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Pneumonia cause by *Streptococcus pneumoniae* (*S. pneumoniae*) is prominent in the young and elderly. Our focus is improving prevention and controlling inflammatory responses during *S. pneumoniae* infection. Using a mouse model, studies tested the efficacy of nasal administration of a nanoparticle-based (NP) vaccine formulation to improve protection against *S. pneumoniae.* Studies also sought to determine the effect of nasal administration of corticotropin releasing hormone (CRH). Results demonstrated that CRH administration decreased mortality compared to Dexamethasone. CRH's effect was associated with significant decrease in inflammatory responses and its protective effect was observed in the absence of neutrophils. A NP based vaccine decreased bacterial growth in lungs correlating with increased IFNγ production. This research suggests efficacious applications of prevention and treatment of pneumococcal disease.

THERAPEUTIC APPROACHES IN THE COMBAT OF PNEUMOCOCCAL INFECTION

THESIS

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

CHAPTER I

INTRODUCTION

1.1. Significance of the Study

In 1985, Garibaldi reported respiratory infections to account for 75 million physician visits per year and an estimated $10 billion in United States (U.S.) health care costs[28]. While recent advances in medicine and public health policy have improved healthcare outcomes related to respiratory disease in the U.S. and worldwide [75], certain groups including the elderly, young and chronically ill remain burdened by high mortality rates [6, 11, 59, 66].

Pneumonia caused by infectious disease is the eighth leading cause of death in the U.S. [57]. Furthermore, individuals with pre-existing chronic respiratory illness (e.g. asthma, chronic obstructive pulmonary disease) are at higher risk for contracting pneumonia. To improve health outcomes, health care providers and biomedical researchers believe that a multi-pronged approach that includes proactive vaccination programs and increased innovation in the development of treatment therapies is needed to confront this health problem [26, 38].

Our laboratory is currently focused on enhancing approaches to optimize immune defense along the respiratory tract. We are particularly interested in identifying novel targets with the potential to mediate adverse cellular immune and inflammatory responses associated

with severe pneumonia. Research has shown that uncontrolled immune and inflammatory responses are a significant contributor to increased mortality risk among pneumonia patients. We are also interested in improving vaccines against respiratory pneumonia caused by *Streptococcus pneumoniae* (*S. pneumoniae)* infection. These areas of research are of critical need despite the availability of a pneumococcal vaccine and the continued risks of pneumonia among immune compromised individuals (e.g. young, elderly and chronically ill).

To begin to address critical areas related to combatting respiratory pneumonia, this research addressed two independent but related topics central to the prevention and improved clinical outcomes associated with respiratory pneumonia caused by *S. pneumoniae*. Specifically, studies were designed to: 1) *Determine the role of corticotropin releasing hormone (CRH), a neuropeptide, in mediating cellular inflammatory responses associated with respiratory S. pneumoniae infection and 2) Test the efficacy of a nanotechnology-based nasal vaccine delivery approach to improve protection against S. pneumoniae.* The subsections that follow provide an overview of the epidemiology and etiology of *S. pneumoniae,* the immunological responses that mediate host defenses, adjuvant treatment modalities used to control immune and inflammatory responses, and immune-based vaccine delivery systems in the prevention of pneumococcal infection.

1.2. *S. pneumoniae* is a major human pathogen

Respiratory pneumonia caused by *S. pneumoniae* is responsible for most pneumonia-related deaths in the U.S. and worldwide among the elderly and other vulnerable populations[12, 26, 76]. *S. pneumoniae* is a gram-positive microorganism (see figure 1). Phenotypically, *S.*

pneumoniae ranges in size from 0.5 to 2 micrometers, is circular (cocci) in shape and grows in chains (streptococcus). As a gram-positive microbe, *S. pneumoniae* contains a cell wall consisting of carbohydrate, lipopolysaccharide constituents, and teichoic acid moieties with antigenic and non-antigenic properties[7]. *S. pneumoniae* is a semi-motile biofilm producer and exists as a facultative anaerobe, allowing it to thrive in low-oxygen environments within lower respiratory airways of the host[17].

S. pneumoniae consists of over 90 known serotypes; many of which colonize the upper respiratory airways (nasal passages). Notably, most serotypes that colonize the upper respiratory tract reside as non-pathogenic commensals. However, certain serotypes have been defined as highly invasive and causative of severe pneumonia. Depending on its invasive phenotype as well as host immune competency, pathogenicity of *S. pneumoniae* results from the translocation of resident asymptomatic *S. pneumoniae* occupying the nasal passages into the sterile confines of the lower respiratory airways and other susceptible tissues (inner ear, cerebral spinal fluid, respiratory sinuses and blood) [66].

Despite a significant reduction in pneumococcal-associated mortalities because of the introduction of the polypeptide and conjugate vaccines, the incidence of pneumococcal disease remains significant. The World Health Organization (WHO) reports that 1.6 million deaths occur each year with significant mortality and hospitalization rates [22]. In 2012, Jinno et al, reported that the cost for patient treatment and hospitalization ranged between $11,000 and $51,000 [38]. In addition, recent evidence suggests that the emergence of virulent serotypes not covered by current vaccines and the notable rise in antibiotic resistance is also to blame [6, 64, 80, 85].

Antibiotics are the standard treatment given to those that develop infectious pneumonia, due to *S. pneumoniae*. In addition, glucocorticoids are usually given as an adjunctive therapy to

suppress excessive inflammation caused by ineffective antibiotic-mediated bacterial clearance. However, because of an emergence of bacterial resistance and complications to short-term and long-term glucocorticoid use, combating pulmonary pneumonias remains a challenge [26]. Thus, without novel approaches to prevent infectious pneumonia as well as advanced therapies to manage and treat pneumococcal infections, respiratory disease will continue to burden healthcare systems in the U.S and globally.

1.3. Mechanisms of host defense against *S. pneumoniae*

The mucosal lining of the respiratory tract constitutes a complex barrier system to prevent the translocation and subsequent colonization of potential pathogens. Here, we summarize four major functional barrier systems including: mucociliary, antimicrobial peptide, pathogen pattern recognition receptors, and the cellular immune response.

a. Mucociliary Responses

The mucociliary response, commonly known as the mucociliary escalator, is the mechanism by which bronchi cleanses itself from foreign pathogens. This component of host defense is an important barrier against infection composed of two aspects, the cilia and mucus. Cilia are hair-like structures that transport mucus in a sweeping manner up and down the throat catching any foreign pathogens within the mucus and preventing their passage to the bronchi. Impairment of mucociliary function could be a contributing factor in the translocation of *S. pneumoniae* into the confines of the lower respiratory airways.

b. Antimicrobial Peptides

Antimicrobial peptides are proteins that behave as an antimicrobial against bacteria, viruses, and fungi. The activity of antimicrobial peptides is to protect the host against bacterial invasion. These peptides kill bacteria in varying ways, such as disruption of membranes, interference with metabolism, and targeting of the cytoplasm. However, because of the capsule expressed within the membrane of *S. pneumoniae* species, antigen recognition is masked which decreases the potential binding of antimicrobial peptides to its surface, reducing killing activity.

c. Pattern Recognition Receptors

Pattern recognition receptors (PRR) recognize distinct patterns on the surface of pathogens which is pertinent in the initiation of the innate immune response. Upon invasion, *S. pneumoniae* is recognized by airway epithelia as well as alveolar macrophages by a class of extracellular and intracellular pattern recognition receptors (PRR) known as Toll-like receptors (TLRs)[38]. TLRs important to pneumococcal infection include TLR-2, which recognizes the peptidoglycan moiety of the cell wall, and TLR-4, which recognizes the pneumolysin toxin that is specific to *S. pneumoniae*[73]. Following recognition of the pathogen by the TLRs, a series of events occurs causing activation of lung epithelial surfaces, leading to downstream activation of NF-κB and subsequent activation of cytokine signaling pathways resulting in the production of pro-inflammatory cytokines e.g. IL-1, IL-18, and TNF-α[77]. Consequently, events including cellular immune recruitment and activation of innate and adaptive cellular immune responses occur with the objective of facilitating anti-microbial reaction.

d. Cellular Immune Responses

Innate and adaptive cellular immune responses play a critical role in determining the outcome of pneumococcal infections. Innate immunity occurs immediately and is non-specific in its response to foreign pathogens. This occurs during the early phase of invasion, the first 3 days or sooner. Antigen-specific responses (e.g. acquired immunity) are mediated by the adaptive cellular immune response. Adaptive immune responses involve the recognition, processing and presentation of antigenic peptides of pathogens by specialized cells which in turn elicit the activation of antigen specific effector cells (e.g. T cells and B cells). Adaptive responses against bacterial pathogens are induced between 7-10 days and generate long-lasting immunity against the inciting pathogen.

The cellular constituents of the innate immune response are many including: mast cells, basophils, natural killer cells, dendritic cells, neutrophils and macrophages. Neutrophils and macrophages however, play a prominent role in the innate defense against pneumococcal disease [77, 80]. These innate immune cells have a direct functional response against *S. pneumoniae* exhibited by their ability to phagocytize and produce toxic levels of reactive oxygen species (ROS), leading to pathogenic destruction. In addition, macrophages and neutrophils can coordinate innate and adaptive immune responses against *S. pneumoniae* through cytokine and chemokine production. This amplifies the recruitment and activation of other cellular immune and non-immune constituents [52]. Due to their potent phenotype, neutrophils and macrophages are considered a “double-edged sword”. While their robust activation is absolutely required to defend against pathogen invasion, uncontrolled activation can lead to harmful, self-inflicted pathogenesis. As introduced in the sections that follow, therapeutic strategies which manage the

aberrant activation of neutrophils and macrophages are critical in reducing the risk of severe respiratory pneumonia that may cause death.

Adaptive immunity is an acquired/pathogen-specific response that typically is induced beyond 3 days following the activation of the innate immune response. The mechanism of activation is a result of the recognition, processing and presentation of antigenic peptides by antigen presenting immune cells (e.g. dendritic cells, macrophages and B cells). $CD4^+$ T cells are the major subset of adaptive immune cells responsible for protection against extracellular bacterial pathogens such as *S. pneumoniae*. $CD4^+$ T cells are typically activated from the presentation of antigen (*S. pneumoniae*) by macrophages or dendritic cells. This leads to the differentiation and activation of a T helper (Th) cellular subpopulation (e.g. Th1, Th2, Th17) that aid in combat of infection through cytokine secretion and support of cell-mediated and humoral antibody defenses. Specifically, during pneumococcal infection Th17 cells produce cytokines such as IL-17A and IL-22 enabling cellular recruitment to the site of infection as well as releasing antimicrobials from local tissue [20]. $CD4^+$ T lymphocytes' release of cytokines also mediate protective humoral responses produced by the secretion of antigen-specific antibodies by activated plasma B cells[58]. Based on this understanding, researchers are investigating potential pneumococcal-associated antigens aimed at developing novel vaccine strategies that preferentially induce $CD4^+$ T cell and humoral B cell responses [43, 45].

1.4 Current treatments used to combat *S. pneumoniae* infection

a. Antibiotics

Penicillin was the first modern antibiotic that was discovered by Alexander Fleming in 1928 and was used for the treatment of syphilis, gangrene, and tuberculosis [31]. Antibiotics are a type of antimicrobial drug that is used in the treatment and prevention of bacterial infections. Different classes of antibiotics include: β-Lactams, tetracylines, aminoglycosides, and cyclic peptides [25]. Various antibiotics used in the treatment of *S. pneumoniae* infection are listed below (Table 1). Antibiotics behave by either killing or inhibiting the growth of bacteria. Bacteriostatic antibiotics are those that stop the growth of bacteria while bactericidal antibiotics kill the invading pathogen. Despite the beneficial use of antibiotics, bacterial pathogens have acquired various mechanisms of resistance. Antibiotic resistance can occur in many ways; however, the most common mechanism is through genetic mutation. These mutations result in bacterial organisms becoming resistant to the action of the antibiotics. When a pathogen such as *S. pneumoniae* becomes resistant to the activity of antibiotics, the inflammatory response becomes robust and uncontrolled. As a result, patients are commonly given an anti-inflammatory such as a glucocorticoid to yield this reaction.

b. Glucocorticoids

Preventing *S. pneumoniae* from colonizing the lower respiratory tract and preventing systemic disease requires robust activation of innate cellular immune and inflammatory responses [58]. However, adverse reactions mediated by highly activated immune responses brought on by severe *S. pneumoniae* infection can increase the risk of death by promoting sepsis.

