and exaggerated inflammation during RSV infection [45]. It has been suggested that the severity of the disease caused by RSV is controlled not only by the replication of the virus but also by the host inflammatory response. Therefore, these two factors together could help us predict the severity of the disease and perhaps establish new treatments aiming to control infection and inflammation [46-48]. An accurate profiling of inflammatory mediators is a promising strategy to explore RSV physiopathology that could contribute to a better management of RSV disease.

Taken together, our *in vitro* and *in vivo* data has shown a correlation between OPN protein expression levels and RSV viral loads. Cells and mice infected with RSV-L19F exhibit increased viral replication that results in a better propagation of the infection compared to infection with RSV-A2. Our results proved that increased levels of OPN protein are sufficient to increase viral loads, thus influencing the onset and severity of infection.

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CHAPTER 6

CONCLUSIONS

To the best of our knowledge, our lab was the first to determine the importance of OPN during RSV infection; however, the mechanism underlying the role of OPN in RSV infection remained to be elucidated. Herein, the results from a series of *in vivo* and *in vitro* experiments show the molecular mechanisms linking OPN expression with increased susceptibility to RSV infection. The salient findings of the present studies are as follows: i) RSV infection leads to increased IL-1β and OPN expression, ii) IL-1β is involved in the regulation of OPN levels during RSV infection, iii) increased OPN expression alone is sufficient to enhance RSV infection and contribute to viral spread, and iv) OPN is a predictive marker of viral loads both *in vitro* and *in vivo*.

In chapter 1 we provided evidence that concomitant expression of IL-1β and OPN upon RSV infection increases susceptibility to RSV. We found a positive correlation between both high IL-1β and high OPN expression levels with increased RSV loads. In addition, we established that IL-1β is partially involved in the increase in OPN expression during RSV infection. Although OPN expression was not exclusively required for RSV infection, early expression of OPN during the infection is associated with a rapid increase in viral loads and spread of the infection among neighboring cells.

In chapter 2 we have shown that RSV replication is not required for OPN expression. We found OPN expression is higher in cells infected with live virus compared

to those infected with UV-inactivated RSV. This finding suggests that RSV infection and replication is a major trigger of OPN expression, although in the absence of viral replication there remained some OPN expression. This suggests that stimulation of the innate immune response by viral genomic material and proteins can also activate OPN expression. While the mechanism of action of OPN during RSV infection is not fully clear, we found that neutralization of OPN receptor (CD44) prior to the infection correlates with a decrease in RSV positive cells. Our data suggest that the interaction between OPN and its receptor CD44 is a key step that facilitates RSV infection process, presumably by altering associated lipid rafts.

In chapter 3 we compared (*in vitro* and *in vivo*) IL-1β and OPN expression in two RSV strains. RSV-L19F is known to induce higher viral loads than RSV-A2. Our results showed that increased levels of RSV-N transcript and viral titers positively correlated with OPN protein levels in cells and mice infected with RSV-L19F. These differences between the non-severe and severe RSV strains showed that OPN expression correlates with increased viral loads in lung homogenates from RSV-infected mice and supernatants from infected cells. This suggests that OPN could be used as a marker for severe infection or to evaluate the effect of treatments during infection.

Together, the results of these studies have pointed to the role of OPN during RSV infection. The first cycle of RSV infection proceeds in an OPN-independent fashion, where the viral nucleic acid is recognized by PRR leading to IL-1β expression, and subsequently to OPN up-regulation. Therefore, the second replication cycle of the virus can be said to be OPN-dependent, wherein the interaction of OPN and its receptor (CD44) results in increased viral fusion to subsequent host cells that results in an increased number of

infected cells within the tissue and therefore increased overall viral progeny production (Fig. 13).

The knowledge acquired from this research could be used in the future to develop new drug targets for treatment and/or prophylaxis of RSV infection since it may lead to development of pharmacological strategies that allow for regulation of IL-1β and OPN expression during infection. As addressed before, inflammation is required for successful viral clearance. However, an exaggerated immune response can be harmful not only because it can lead to tissue damage and exaggerated mucus production that leads to airway obstruction, but also because an improper immune response can be associated with increased viral loads and dissemination of the infection. Our study has laid the foundation for future prospective research designed to better understand the complex mechanism of severe RSV infection and better design effective treatments able to modulate infection and lead to a proper control of the inflammation.