Sepsis occurs when severe inflammation produces tissue damage allowing *S. pneumoniae* to disseminate (e.g. escape) from the initial site of infection into the blood (bacteremia). In such cases, abnormal inflammatory responses (e.g. cytokine storm), primarily caused by uncontrolled activation of neutrophils and monocytes, results in immune-mediated pathogenesis that can exacerbate disease and lead to severe pulmonary injury and even death [81]. This is commonly observed under circumstances of persistent infection often a result of ineffective antibiotic treatment and/or insufficient immune function.

The central nervous system's (CNS) control of cortisol secretion from the adrenal glands has a sentinel role in restoring homeostasis in response to infection. This occurs by inducing cortisol-mediated global suppression of immune and inflammatory responses. Knowledge of the physiological action of cortisol, an endogenous glucocorticoid, is why glucocorticoids are utilized to reduce inflammatory responses[69.] Based on glucocorticoid function, their pharmacologic use is a standard of care in the treatment of severe inflammatory pneumonia [2, 36, 37, 67, 76, 71, 72]. Glucocorticoids exert their immunosuppressive properties on both cells and its cellular environment (e.g. proteins, membranes, and organelles) [60]. They affect these cells by binding to the corresponding glucocorticoid receptor. Once these receptors are activated, this causes an increase in the expression of anti-inflammatory proteins while repressing the expression of pro-inflammatory proteins. This mechanism protects against extended tissue damage and restores homeostatic conditions.

The pharmacologic use of glucocorticoids also has certain drawbacks including increased risk to secondary infections caused by a non-specific immune suppression as well as off-target metabolic side effects [49]. Pertaining to its use in the treatment of bacterial pneumonia, controversy exists in their effectiveness in reducing mortality rates during *S. pneumoniae*

infection [48] [71, 72, 74, 79]. Specifically, Skrupky et al, states that no exclusive conclusions can be drawn from the utilization of glucocorticoids and the resolution of disease and/or sepsis [73]. In contrast, Gonzalez-Rey et al demonstrated that endogenous peptides, such as Urocortin, as promising therapeutics demonstrating reduced mortality [30]. Based on these and other reports, there remains a critical need to identify alternative approaches to glucocorticoid treatment to aid in the management of inflammation during severe pneumococcal infection.

Pharmacologic approaches that dampen excessive inflammatory responses without jeopardizing the host immune integrity are envisioned to be an optimal treatment paradigm. Given the significant role that neuropeptides play in regulation of cellular immune and inflammatory responses, a further identification of neuropeptides' cellular immune targets may reveal novel alternative treatments. As mentioned above, Urocortin was found to have beneficial effects in reducing mortality risks among septic patients. Urocortin is a homologue of corticotropin releasing hormone (CRH), a 41 amino acid neuropeptide released within the hypo-pituitary-axis (HPA) that controls central nervous system function. This hormone serves as an important mediator of adaptive physiological responses to stress (e.g. physical, psychological and environmental) by mediating downstream release of cortisol [18, 27, 42, 56]. Regulation by CRH is typically involved in controlling immune and inflammatory responses through cortisol, but has been found to have direct impact within inflamed tissues through binding of two cellular receptors, CRH receptor 1 (CRH-R1) and CRH receptor 2 (CRH-R2) [50, 65, 82]. CRH binds primarily to CRH-R1, while Urocortin binds exclusively to CRHR2. In addition to its role in inducing downstream cortisol release that in turn regulates immune suppression, CRH has also been found at peripheral sites of inflammation [8, 13, 21, 62, 82, 84, 87]. However, its mechanisms of action on cellular immune function remain unclear. Importantly, how CRH may impact

pulmonary immune defenses against bacterial infections (e.g. *S. pneumoniae*) is largely unknown.

Control of inflammatory responses is a critical determinant of disease outcome from *S. pneumoniae* infection. Specifically, understanding the mechanism of action of CRH on particular inflammatory cells could provide novel insight of how its use may prove effective in mediating cellular immune/inflammatory responses caused by severe bacterial infections. Previous studies have demonstrated the efficacy of CRH receptor antagonists (e.g. Antalarmin and Astressin2B) to manipulate CRH activity [14, 15, 16, 32]. We will take advantage of the CRH receptor 1 antagonist, Antalarmin, to address its role in mediating *S. pneumoniae* infection.

c. Vaccination

Protective immunity is a hallmark of public health. Infants, the elderly, and the immunocompromised are routinely vaccinated against pneumonia. Despite proactive vaccination programs, there remains a large percentage of the population not protected by the currently available conjugate and poly-valent vaccines. The significance of this research is underscored by the large morbidity and mortality associated with Community-acquired pneumonia (CAP) worldwide despite advances in vaccination [39].

i) Conjugate and Poly-valent vaccines: The current pneumococcal vaccines are the Pneumococcal Conjugate Vaccine, known as the PCV13 and the Pneumococcal Polysaccharide Vaccine, known as the PPSV. PCV13 is intended for young children protecting against the most serious types of infection including meningitis, pneumonia, and bacteremia. The PPSV23 is intended for the elderly preventing invasive infections including meningitis and bacteremia. The numerical value in the titles of these vaccines indicates the different serotypes of

S. pneumoniae covered by these vaccines. Specifically, PCV 13 covers 13 serotypes while the PPSV23 covers 23. Although conjugate and polysaccharide vaccines have been central to the prevention of *S. pneumoniae*-induced CAP, the success of vaccination particularly among the elderly has been less than expected due to a status quo in mortality rates [11]. This lack in vaccine efficacy is believed to be attributed to less than optimal immune responses generated against current conjugate and polysaccharide vaccine constructs [36]. Several explanations for their ineffectiveness likely include the premature immune system of the young and the age-associated decline of immune system function in the elderly [61, 68].

d. Delivery Systems to improve vaccination

Nanotechnology-based vaccine constructs: Nanotechnology is emerging as a feasible approach to target activation of immune responses and promises to be an effective strategy to overcome the current limitations associated with vaccines [37, 71]. Recently it has been shown that nanoparticle (NP) constructs provide a vehicle to carry specific antigenic payloads as well as target immune responses by functionizing NP surfaces through antibody-surface coating. Therefore, affording the ability to target the activation of robust antigen-specific and lasting immune response against various pathogens [55]. Several types of NPs are utilized for drug delivery including: liposomes, silica (magnetic NPs), solid lipid NPs, polymeric NPs, dendrimers, and carbon NPs [83].

Given the promise of NP technology, interest has been raised in determining its effectiveness in the prevention of respiratory infections [9, 35]. Specifically, an approach which targets the local respiratory tract could be advantageous in reducing pneumonia caused by *S.*

pneumoniae. To date, various studies have evaluated the efficacy of intranasal vaccine delivery systems. However, there is a further need to characterize experimental approaches using Poly(lactic-co-glycolic acid) or PLGA NP technology. We envision that a reduction in CAP-related deaths will be difficult to realize without improved strategies to augment immunity. We believe that targeted delivery of vaccines across the respiratory tract, the primary site of bacterial pneumonia, is crucial to this strategy. Utilizing this therapeutic drug delivery technique, we investigated an immunization strategy using heat-killed *S. pneumoniae* antigen (Ag) and PLGA NPs (Figure 2). The use of PLGA nanoparticles for targeting immune therapies provides numerous advantages including: biocompatibility, biodegradability, size specificity, composition, and ease of production.

1.5 The Study

This research, demonstrated in our published papers, addressed two independent but related topics central to the prevention and improved clinical outcomes associated with pneumococcal disease. Herein, studies were designed to: 1) *Determine the role of corticotropin releasing hormone (CRH) in mediating cellular inflammatory responses associated with S. pneumoniae infection and 2) Test the efficacy of a nanotechnology-based nasal vaccine delivery approach to improve protection against S. pneumoniae.*

a. HYPOTHESIS

I hypothesized that CRH administered to the respiratory tract will increase both survival and cellular anti-inflammatory responses during *S. pneumoniae* infection in the lungs through the CRH receptor 1 pathway. This is contrasted with the current glucocorticoid therapy which

globally suppresses the immune system, allowing for possible secondary infections. Positive results could potentially lead to an innovative and novel regimen for physicians.

Additionally, I hypothesized that an intranasal nanoparticle-based delivery system would enhance protection against pneumococcal infection by enhancing cell-mediated immune responses.

b. SPECIFIC AIMS

The hypotheses were addressed, using a CD-1 mouse model, with the following objectives:

Specific Aim 1: To determine the relationship of intranasal administration of CRH and CRH receptor 1 during *S. pneumoniae* infection in the lung.

Specific Aim 2: To determine the efficacy of intranasally administrated PLGA nanoparticles encapsulated with *S. pneumoniae*-whole antigen in the lung.

c. RATIONALE

Excessive inflammation is believed to be a major contributing factor of mortality risk caused by pneumococcal infection. Under such circumstances, glucocorticoids are commonly used as adjuvant treatment in attempt to suppress overt inflammation in the presence of antibiotics. The expected benefit of glucocorticoid treatment is to reduce inadvertent tissue damage by suppression of immune-mediated inflammatory responses. However, systemic immune suppression by glucocorticoids can also lead to secondary infections among susceptible individuals. The development of approaches that can tailor immune and inflammatory responses

without overt suppression during uncontrolled infection may hold promise in reducing mortality due to *S. pneumoniae*-induced sepsis.

Previous findings and our own published studies, support the overarching hypothesis that CRH, as a known mediator of inflammatory responses in peripheral tissues, is a plausible target for tailoring immune and inflammatory responses [15, 34, 86]. For example, studies by Radulovic et al, demonstrated the role of CRH-R1 activity to mediate inflammatory response associated with the exacerbation of a murine model of colitis [63]. Additional studies have demonstrated the potential of CRH and CRH receptor expression to mediate rheumatoid arthritis and asthma [13, 21, 84]. Most relevant to the current study, Kim et al demonstrated a unique dichotomous relationship between CRH-R1 and CRH-R2 activation associated with the susceptibility to *S. pneumoniae* given external aversive stress (e.g. physical restraint). The findings of Kim et al showed that antagonizing CRH-R1 decreased host survival while CRH-R2 antagonism promoted host survival during *S. pneumoniae* infection [44]. Such findings provide significant evidence of CRH's role in mediating cellular immune and inflammatory responses.

A number of unresolved questions exist regarding the mechanisms through which CRH may be mediating immune and inflammatory responses. For example, the studies by Kim et al involved the use of a "stress paradigm" in which mice underwent consecutive days of restraint stress to understand the role of the central nervous system in regulation of pulmonary immune defenses. Therefore, the results were difficult to distinguish between the effect of the aversive stress and that of the stress caused by infection as contributing to the role of CRH and its receptor activity. In addition, previous studies have yet to define the role of CRH and its receptors on specific immune cell types and their function. Furthermore, the local effect that CRH has on cellular and inflammatory response in the lungs during *S. pneumoniae* infection

remains unknown. The studies described in Chapter II begin to address these questions, demonstrating the importance of CRH-R1 activity in mediating survival and its influence on pulmonary cellular immune inflammatory responses.

The availability of vaccines has led to a significant reduction in pneumococcal disease worldwide [54]. These vaccines have proven to be very successful in preventing disease by most invasive serotypes of *S. pneumoniae* species [10, 51]. Epidemiological studies however, are beginning to reveal an emergence of *S. pneumoniae* serotypes not covered by current vaccines to be the cause of invasive pneumococcal disease [5]. In addition, a high mortality rate still exists among previously immunized populations (e.g. elderly and chronically ill). These findings signify a further need to improve vaccine treatments to prevent the contraction of pneumococcal infection for those high-risk populations.

Targeting the lung by nasal delivery techniques offers promise for improved protection against respiratory pathogens including *S. pneumoniae*. Ongoing studies demonstrate the efficacy of intranasal vaccines [23, 24]. These studies support the idea that targeting resident cellular immune responses along the respiratory tract could promote increased and sustained immune protection compared to current systemically administered vaccines [53]. Specifically, literature shows that nasal delivery may be ideal when rapid onset of action is needed due to their high permeability and highly absorptive surface area [40].

Nanoparticle technologies have emerged with significant promise in the development of novel vaccine delivery systems [4]. PLGA NPs offer many advantages for vaccine delivery systems including: their ease of production [1], their bioavailability [3] and ability to encapsulate whole *S. pneumoniae* antigen (lysate) due to their negative charge [19] compared to other nanoparticle composites [48]. Our rationale is that by utilizing such an ideal delivery system, NP

technology will target antigen more efficiently across the respiratory airways resulting in heightened and sustained immune response, resulting in greater protection against *S. pneumoniae.*

Overall, the rationale for the studies described in the following chapters is two-fold. First, studies in Chapter II sought to elucidate the role of CRH and CRH-R1 in mediating cellular immune and inflammatory responses associated with *S. pneumoniae* infection. We proposed to manipulate them as a potential novel approach to tailor immune and inflammatory immune responses in order to overcome overt suppression that occurs with glucocorticoid use. Second, the studies in Chapter III tested the efficacy of nasal delivery utilizing a nanoparticle-based vaccine as an advanced approach to increase protective immune responses against pneumococcal infection that may be useful for at risk populations with compromised immune systems.

Figure 1.

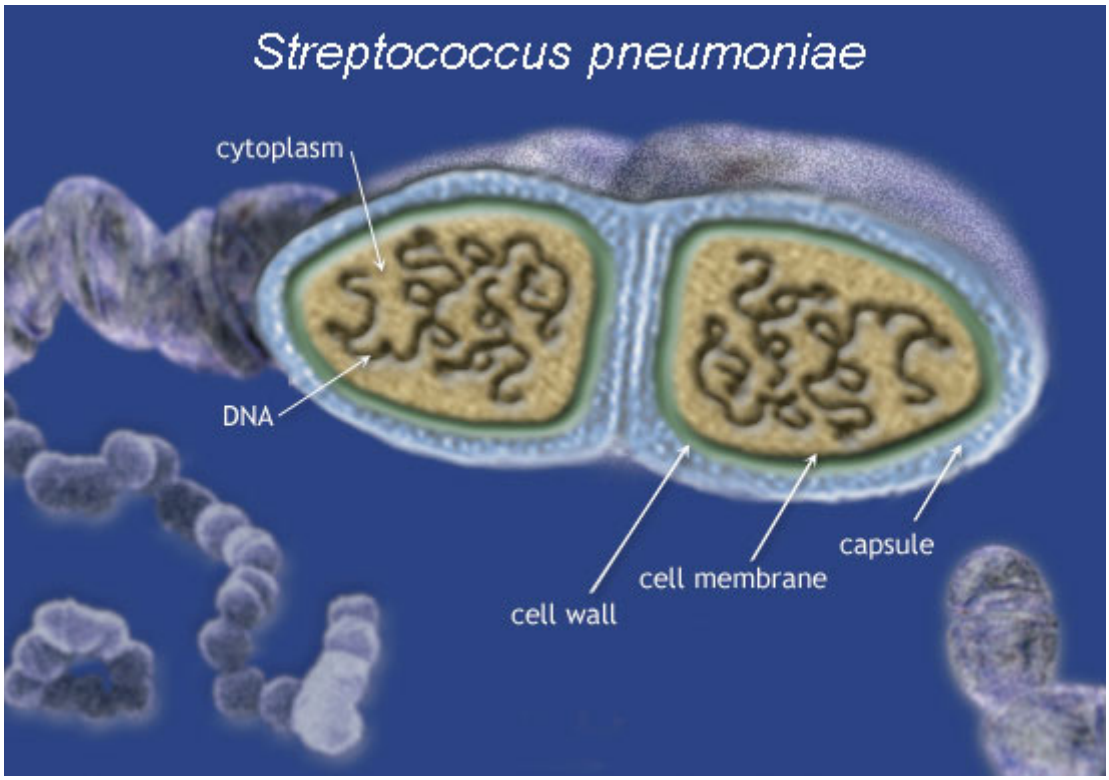

Figure 1: ***Streptococcus Pneumoniae;*** **Courtesy of chori.org**

Table 1.

ANTIBIOTICS TO BE USED FOR SUSCEPTIBLE PNEUMOCOCCAL BACTERIA	CLASSIFICATION OF ANTIBIOTIC	TARGET
Penicillin (Ampicillin and Amoxicillin)	B-Lactam	Cell Wall
Cephaloporins I,II	B-Lactam	Cell Wall
Erythromycin	Macrolides	Protein Synthesis
ANTIBIOTICS TO BE USED FOR PENICILLIN RESISTANT BACTERIA		
Cephalosporins III (ex. Cefotaxime, ceftriaxone)	B-Lactam	Cell Wall
ALTERNATIVE ANTIBIOTICS		
Vancomycin	Cyclic Peptide	Cell Wall

Table 1: Antibiotics specific to treatment for *S. pneumoniae* infection.

Table 1: Antibiotics Utilized for *S. pneumoniae* Treatment

Figure 2.

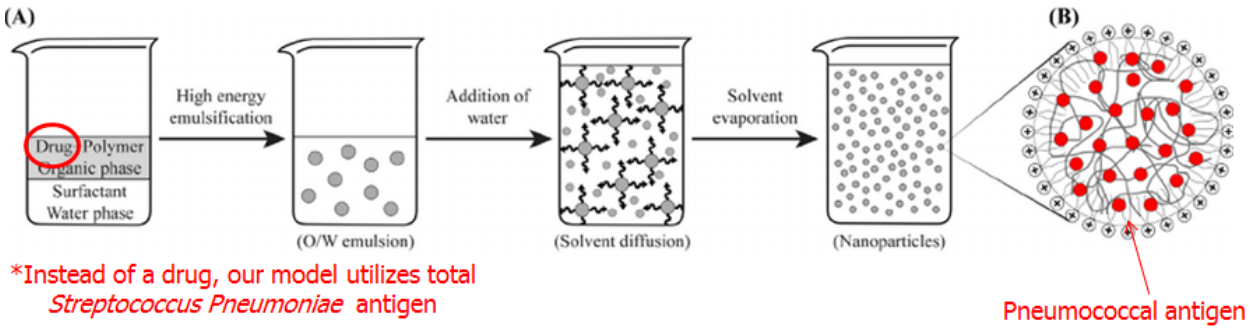

Figure 2: Schematic of the production of PLGA nanoparticles [33].

CHAPTER II

CORTICOTROPIN RELEASING HORMONE IMPROVES SURVIVAL IN PNEUMOCOCCAL PNEUMONIA BY REDUCING PULMONARY INFLAMMATION

INTRODUCTION

The use of glucocorticoids to reduce inflammatory responses is largely based on knowledge of the physiological action of the endogenous glucocorticoid, cortisol. Glucocorticoids can exert their immunosuppressive properties on both cells and its cellular environment (e.g. proteins, membranes, and organelles) (11). In response to infection, cortisol secretion from the adrenal glands acts in part as a global suppressor of immune and inflammatory responses. This mechanism protects against extended tissue damage and restores homeostatic conditions. However, its use can also result in an increased risk to secondary infections caused by a non-specific immune suppression.

Cortisol is a human glucocorticoid whose release is mediated by corticotropin releasing hormone (CRH). CRH is a neuropeptide released from the hypothalamic-pituitary-adrenal (HPA)

axis of the central nervous system. This hormone serves as an important mediator of adaptive physiological responses to stress (e.g. physical, psychological and environmental). In addition to its role in inducing downstream cortisol release that in turn regulates immune suppression, CRH has also been found at peripheral sites of inflammation (41, 17, 45). Previous findings suggest that CRH, through its ligation to its receptors, can modulate cellular immune and inflammatory responses (12, 46, 51, 61). However, its mechanisms of action on cellular immune function remain unclear. Importantly, few studies have demonstrated how CRH may impact innate pulmonary immune defenses against bacterial infections (e.g. *Streptococcus. pneumoniae*) (19, 29, 35). Determining the mechanism of action of CRH on particular inflammatory cells will provide a novel understanding of mechanisms mediating cellular immune inflammatory responses caused by severe bacterial infections.

Streptococcus pneumoniae (*S. pneumoniae*) is a microorganism commonly present among the commensal microflora along the upper respiratory tract. Among the 92 known serotypes of *S. pneumoniae*, few are considered pathogenic. Rather, transmission of disease stems from the resident asymptomatic pneumococcus along the nasal passages. Still, a majority of deaths are due to complications from respiratory pneumonia caused by *S. pneumoniae* (13, 28, 44). For many years routine vaccination, antibiotic use, and adjunctive therapies have proven efficacious in certain settings and for certain groups. For example, immunization programs have proven successful in reducing pneumonia and are predicted to reduce child mortality by 30% by 2015 globally (6,16). However, the emergence of virulent serotypes not covered by current vaccines and the notable rise in antibiotic resistance raises concern for increased mortality risk caused by *S. pneumoniae*.

Individuals particularly at risks include, the very young (<6 months to 3 years), the elderly (> 65 years), and individuals with chronic disease (e.g. emphysema, chronic obstructive pulmonary disease, immunodeficiency) (2, 50). The risk associated with these groups is believed to be in part due to their lack of immune competency. During the early stages of *S. pneumoniae* infection, robust innate cellular immune and inflammatory responses play a critical role in eradicating *S. pneumoniae* from the lower-respiratory tract as the host's response in preventing persistent infection and disseminating systemic disease. However, under conditions whereby the host immune response fails to clear the ensuing infection, immune-mediated inflammatory responses can have detrimental effects, particularly where antibiotic treatment is also ineffective. To avoid such outcomes, suppression of inflammatory responses through glucocorticoids is used as an adjunctive therapy during severe infection (40, 64). Dexamethasone is a common adjuvant therapy used to reduce inflammatory responses in patients with bacterial pneumonia (1, 25, 26, 59). The purpose of suppressing inflammatory responses during severe infection is to avoid tissue damage, leading to both sepsis and death. *S. pneumoniae*-associated sepsis occurs by dissemination from the initial site of infection (e.g. the lung) due to tissue injury caused by severe inflammatory reactions, leading to bacteremia in blood. Although dexamethasone is commonly used in cases of severe bacterial infections (e.g. meningitis, community-acquired pneumonia), controversy exists in its effectiveness in reducing mortality rates during *S. pneumoniae* infection (36, 44, 47, 48). This paper will determine the role of two CRH and dexamethasone in mediating mortality, and how they influence inflammatory responses during pneumococcal infection.

MATERIALS AND METHODS

Animals

Adult (6–8 weeks of age) female CD-1 mice (Harlan Sprague–Dawley, Indianapolis, Indiana) were used in all studies. Mice were maintained under specific pathogen-free conditions on a 12:12 light/dark cycle (7:00 p.m. –7:00 a.m.). Mice were kept under optimal temperature and humidity controlled conditions. The University of North Texas Health Science Center's Institutional Animal Care and Use Committee (IACUC) approved these studies.

Intranasal infection and administration of pharmacologic agents

S. pneumoniae strain #6301 (ATCC, Manassas, VA) was grown for 16 hours (hr) to obtain mid-log phase cultures on blood agar plates (Thermo Fisher Scientific, Lenexa, KS). Mice were infected with 1 x 10^5 colony forming units (CFUs) (LD_{50}) of *S. pneumoniae* strain #6301 (ATCC, Manassas, VA) by intranasal route in a volume of 40 microliters (µl) of Brain–Heart Infusion Broth (EMD, EMD Chemicals Inc. Gibbtown, NJ) or broth (e.g., sham infection) after anesthesia (100-150 µl ketamine/xylazine intraperitoneally).