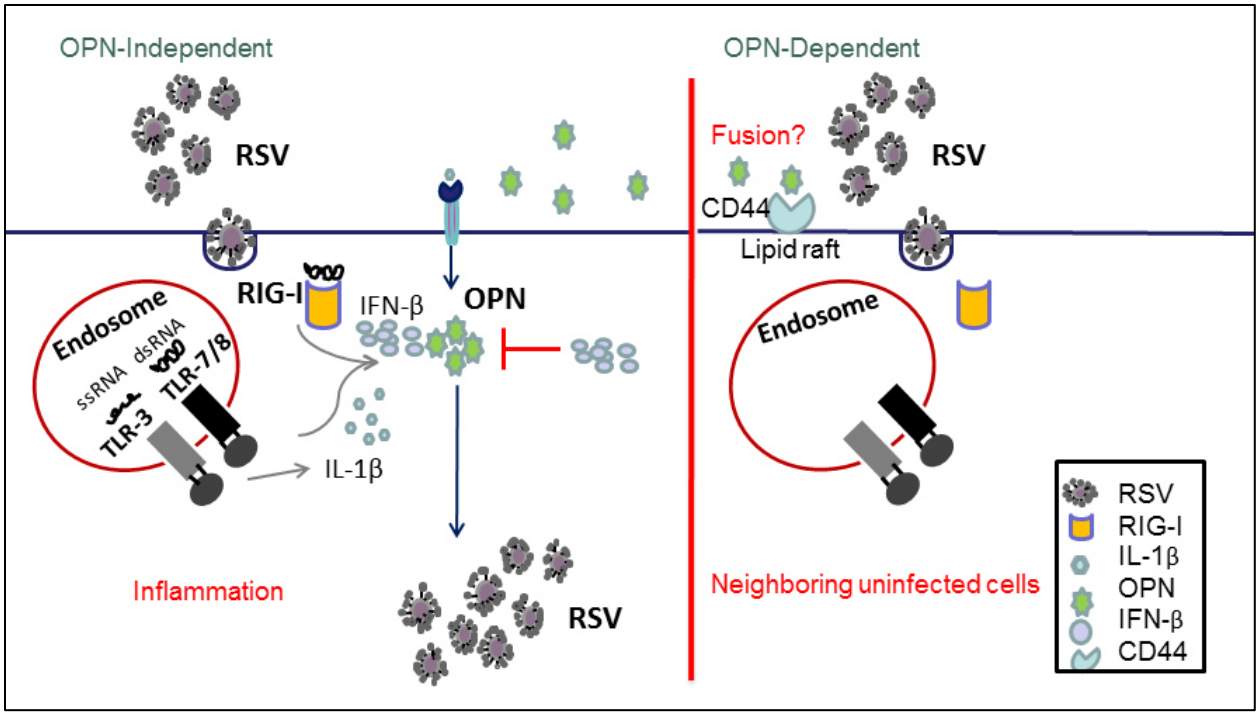

Figure 13. Model for the role of OPN during RSV infection. (Left panel) OPN-independent model. During RSV infection, the virus is recognized by the host cells through PRR (TLR-3, RIG-I, TLR-7); recognition of the virus leads to activation of the innate immune response which leads to release of anti-viral and pro-inflammatory cytokines like IFN-β and IL-1β. Expression of IL-1β leads to up-regulation of OPN whereas expression of IFN-β down-regulates OPN expression. (Right panel) OPN-dependent model. Fusion of RSV is accelerated in the presence of OPN and its receptor (CD44), thus leading to an increased number of infected cells, which results in increased viral loads.

APPENDIX A

IACUC APPROVAL FOR ANIMAL RESEARCH

USF UNIVERSITY OF SOUTH FLORIDA

DIVISION OF RESEARCH INTEGRITY AND COMPLIANCE
INSTITUTIONAL ANIMAL CARE_USE COMMITTEE

MEMORANDUM

TO:	Shyam Mohapatra, Ph.D. Dept. of Internal Medicine MDC019
FROM:	Jay B. Dean, Ph.D, Chairperson Institutional Animal Care & Use Committee Division of Research Integrity and Compliance
DATE:	5/21/2014
PROJECT TITLE:	Development of Nanotherapeutics for RSV-Induced Disease
AGENCY/SOURCE OF SUPPORT:	Department of Veterans Affairs
IACUC PROTOCOL#:	**V 4393**
PROTOCOL STATUS:	**APPROVED**

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC requested modifications/further information in response to that review and has received the required information. The IACUC **APPROVED** your request to use the following animals in your protocol for a one-year period **beginning 5/21/2014 :**

- 2304 Mice, male/female

ABOUT THE AUTHOR

Viviana Sampayo-Escobar was born in Barranquilla, Colombia. After attending the Universidad Del Norte (Barranquilla, Colombia), she received her Bachelor of Science in Microbiology (2006) and her Master of Science in Medical Sciences (2011). After participating in graduate research focusing on Respiratory Syncytial Virus at the Univerdidad Del Norte College of Medicine, she was awarded the Fulbright-Becas Caldas Doctoral Fellowship in 2011. After receiving her award, she moved to Tampa, FL to continue her graduate training as a Ph.D. student in the Integrated Biomedical Science program at the University of South Florida (USF). Viviana performed her research in the Department of Molecular Medicine under the mentorship of Dr. Shyam S. Mohapatra. While at USF, she earned her second Master of Science in Medical Sciences in the fall of 2014. During her doctoral studies, she published four co-authored manuscripts on Respiratory Syncytial Virus infection. While at USF, Viviana presented multiple posters at the annual USF Health Research Day and Veterans Affairs Research Day conferences, receiving the Outstanding Doctoral Student Poster Presentation in Allergy, Immunology, and Infectious Diseases (SIPAIID) award in 2017.