Human Corticotropin Releasing Hormone (Sigma-Aldrich, St. Louis, MO), antalarmin (Sigma-Aldrich), and dexamethasone (Sigma-Aldrich). Optimal doses of CRH (1mg/kg), antalarmin (1mg/kg) and dexamethasone (1mg/kg) were administered by intranasal route based on previous published results (29)

Brochoalveolar lavage fluid (BALF) Isolation

BALF was prepared by intratracheal perfusion of 1 ml of sterile 1x Phosphate Buffered Saline (PBS) solution using a 25 gauge (G) blunt-end needle. After removing cells by centrifugation, the total number of leukocytes were collected from BALF. BALF cells were quantified by viable cell trypan blue staining and hemocytometer techniques using light microscopy under 40X magnification. The non-cellular BALF samples were stored at ˉ80° C until analysis.

CXCL1 chemokine detection in the BALF

CXCL1 chemokine and IL-17A production was determined by sandwich ELISA method from BALF. All procedures were performed as described by the manufacturer (R&D Systems Inc. Minneapolis, MN). Briefly, flat-bottomed 96-well plates were coated with an optimal titration of capture antibody followed by overnight blocking using 10 % FBS in PBS to deter non-specific binding. After incubation of samples at 4˚C for 16 hr, plates were incubated with biotin-conjugated detection antibody and streptavidin-HRP (horseradish peroxidase). Tetramethylbenzidine (TMB) peroxidase substrate solution (Rockland Immunochemicals, Inc. Gilbertsville, PA) was added to each well for colorimetric determination of concentration of each cytokine according to standard curve generated by reference concentration of cytokine at wavelength of 450 nm detected by colorimetric plate reader (Bioteck Instruments Inc. Winooski, VT). ELISA antibody set and recombinant cytokine and for standard were purchased from R&D Systems (R&D Systems Inc. Minneapolis, MN).

Neutrophil Depletion

24 hr prior to infection, all treatment groups were administered a single injection of 0.5 milligrams (mg) of 1A8 depletion antibody (Bio X cell, West Lebanon, NH) in a 200 µl volume intraperitoneally. The depletion antibody was prepared in 1x PBS immediately preceding administration to animals. Efficiency of neutrophil depletion was confirmed by flow cytometry using LY6G antibody labeling of lung leukocytes.

Statistical analysis

Statistical analysis was performed using GraphPad Prism Version 5.0p (GraphPad Software, San Diego, USA) and Stata 14 (StataCorp LP, College Station, USA). Log-rank (Mantel-Cox) test was used for survival analysis. For multi-experimental group analysis, data were subjected to one-way ANOVA (analysis of variance). Rodger's method was utilized for post-hoc analysis. For two-group comparisons, two-sample proportion test were used. All data are expressed as mean ± standard error of mean (SEM). The two-tailed level of significance was set to at a p value of $p \leq 0.05$ for group difference.

RESULTS

Reducing bacterial burden within a patient diagnosed with pneumococcal pneumonia is the main objective when determining treatment. However, during severe infection, the inability to clear infections due to deficient immune responses and ineffective antimicrobial treatment can

lead to unwanted inflammatory reactions that require adjunctive anti-inflammatory therapy. Under such circumstances, dexamethasone is often used as an adjuvant yet evidence suggests its use has not reduced mortality rates among pneumonia patients caused by infection (13, 17, 33). Our laboratory has previously demonstrated that intraperitoneal (IP) administration of antalarmin, a CRH-R1 specific antagonist reduces survival using a model of aversive stress plus *S. pneumoniae* infection (29). Though changes in disease outcome were seen when antagonists were administered IP, no studies have investigated how targeting CRH and CRH-R1 activity in the respiratory tract impacts disease outcome. Using an experimental model of murine respiratory *S. pneumoniae* infection, we compared sham-treated infected mice to experimental groups of mice receiving intranasal administration of CRH, a CRH-R1 selective antagonist (antalarmin) or Dexamethasone (**Fig 1)**. Dexamethasone is the pharmacologic glucocorticoid analogue currently used clinically (7, 30, 34).

Initial studies investigated the time-associated effect of CRH administration during pneumococcal infection. **Fig 2** shows the results of a survival study where groups were administered treatment at 9 (A), 18 (B), or 30 (C) hr, following infection. As shown in **fig 2A**, survival was greatest among untreated mice. However, no significant differences in mortality rates were observed between experimental groups compared to sham-treated mice. In contrast, mice administered CRH 18 hr after infection resulted in a significantly higher survivorship compared to all experimental groups. Interestingly, intranasal administration of antalamin significantly reduced the protective effect of CRH, suggesting that endogenous ligation of CRH to its cognate receptor, CRH-R1 could be mediating responses during pneumococcal infection. (**Fig 2B**). No significant differences in outcome were found between experimental groups at 30 hr. (**Fig 2C**).

We next tested the hypothesis that survival is predicted by the type of cellular inflammatory response produced during infection given intranasal administration of CRH. We determined the leukocyte response to respiratory *S. pneumoniae* infection by enumerating the total leukocyte numbers in the BALF given intranasal administration of CRH, antalarmin or dexamethasone. Mice administered CRH demonstrated a significant decrease in total leukocyte numbers compared to sham-treated mice as well as mice administered dexamethasone. Accordingly, administration of antalarmin led to a significantly higher increase in the total leukocyte count compared to CRH and infection alone. Thus, affirming endogenous CRH ligation to CRH-R1 as a mechanism of action (**Fig 3**).

The above results suggest that CRH through triggering of CRH-R1 could be a mechanism controlling cellular recruitment. CXCL1 is a chemokine released after the activation of epithelial cells and has been shown to preferentially lead to the recruitment of monocyte populations during pulmonary infection. We therefore determined how CRH and blocking its receptor would impact CXCL1 production along the respiratory airways. We found significantly lower levels of CXCL1 in the BALF among mice given CRH. Importantly, administration of the CRH-R1 selective antagonist antalarmin, significantly increased CXCL1 levels below that found in the BALF of infected untreated mice and mice receiving Dexamethasone (**Fig 4**). These results demonstrate a relationship between CXCL1-mediated neutrophil/monocyte recruitment and a CRH/CRH-R1 mode of action. IL-17A is also an important mediator of monocyte (e.g. macrophages and neutrophils) recruitment along the respiratory tract (5, 67). However, IL-17A was not detected in the BALF among all experimental groups (data not shown).

Neutrophils and monocyte/macrophage cells play a major role in mediating respiratory bacterial infections including *S. pneumoniae* (10, 62). Most intriguing of these immune cell-

types are their complex functional roles including the induction of potent pro-inflammatory and oxidative stress responses as well as their capacity to mediate anti-inflammatory responses involved in disease resolution (55, 66). We have previously demonstrated a role for CRH in modulating leukocyte responses in lungs given restraint stress and *S. pneumoniae* infection (29). Specifically, those findings demonstrated divergent effects of CRH receptor antagonism on the influence of neutrophil and monocyte/macrophage infiltration to the lungs in response to infection. Due to their significant involvement in mediation pneumococcal infection, neutrophil depletion studies were performed to ascertain the potential relationship between CRH and neutrophil-mediated responses associated with disease outcome. **Fig 5** demonstrated that survival could be significantly increased by CRH administration in immunocompetent mice. Interestingly, CRH administration in the absence of neutrophils resulted in the highest survival rate compared to all experimental conditions. This suggests that CRH's mode of action on neutrophil function may in part be a determinant of mortality risks (**Fig 5**).

DISCUSSION

CRH is produced by the HPA. Its ability to influence immune function is commonly associated with the release of Adrenal Corticotropic Hormone (ACTH) from the adrenal glands, resulting in cortisol release that in turn translates into non-specific immune suppression (44). Alternatively, CRH is also secreted in peripheral tissues (e.g. synovial tissue, gastrointestinal tract, placenta), where it is believed to modulate cellular immune and inflammatory responses through preferences in CRH receptor 1 and 2 activity (37, 68, 31). To date, few studies have defined the role of CRH in the regulation of pulmonary cellular immune and inflammatory responses (36). A previous study also demonstrated that preferences in CRH receptor expression could be associated with asthma severity (14). We have previously determined that

intraperitoneal injection of CRH receptor antagonists can modulate severity of pneumococcal pneumonia in the presence of aversive stress. The results from this study demonstrate for the first time, the protective effect of intranasal CRH administration through modulation of cellular inflammatory responses across the respiratory tract.

S. pneumoniae is the leading causative agent of community-acquired pneumonia worldwide and is responsible for the highest mortality rates among the elderly, young and immunocompromised (13). In addition to antibiotic resistance, aberrant immune and inflammatory responses are believed to be a key determinant of disease outcome (11). Specifically, studies have documented uncontrolled inflammatory reactions produced by neutrophils and monocytes to be active participants in exacerbated responses in an effort to eradicate *S. pneumoniae* infection (38). We hypothesized that targeting CRH-mediated effects to the respiratory tract will predict disease outcome through modulation of immune and inflammatory responses. Initial studies determined the timing of intranasal CRH administration following *S. pneumoniae* infection to effect survival. **Fig 2A, 2B**, and **2C** demonstrate the effect of intranasal administration of CRH on survival when given at 9, 18, and 30 hours after *S. pneumoniae* infection, respectively. **Fig 2A** shows that CRH as well as dexamethasone administered at 9 hr has no significant effect on survival compared to untreated mice. This outcome suggests that host responses presumably innate cellular mechanisms of immune defense are unresponsive to CRH and dexamethasone administered at this time point of *S. pneumoniae* infection. Our finding that administration of the CRH-R1 selective antagonist, antalarmin also did not impact outcome suggests that endogenous CRH is at most having a negligible role during the early stages of infection; supportive of CRH's inability to impact survival given 9 hr after infection. In contrast, **Fig 2B** demonstrates a significant protective effect of CRH administered

at 18 hours after infection compared to dexamethasone which demonstrated the lowest survival outcome. Furthermore, in that no significant differences in survival were demonstrated between all experimental groups when CRH, dexamethasone or antalarmin was administered at 30 hr further illustrates the significance in defining the specificity of temporal host responses against *S. pneumoniae* infection as a determinant of CRH's protective efficacy. Thus, the influence of CRH could be predicted by the type and intensity of inflammatory mediators involved during specific times of an ensuing infection. We have previously shown that blocking CRH-R1 activity by I.P. infection improves survival in mice subjected to *S. pneumoniae* infection under aversive restraint stress (29). This finding contrasts with our current findings suggesting that route of administration (e.g. nasal versus I.P.) can define CRH's mode of action. One potential explanation could be the distinct differences known between mucosal immune environment and that of systemic immune responses. Future studies are required for in depth determination of preferential CRH receptor expression along respiratory and non-mucosal tissues.

Previous studies have demonstrated the importance of identifying the optimal window of therapeutic efficacy pertaining to pharmacologic use in the management of aberrant inflammatory responses (7, 33, 49). A robust cellular immune response is absolutely required in early host protection against invading *S. pneumoniae* infection (38, 22, 58). Therefore, studies were performed to correlate cellular immune responses against *S. pneumoniae* with survival given intranasal administration of CRH. In that intranasal administration of CRH significantly reduced the number of total leukocytes found in the BALF compared to untreated mice demonstrates CRH's ability to modulate cellular inflammatory responses (**Fig 3**). Conversely, intranasal administration of the CRH-R1 antagonist antalarmin significantly increased leukocytes numbers. Thus, reinforcing CRH's role as a modulator of respiratory cellular responses based on

CRH-R1 specificity of action. Interestingly, intranasal administration of dexamethasone did not reduce total BALF numbers. If fact, BALF leukocyte numbers in dexamethasone-treated mice were higher than untreated mice. Chemotactic factors play a major role in cellular recruitment and therefore are critical determinants of inflammatory responses. One possible mechanism through which CRH may mediate cellular immune responses could be the regulation of chemokine function. To further substantiate the role of CRH as a mediator of immune and inflammatory responses along respiratory tissues, we compared the level of CXCL-1 and IL-17A in the BALF between naïve (control) and experimental groups (infection and/or treated animals). CXCL-1 and IL-17A are preferential mediators involved in the recruitment of neutrophils and monocyte subpopulations to sites of infection (4). Although IL-17A was not detected in BALF, **Fig 4** shows that infected mice administered CRH at 18 hours results in a significant decrease in CXCL1. This finding correlated with CRH-induced decrease in total BALF leukocytes, demonstrating a mechanistic link between CRH and cellular immune and inflammatory responses associated with respiratory *S. pneumoniae* infection. Such findings are impactful in light of the importance in managing inflammatory reactions during severe pneumococcal disease, particularly for at-risk populations. These findings suggest that CRH was more effective in inducing an anti-inflammatory phenotype than dexamethasone, a known suppressor of inflammatory responses. While inhaled glucocorticoids have proven therapeutic in management of respiratory inflammatory disease (15, 32, 39), CRH may also me an effective alternative. Future studies that determine the influences of CRH on the broader array of cytokine and chemokine mediators will provide further understanding of how CRH and its cognates receptors could be useful in manipulating pro- versus anti-inflammatory responses.

There is emerging debate of whether neutrophils are absolutely necessary to purge the system of foreign invaders during early pneumococcal infection (37, 52), or if neutrophils have a greater potential to promote detrimental effects during early pneumococcal infection (3, 10, 24). The generation of the 1A8 neutrophil-neutralizing antibody (57) affords the ability to distinguish the roles of neutrophils against bacterial infection. Here, we took advantage of the 1A8 antibody to determine the contribution of neutrophils in survival against pneumococcal infection and whether its influence could be linked to CRH's protective role. In that depletion of neutrophils did not result in a significant difference in overall survival compared to neutrophil competent mice, raises an important question relating their contribution to protection against pneumococcal infection. In support, previous studies have raised a similar argument related to their role against *S. pneumoniae* and other respiratory infectious disease. For example, Cooper PR et. al., suggest that the requirement for neutrophils in protection against pneumococcal infection may depend on disease severity (9, 14, 23). Consistent with **Fig 2** above, intranasal administration of CRH to neutrophil-competent mice increased survival compared to untreated mice. Most intriguing however, was the observation that CRH administration compensated for the absence of neutrophils, resulting in a significantly higher survivorship compared to neutrophil competent mice administered CRH. This finding suggests that neutrophils may be expendable in protection against *S. pneumoniae* infection. Alternatively, one might consider that the necessity of neutrophils during infection is tightly linked to disease status (e.g. bacterial burden, inflammatory condition). To date, the direct effect of CRH on neutrophils and other leukocyte populations remain largely unknown. Our preliminary studies of their influence in lung parenchymal tissue suggests neutrophils and other monocyte lineages are responsive to CRH (our preliminary findings). Knowledge of how CRH modulates leukocyte subpopulations'

function will benefit our understanding of CRH as a mediator of anti-inflammatory inflammatory responses.

In conclusion, our studies reveal the potential novel use of nasal delivery of CRH in control of overt inflammatory responses localized along respiratory tissues with the potential in reducing mortality risks associated with pneumococcal infection. Importantly, our results provide evidence of neutrophils' dispensable role during pneumococcal infection, particularly when considering adjuvant therapy. To date, dexamethasone is a primary standard of care in adjuvant treatment of respiratory-related and the management of systemic pneumococcal disease (52). However, its efficacy in reducing mortality, particularly for certain populations remains uncertain. For example, Remmelts, H.H et. al., found that dexamethasone use among certain individuals with community-acquired pneumonia produce very diverse cytokine responses with potential for adverse disease outcome (51). We believe that the development of novel approaches which tailor cellular immune and inflammatory responses is needed and a further understanding of the mechanisms through which CRH regulated immune and inflammatory responses may reveal improved adjuvant treatment that will eliminate mortality risks associated with pneumococcal infection.

Figure 1.

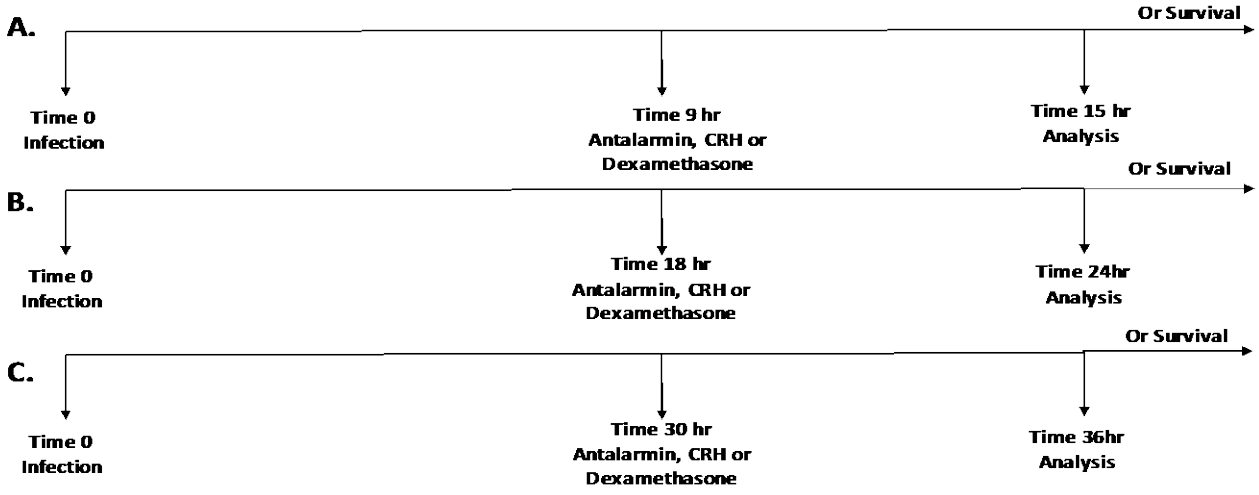

Figure 1 Experimental Design. All mice were infected with 1 x 10^5 colony forming units (CFUs) of *S. pneumoniae* by intranasal route. Selected groups of mice were administered PBS (placebo), Antalarmin (1mg/kg), CRH (1mg/kg) or dexamethasone (1mg/kg) by intranasal route at designated time points 9 hr (A), 18 hr (B) and 30 hr (C). Selected groups of mice were sacrifice 6 hr after administration of drug for analysis. Additional groups of mice were monitored for survival.

Figure 2.

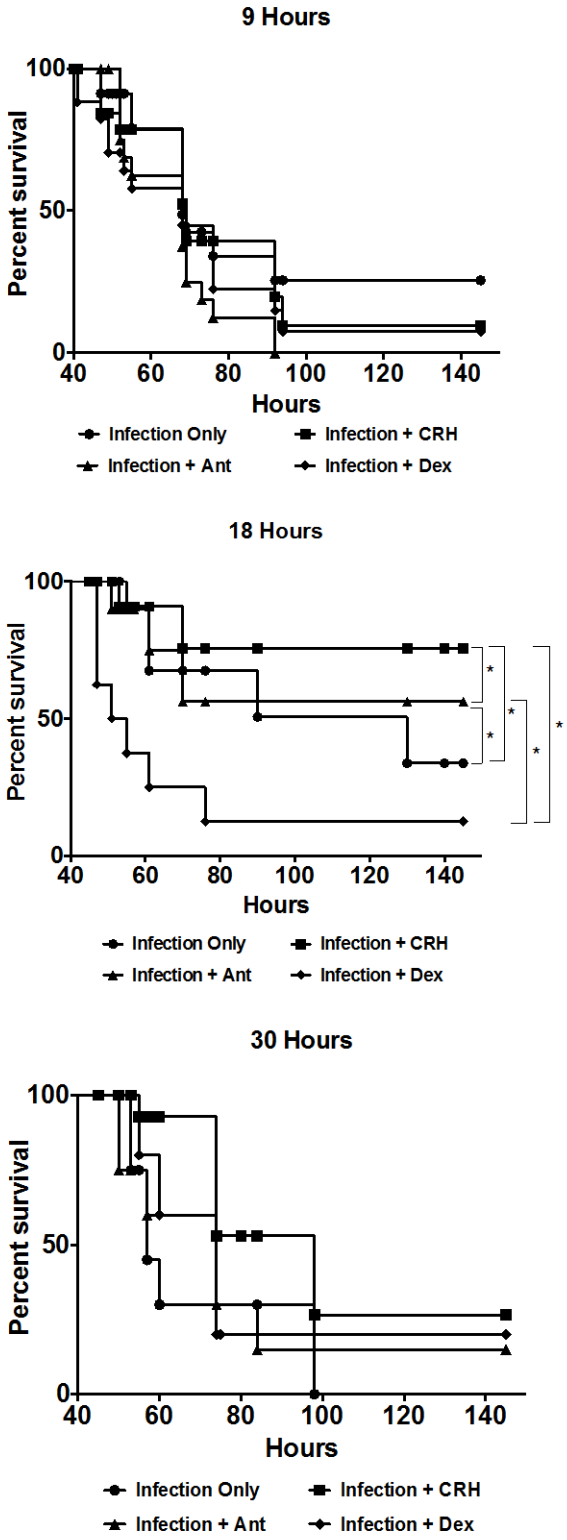

Figure 2 Time-dependent Administration of CRH Effect on Survival. Groups were administered Antalarmin (1mg/kg) CRH (1mg/kg), or Dexamethasone at a concentration of 1mg/kg. All groups were compared with the infection only group that received placebo treatment. A. 9 hours after infection B. 18 hours after infection C. 30 hours after infection, n=10 mice/group; ** $p \leq 0.05$ log rank test or no significance. Software: Graph Prism.

Figure 3.

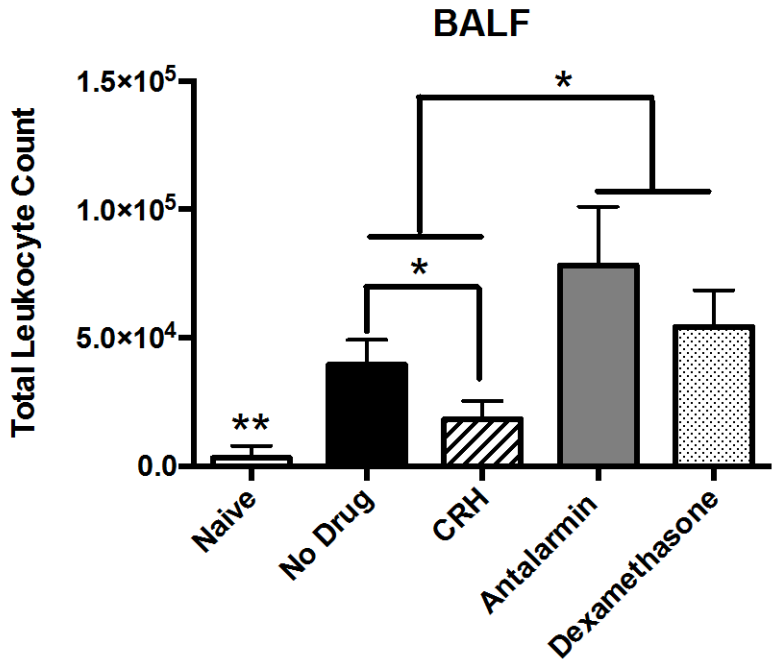

Figure 3. CRH decreases total leukocyte numbers in the Bronchoalveolar lavage fluid (BALF) at 24 hours, following treatment at 18 hours. CRH (1mg/kg), antalarmin (1mg/kg) or dexamethasone (1mg/kg) was administered by intranasal route 18 hr after infection. Total leukocytes numbers were determined in BALF of naïve and experimental groups of mice (n=5). Asterisks (**) indicate significant $p \leq 0.05$ differences from all experimental groups. Asterisk (*) indicates significant $p \leq 0.05$ differences between experimental groups determined by ANOVA. Rodger's method was utilized for post-hoc analysis. Software: Graph Prism.

Figure 4.

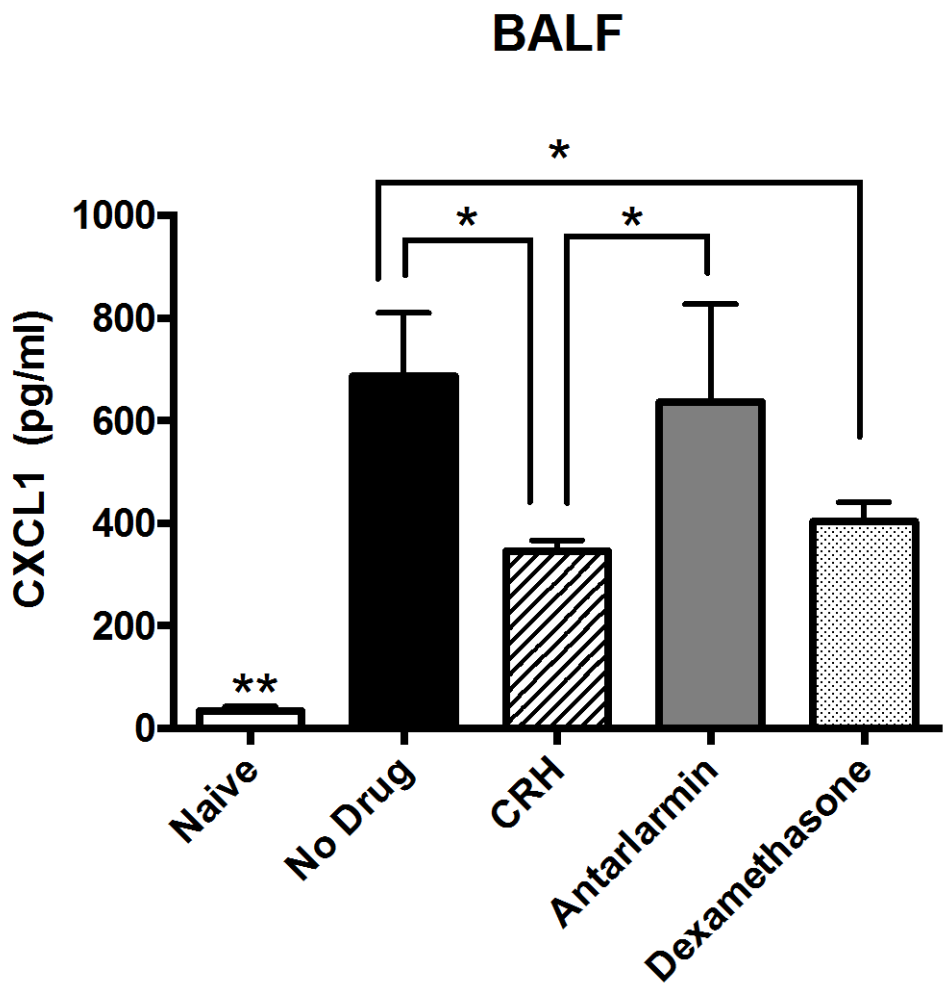

Figure 4 Production of CXCL1 in the Bronchoalveolar lavage fluid (BALF) decreases with CRH at 24 hours following administration at 18 hours. Naïve or Following treatment, BALF was harvested from mice for chemokine evaluation. Using ELISA, CXCL1 production was evaluated. n=5, statistical significance indicated by *. Asterisks (**) indicate significant $p \leq 0.05$ differences from all experimental groups. Asterisk (*) indicates significant $p \leq 0.05$ differences between experimental groups determined by ANOVA. Rodger's method was utilized for post-hoc analysis. Software: Graph Prism.

Figure 5.

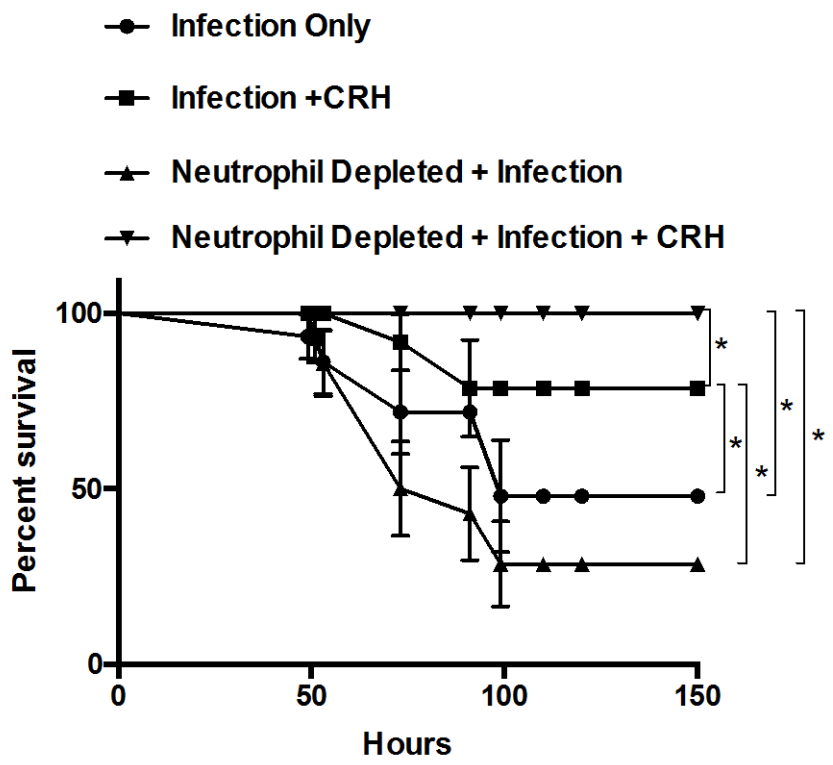

Figure 5 Elimination of Neutrophils Enhances Survival with CRH. Mice were administered placebo or 1A8 neutralizing antibody (0.5 milligrams) by intraperitoneal route one day prior to S. *pneumoniae* infection (1 x 10^5 CFU). Selected groups received CRH (1mg/kg) by intranasal route18 hours after infection. All groups were compared with the infection only group that received placebo treatment. Data represent (n=5 or10 mice/group) Asterisk (*) indicates significant ($p \leq 0.05$) differences between experimental groups determined by Log Rank Test. Software: Graph Prism.

REFERENCES

1. Adcock IM, Caramori G, Kirkham PA. Strategies for improving the efficacy and therapeutic ratio of glucocorticoids. Curr Opin Pharmacol. 2012 Mar 23.

2. Almagro P, Cabrera FJ, Diez J, Boixeda R, Ortiz B, Murio C, Soriano JB; Working Group on COPD of the Spanish Society of Internal Medicine. Co morbidities and short-term prognosis in patients hospitalized for acute exacerbation of COPD. The ESMI study. Chest. 2012 Apr 5.

3. Balamayooran, G et al., Mechanisms of neturophil accumulation in the lungs against bacteria, Am J Respir Cell Mol Biol. 2010 Jul;91):5-16

4. Bigorgne, A. E., John, B., Ebrahimkhani, M. R., Shimizu-Albergine, M., Campbell, J. S., & Crispe, I. N. (2016). TLR4-dependent secretion by hepatic stellate cells of the neutrophil-chemoattractant CXCL1 mediates liver response to gut microbiota. PloS One, 11(3), e0151063. doi:10.1371/journal.pone.0151063

5. Bi Y, Zhou J, Yang H, Wang X, Zhang X, Wang Q, Wu X, Han Y, Song Y, Tan Y, Du Z, Yang H, Zhou D, Cui Y, Zhou L, Yan Y, Zhang P, Guo Z, Wang X, Liu G, Yang R. IL-17A produced by neutrophils protects against pneumonic plague through orchestrating IFN-γ-activated macrophage programming. J Immunol. 2014 Jan 15;192(2):704-13. doi: 10.4049/jimmunol.1301687. Epub 2013 Dec 13. PMID: 24337746

6. Boyce, W.T., et al., Psychobiologic reactivity to stress and childhood respiratory illnesses: results of two prospective studies. Psychosom Med, 1995. 57(5): p. 411-22.

7. Calogero AE, Liapi C, Chrousos GP. Hypothalamic and suprahypothalamic effects of prolonged treatment with dexamethasone in the rat, J Endocrinol Invest. 1991 Apr;14(4):277-86.

8. Carrarelli P, Yen CF, Funghi L, Arcuri F, Tosti C, Bifulco G, Luddi A, Lee CL, Petraglia F., Expression of Inflammatory and Neurogenic Mediators in Adenomyosis: A Pathogenetic Role, Reprod Sci. 2016 Jul 20. pii: 1933719116657192.

9. Cooper PR, Palmer LJ, Chapple IL. Neutrophil extracellular traps as a new paradigm in innate immunity: friend or foe? Periodontol 2000. 2013 Oct;63(1):165-97. doi: 10.1111/prd.12025. Review. PMID: 23931060

10. Costantini C, Calzetti F, Perbellini O, Micheletti A, Scarponi C, Lonardi S, Pelletier M, Schakel K, Pizzolo G, Facchetti F, Vermi W, Albanesi C, Cassatella MA. Human neutrophils interact with both 6-sulfo LacNAc+ DC and NK cells to amplify NK-derived IFN{gamma}: role of CD18, ICAM-1, and ICAM-3, Blood. 2011 Feb 3;117(5):1677-86. Epub 2010 Nov 22.

11. Culpitt SV, Maziak W, Loukidis S, Nightingale JA, Matthews JL, Barnes PJ:, Effect of high dose inhaled steroid on cells, cytokines and proteases in induced sputum in Chronic Obstructive Pulmonary Disease. Am J Respir Crit Care Med 1999, 160:1635-1639

12. De Miguel, Z., Vegas, O., Garmendia, L., Arregi, A., Beitia, G., & Azpiroz, A. (2011). Behavioral coping strategies in response to social stress are associated with distinct neuroendocrine, monoaminergic and immune response profiles in mice. Behavioural Brain Research, 225(2), 554-561. doi:10.1016/j.bbr.2011.08.011; 10.1016/j.bbr.2011.08.011

13. De Pascale G, Bello G, Antonelli M., Steroids in severe pneumonia: a literature review. Minerva Anestesiol. 2011 Sep;77(9):902-10.

14. Drescher B, Bai F. Neutrophil in viral infections, friend or foe? Virus Res. 2013 Jan;171(1):1-7. doi: 10.1016/j.virusres.2012.11.002. Epub 2012 Nov 21. Review. PMID: 23178588

15, Finney L, Berry M, Singanayagam A, Elkin SL, Johnston SL, Mallia P. Inhaled corticosteroids and pneumonia in chronic obstructive pulmonary disease. Lancet Respir Med. 2014 Nov;2(11):919-32. doi: 10.1016/S2213-2600(14)70169-9. Epub 2014 Sep 17. Review. PMID: 25240963

16. Frei CR, Bell AM, Traugott KA, Jaso TC, Daniels KR, Mortensen EM, Restrepo MI, Oramasionwu CU, Ruiz AD, Mylchreest WR, Sikirica V, Raut MR, Fisher A, Schein JR. A clinical pathway for community-acquired pneumonia: an observational cohort study. BMC Infect Dis. 2011 Jul 6;11:188.

17. Ganceviciene R, Graziene V, Fimmel S, Zouboulis CC. Involvement of the corticotropin-releasing hormone system in the pathogenesis of acne vulgaris., Br J Dermatol. 2009 Feb;160(2):345-52. doi: 10.1111/j.1365-2133.2008.08959.x. Epub 2008 Dec 10. PMID: 19077080

18. Georges G, Kraft M, Vianna EO, Martin RJ., Human corticotropin-releasing hormone improves overnight FEV1 in nocturnal asthma. J Asthma. 1998;35(3):261-5.

19. Gonzales, X. F., Deshmukh, A., Pulse, M., Johnson, K., & Jones, H. P. (2008). Stress-induced differences in primary and secondary resistance against bacterial sepsis corresponds

with diverse corticotropin releasing hormone receptor expression by pulmonary CD11c+ MHC II+ and CD11c- MHC II+ APCs. Brain, Behavior, and Immunity, 22(4), 552-564. doi:10.1016/j.bbi.2007.11.005; 10.1016/j.bbi.2007.11.005

20. Gruenberg, D. A., Wright, R. J., Visness, C. M., Jaffee, K. F., Bloomberg, G. R., Cruikshank, W. W., et al. (2015). Relation between stress and cytokine responses in inner-city mothers. Annals of Allergy, Asthma & Immunology : Official Publication of the American College of Allergy, Asthma, & Immunology, 115(5), 439-445.e3. doi:10.1016/j.anai.2015.07.021

21. Gudina EK, Tesfaye M, Adane A, Lemma K, Shibiru T, Wieser A, Pfister HW, Klein M., Adjunctive dexamethasone therapy in unconfirmed bacterial meningitis in resource limited settings: is it a risk worth taking? BMC Neurol. 2016 Aug 26;16(1):153. doi: 10.1186/s12883-016-0678-0.

22. Huang Y, Wang Z, Jin C, Wang L, Zhang X, Xu W, Xiang Y, Wang W, He X, Yin Y, He Y. TLR2 promotes macrophage recruitment and S.pneumoniae clearance during mouse otitis media.

Pediatr Res. 2016 Jul 27. doi: 10.1038/pr.2016.154. [Epub ahead of print] PMID: 27463737

23. Herrod HG. Pulmonary defense mechanisms: friend and foe. South Med J. 1984 Aug;77(8):1007-9. Review. No abstract available. PMID: 6379890

24. Isailovic, N., Daigo, K., Mantovani, A., & Selmi, C. (2015). Interleukin-17 and innate immunity in infections and chronic inflammation. Journal of Autoimmunity, 60, 1-11. doi:S0896-8411(15)00059-1

25. Jain, R., et al., Corticotropin-releasing factor modulates the immune response to stress in the rat. Endocrinology, 1991. 128(3): p. 1329-36.

26. Jessop, D.S., M.S. Harbuz, and S.L. Lightman, CRH in chronic inflammatory stress. Peptides, 2001. 22(5): p. 803-7.

27. Jin, L., Batra, S., Douda, D. N., Palaniyar, N., & Jeyaseelan, S. (2014). CXCL1 contributes to host defense in polymicrobial sepsis via modulating T cell and neutrophil functions. Journal of Immunology (Baltimore, Md.: 1950), 193(7), 3549-3558. doi:10.4049/jimmunol.1401138

28. Jinno S, Jacobs MR. Pneumonia Due to Drug-Resistant Streptococcus pneumonia, Curr Infect Dis Rep. 2012 Jun;14(3):292-9

29. Kim, B. J., Kayembe, K., Simecka, J. W., Pulse, M., & Jones, H. P. (2011). Corticotropin-releasing hormone receptor-1 and 2 activity produces divergent resistance against stress-induced pulmonary streptococcus pneumoniae infection. Journal of Neuroimmunology, 237(1-2), 57-65. doi:10.1016/j.jneuroim.2011.06.016; 10.1016/j.jneuroim.2011.06.016

30. Kruisbeek, A. M. (2001). Isolation of mouse mononuclear cells. Current Protocols in Immunology / Edited by John E.Coligan ...[Et Al.], Chapter 3, Unit 3.1. doi:10.1002/0471142735.im0301s39

31. Liu Y, Fang X, Yuan J, Sun Z, Li C, Li R, Li L, Zhu C, Wan R, Guo R, Jin L, Li S., The role of corticotropin-releasing hormone receptor 1 in the development of colitis-associated cancer in mouse model, Endocr Relat Cancer. 2014 Aug;21(4):639-51. doi: 10.1530/ERC-14-0239.

32. McKeever T, Harrison TW, Hubbard R, Shaw D. Inhaled corticosteroids and the risk of pneumonia in people with asthma: a case-control study. Chest. 2013 Dec;144(6):1788-94. doi: 10.1378/chest.13-0871. PMID: 23990003

33. Meijvis SC, Hardeman H, Remmelts HH, Heijligenberg R, Rijkers GT, van Velzen-Blad H, Voorn GP, van de Garde EM, Endeman H, Grutters JC, Bos WJ, Biesma DH. Dexamethasone and length of hospital stay in patients with community-acquired pneumonia: a randomised, double-blind, placebo-controlled trial, Lancet. 2011 Jun 11;377(9782):2023-30. Epub 2011 Jun 1.

34. Mogensen, T. H., Berg, R. S., Paludan, S. R., & Ostergaard, L. (2008). Mechanisms of dexamethasone-mediated inhibition of toll-like receptor signaling induced by neisseria meningitidis and streptococcus pneumoniae. Infection and Immunity, 76(1), 189-197. doi:IAI.00856-07

35. Murray, S. E., Lallman, H. R., Heard, A. D., Rittenberg, M. B., & Stenzel-Poore, M. P. (2001). A genetic model of stress displays decreased lymphocytes and impaired antibody responses without altered susceptibility to streptococcus pneumoniae. Journal of Immunology (Baltimore, Md.: 1950), 167(2), 691-698.

36. O'Kane, M., Murphy, E. P., & Kirby, B. (2006). The role of corticotropin-releasing hormone in immune-mediated cutaneous inflammatory disease. Experimental Dermatology, 15(3), 143-153. doi:10.1111/j.1600-0625.2006.00382.

37. Peracoli MT et al., Increased reactive oxygen species and tumor necrosis factor-alpha production by monocytes are associated with elevated levels of uric acid in pre-eclamptic women. Am J reprod Immunol. 2011 May 27.

38. Pichichero ME, Almudevar A. Inflammation-associated cytokine analysis identifies presence of respiratory bacterial pathogens in the nasopharynx.Pathog Dis. 2016 Aug;74(6). pii: ftw064. doi: 10.1093/femspd/ftw064. Epub 2016 Aug 4. PMID: 27493063

39. Poulos LM, Ampon RD, Marks GB, Reddel HK. Inappropriate prescribing of inhaled corticosteroids: are they being prescribed for respiratory tract infections? A retrospective cohort study. Prim Care Respir J. 2013 Jun;22(2):201-8. doi: 10.4104/pcrj.2013.00036. PMID: 23616052

40. Refojo D, Holsboer F, CRH signaling, Molecular specificity for drug targeting in the CNS, 2009, Ann N Y Acad Sci; Oct 1179; 106-19.

41. Rassouli O, Liapakis G, Lazaridis I, Sakellaris G, Gkountelias K, Gravanis A, Margioris AN, Karalis KP, Venihaki M., A novel role of peripheral corticotropin-releasing hormone (CRH) on dermal fibroblasts. PLoS One. 2011;6(7):e21654. doi: 10.1371/journal.pone.0021654. Epub 2011 Jul 13. PMID:21765902

42. Remmelts HH, Meijvis SC, Heijligenberg R, Rijkers GT, Oosterheert JJ, Bos WJ, Endeman H, Grutters JC, Hoepelman AI, Biesma DH. Biomarkers define the clinical response to dexamethasone in community-acquired pneumonia, J Infect. 2012 Jul;65(1):25-31. doi: 10.1016/j.jinf.2012.03.008.

43. Remmelts HH, Meijvis SC, Kovaleva A, Biesma DH, Rijkers GT, Heijligenberg R., Changes in serum cortisol levels during community-acquired pneumonia: the influence of dexamethasone., Respir Med. 2012 Jun;106(6):905-8. doi: 10.1016/j.rmed.2012.02.008.

44. Rodgers, G. L., & Klugman, K. P. (2011). The future of pneumococcal disease prevention. Vaccine, 29 Suppl 3, C43-8. doi:10.1016/j.vaccine.2011.07.047; 10.1016/j.vaccine.2011.07.047

45. Saunders PR, Santos J, Hanssen NP, Yates D, Groot JA, Perdue MH. Physical and psychological stress in rats enhances colonic epithelial permeability via peripheral CRH. Dig Dis Sci. 2002 Jan;47(1):208-15. PMID: 11852879

46. Sasayama, D., Hori, H., Iijima, Y., Teraishi, T., Hattori, K., Ota, M., . . . Kunugi, H. (2011). Modulation of cortisol responses to the DEX/CRH test by polymorphisms of the interleukin-1beta gene in healthy adults. Behavioral and Brain Functions : BBF, 7, 23-9081-7-23. doi:10.1186/1744-9081-7-23; 10.1186/1744-9081-7-23

47. Schuster, K. M., Macleod, J. B., Fernandez, J. B., Kumar, M., & Barquist, E. S. (2012). Adrenocorticotropic hormone and cortisol response to corticotropin releasing hormone in the critically ill-a novel assessment of the hypothalamic-pituitary-adrenal axis. American Journal of Surgery, 203(2), 205-210. doi:10.1016/j.amjsurg.2010.11.015; 10.1016/j.amjsurg.2010.11.015

48. Silverman, E. S., Breault, D. T., Vallone, J., Subramanian, S., Yilmaz, A. D., Mathew, S., . . . Majzoub, J. A. (2004). Corticotropin-releasing hormone deficiency increases allergen-induced airway inflammation in a mouse model of asthma. The Journal of Allergy and Clinical Immunology, 114(4), 747-754. doi:10.1016/j.jaci.2004.06.055

49. Skrupky, L. P., Kerby, P. W., & Hotchkiss, R. S. (2011). Advances in the management of sepsis and the understanding of key immunologic defects. Anesthesiology, 115(6), 1349-1362. doi:10.1097/ALN.0b013e31823422e8; 10.1097/ALN.0b013e31823422e8

50. Sternberg EM, Licinio J. Overview of neuroimmune stress interactions. Implications for susceptibility to inflammatory disease, Ann N Y Acad Sci. 1995 Dec 29;771:364-71.

51. Sternberg, E. M., Chrousos, G. P., Wilder, R. L., & Gold, P. W. (1992). The stress response and the regulation of inflammatory disease. Annals of Internal Medicine, 117(10), 854-866.

52. Sutherland, T. E., Logan, N., Ruckerl, D., Humbles, A. A., Allan, S. M., Papayannopoulos, V., et al. (2014). Chitinase-like proteins promote IL-17-mediated neutrophilia in a tradeoff between nematode killing and host damage.Nature Immunology, 15(12), 1116-1125. doi:10.1038/ni.3023.

53. Tsilioni I, Russell IJ, Stewart JM, Gleason RM, Theoharides TC., Neuropeptides CRH, SP, HK-1, and Inflammatory Cytokines IL-6 and TNF Are Increased in Serum of Patients with Fibromyalgia Syndrome, Implicating Mast Cells., J Pharmacol Exp Ther. 2016 Mar;356(3):664-72. doi: 10.1124/jpet.115.230060.

54. Uchoa ET, Aguilera G, Herman JP, Fiedler JL, Deak T, de Sousa MB., Novel aspects of glucocorticoid actions, J Neuroendocrinol. 2014 Sep;26(9):557-72. doi: 10.1111/jne.12157.

55. Vakharia K, Hinson JP. Lipopolysaccharide directly stimulates cortisol secretion by human adrenal cells by a cyclooxygenase-dependent mechanism. Endocrinology. 2005 Mar;146(3):1398-402. Epub 2004 Nov 24.

56. Vasiadi M, Therianou A, Sideri K, Smyrnioti M, Sismanopoulos N, Delivanis DA, Asadi S, Katsarou-Katsari A, Petrakopoulou T, Theoharides A, Antoniou C, Papadavid E, Stavrianeas N, Kalogeromitros D, Theoharides TC., Increased serum CRH levels with

decreased skin CRHR-1 gene expression in psoriasis and atopic dermatitis, J Allergy Clin Immunol.,2012 May;129(5):1410-3. doi: 10.1016/j.jaci.2012.01.041.

57. Verbanac KM, Gross UM, Rebellato LM, Thomas JM. Production of stable rabbit-mouse heterohybridomas: characterization of a rabbit monoclonal antibody recognizing a 180 kDa human lymphocyte membrane antigen. Hybridoma. 1993 Jun;12(3):285-95. PMID: 8359823

58. Vissers M, Ahout IM, van den Kieboom CH, van der Gaast-de Jongh CE, Groh L, Cremers AJ, de Groot R, de Jonge MI, Ferwerda G. High pneumococcal density correlates with more mucosal inflammation and reduced respiratory syncytial virus disease severity in infants., BMC Infect Dis. 2016 Mar 17;16:129. doi: 10.1186/s12879-016-1454-x. PMID: 26983753

59. Vlahos R, Wark PA, Anderson GP, Bozinovski S.Glucocorticosteroids Differentially Regulate MMP-9 and Neutrophil Elastase in COPD. PLoS One. 2012;7(3):e33277. Epub 2012 Mar 7.

60. Wang WC, Zhang XZ, Liu DW, Qiu J, Wang XH, Zhou JH., Corticotropin-releasing hormone and urocortin promote phagocytosis of rat macrophages through convergent but distinct pathways.

Life Sci. 2015 Feb 1;122:100-7. doi: 10.1016/j.lfs.2014.12.015.

61. Wang, W., Zhang, X., Yang, L., Liu, D., Liu, G., & Zhou, J. (2012). Lipopolysaccharide upregulates the expression of corticotropin-releasing hormone via MAP kinase pathway in rat peritoneal macrophages. Molecular and Cellular Biochemistry, 361(1-2), 1-7. doi:10.1007/s11010-011-1080-2; 10.1007/s11010-011-1080-2

62. Whale, T.A. and P.J. Griebel, A sheep in wolf's clothes: can neutrophils direct the immune response? Vet J, 2009. 180(2): p. 169-77

63. Williams, A. E., Jose, R. J., Brown, J. S., & Chambers, R. C. (2015). Enhanced inflammation in aged mice following infection with streptococcus pneumoniae is associated with decreased IL-10 and augmented chemokine production. American Journal of Physiology.Lung Cellular and Molecular Physiology, 308(6), L539-49. doi:10.1152/ajplung.00141.2014

64. Wunderink RG, Adjunctive therapy in community-acquired pneumonia. Semin Respir Crit Care Med. 2009 Apr;30(2):146-53. Epub 2009 Mar 18.

65. Yang C, Gao W, Yang X, Wang H, Du J, Zhong H, Zhou L, Zhou J, Zhang Y, Jiang J., Surgery. CRH knockout inhibits the murine innate immune responses in association with endoplasmic reticulum stress after thermal injury., 2015 Jul;158(1):255-65. doi: 10.1016/j.surg.2015.01.024.

66. Zhao, H., et al., Reactive oxygen species- and nitric oxide-mediated lung inflammation and mitochondrial dysfunction in wild-type and iNOS-deficient mice exposed to diesel exhaust particles. J Toxicol Environ Health A, 2009. 72(8): p. 560-70.

67. Zizzo G, Cohen PL. IL-17 stimulates differentiation of human anti-inflammatory macrophages and phagocytosis of apoptotic neutrophils in response to IL-10 and glucocorticoids. J Immunol. 2013 May 15;190(10):5237-46. doi: 10.4049/jimmunol.1203017. Epub 2013 Apr 17. PMID: 23596310

68. Zhu, H., Wang, J., Li, J., & Li, S. (2011). Corticotropin-releasing factor family and its receptors: Pro-inflammatory or anti-inflammatory targets in the periphery? Inflammation

Research: Official Journal of the European Histamine Research Society ...[Et Al.], 60(8), 715-721. doi:10.1007/s00011-011-0329-2; 10.1007/s00011-011-0329-2.

CHAPTER III

INTRANASAL DELIVERY OF NANOPARTICLE-BASED VACCINE INCREASES PROTECTION AGAINST *S. PNEUMONIAE*

Introduction

Community-acquired pneumonia (CAP) remains the leading cause of morbidity and mortality worldwide despite advances in antimicrobial therapy and advances in the management of severe infections. *Streptococcus pneumoniae* (*S. pneumoniae)* in particular, is responsible for an estimated 2 million deaths annually, making it the most common etiological risk for CAP [25]. Although conjugate and polysaccharide vaccines have been central to the prevention of *S. pneumonia*-induced CAP, the impact of vaccination particularly among the elderly has been less than expected due to a status quo in mortality rates. This lack in vaccine efficacy is believed to be attributed to less than optimal immune responses generated against current conjugate and polysaccharide vaccine constructs [24]. Thus, a reduction in CAP-related deaths will be difficult to realize without improved strategies to augment immunity.

Evidence suggests that induction of immune responses locally across the respiratory mucosa enhances protective immunity against pathogens of the respiratory tract. Previous studies have shown that nasal immunization is advantageous in inducing protective immunity against

respiratory, viral, and bacterial infectious disease through the induction of local and systemic immune responses [5, 6, 11, 20]. On the contrary, consideration of the potential limitations associated with nasal immunization is also of significant importance. In general, the respiratory mucosa is a volatile environment not unlike other mucosal sites (e.g. gastrointestinal) that could compromise the induction of immune responses resulting from the enzymatic destruction in vaccine components [7]. Also of concern is the potential for immune tolerance as routinely demonstrated in models of oral vaccination [1, 8, 10].

Nanotechnology is emerging as a feasible approach to target activation of immune responses and promises to be an effective strategy to overcome current limitations mentioned above [10, 21]. We are particularly interested in the use of PLGA NPs. The use of PLGA NPs for targeting immune therapies provides numerous advantages including: biocompatibility, biodegradability, size specificity, composition and ease of production [1, 9, 13]. Recently it has been shown that NP therapy provides a vehicle to activate a robust and lasting immune response against various pathogens [16]. In the current study, we determined the efficacy of NP-HKSP-based vaccine against *S. pneumoniae* infection by targeting the respiratory mucosa for the induction of protective immunity. Our results demonstrate that intranasal administration of NP-HKSP produces enhanced protection against pneumococcal disease that is supported by a robust antigen-specific cell-mediated immune response.

Materials and Methods

Animals

Adult (6-8 weeks of age) female CD-1 mice (Harlan Sprague-Dawley, Indianapolis, Indiana) were used in all studies. Mice were maintained under specific pathogen-free conditions

on a 12:12 light/dark cycle (7:00 PM to 7:00 AM). Mice were kept under optimal temperature and humidity controlled conditions. All studies were approved by the University of North Texas Health Science Center's Institutional Animal Care and Use Committee (IACUC).

Nanoparticle construction

The PLGA NPs encapsulating HKSP were prepared by double emulsion method as previously described [22, 26]. Briefly, 1 $X10^6$ HKSP were lysed by sonication in 200 µl of phosphate-buffered saline (PBS) and 70 mg of PLGA (Lakeshore Biomaterials AL, USA) was dissolved in 1 ml of ethyl acetate. These two solutions were mixed and vortexed at maximum speed for 1 minute to form primary water-in-oil emulsion. The primary emulsion was then mixed with 3 ml of 1% polyvinyl alcohol (PVA) solution. This solution was sonicated using an ultrasonic processor UP200H system (Hielscher Ultrasonics GmbH, Germany) at 40% amplitude for 2 minutes on continuous mode, in a clean glass vial immersed in ice, to prepare HKSP encapsulating PLGA NPs. The solution was further diluted to 20 ml with autoclaved water (0.22 µ filter sterilized) and stirred for 1 h at room temperature under mild vacuum to evaporate ethyl acetate. The solution was then centrifuged to collect NPs and this process was repeated twice to remove excess PVA. The nanoparticle pellet was resuspended in 500 µl of autoclaved water and lyophilized using an ATR FD 3.0 system (ATR Inc., MO, USA). The final NPs were stored at -20° C until further use.

To make fluorescently labeled HKSP NPs, nile red was premixed with PLGA solution in ethyl acetate (5% v/v of 10mg/ml nile red stock solution in ethyl acetate) and the remainder of the procedure was performed as explained above. Nile red is a lipophilic dye which partition into the organic phase and form complex with PLGA polymer. The excess of nile red is remove during the washes as the surfactant PVA helps in solubilization and removal of dye which is

uncomplexed with PLGA [23]. Nile red is a small molecule dye and does not affect the size of nanoparticles. Particle size of nanoparticles was determined by dynamic light scattering method (NanoTrac ULTRA) using aqueous suspension (1 mg/ml).

Intranasal immunization and infection

Mice were immunized on day 0 with either HKSP or NP-HKSP prepared from heat-killed *S. pneumoniae* by heating live bacteria in a water bath, set at 85° C, for 45-60 minutes [15]. Following dilution with PBS, immunization consisted of administrating a single dose of 4 X 10^5 colony forming unit (CFUs) equivalents of HKSP or NP-HKSP by intranasal route in two successive 40 µl volumes of 2 X 10^5 CFUs. This dose was determined by back-calculated based on the encapsulation efficiency.

S. pneumoniae strain #6301 (ATCC, Manassas, VA) was grown for 16 h to obtain mid-log phase cultures on blood agar plates. Mice were infected with 2 X 10^5 CFUs (LD_{80}) of *S. pneumoniae s*train #6301 (ATCC, Manassas, VA) by intranasal route in a volume of 40 µl of Brain-Heart Infusion Broth (EMD, EMD Chemicals Inc. Gibbtown, NJ) or broth (e.g. sham infection) after anesthesia.

Determination of nanoparticle distribution in lungs

To visualize and confirm the distribution of NPs, we performed *ex-vivo* imaging of mice lungs. Fluorescent NPs were prepared as explained in the nanoparticle construction section. Briefly, nile red stained NP-HKSP were suspended in autoclaved water at a concentration of 4 mg/ml and 80 µl of NPs were administered via intranasal route as described above. Mice were sacrificed on days 1 and 11 to visualize and confirm NP deposition the lungs based on

preliminary evidence demonstrating NP retention (data not shown). Briefly, isolated lungs were perfused and washed with PBS before imaging. The isolated lungs were placed in a petri dish and imaged using Lumina XR imaging system (Caliper Life Sciences, CA). The background due to autofluorescence was collected and subtracted by the IVIS lumina XR imaging system software, which collects and subtracts autofluorescence while constructing the image and the intensity was normalized to compare the fluorescence between days 1 and 11.

Lung leukocyte isolation

PBS-perfused lungs were harvested and finely minced after separation into single lobes and incubated in collagenase (Type II collagenase, Sigma-Adrich, St. Louis, MO) digestion media containing 300 units/ml of DNase (Sigma-Adrich) in RPMI 1640 culture media for 1 h and 30 minutes After digestion, lungs were passed through a nylon mesh filter (LabPak, Depew, NY) into sterile 50 ml conical tubes and washed twice in wash media (Hyclone, Logan, UT). Lung mononuclear cells were prepared by ficoll-hypaque (Lymphocyte M, Cedarlane, Laboratories, Ltd.; Ontario, CA) centrifugation. Contaminating RBCs were removed using ACK lysis buffer as previously described [18].

In vitro IFN-γ cytokine determination by total lung leukocytes

IFN-γ cytokine production was determined by sandwich ELISA method from lung supernatants cultured *ex-vivo* for 4 days. All procedures were performed as described by the manufacturer. Briefly, flat-bottomed 96-well plates were coated with an optimal titration of capture antibody followed by overnight blocking using 10% FBS in PBS to deter non-specific binding. After incubation of samples at 4° C for 16 h, plates were incubated with biotin-

conjugated detection antibody and streptavidin-HRP (Horseradish peroxidase). Tetramethylbenzididne (TMB) peroxidase substrate solution (Rockland Immunochemicals, Inc. Gilbertsville, PA) was added to each well for colorimetric determination of concentration of each cytokine according to standard curve generated by reference concentration of cytokine at wavelength of 450 nm detected by colorimetric plate reader (Bioteck Instruments Inc. Winooski, VT). ELISA antibody set and recombinant cytokine for standard were purchased from R&D Systems (R&D Systems Inc. Minneapolis, MN) for recombinant IFN-γ.

Determination of Bacterial Colonization

To access bacterial growth, lung tissues were harvested and homogenized in sterile PBS. Ten-fold dilutions of sample homogenates were plated in triplicate onto blood agar plates and incubated at 37° C with 5% CO_2 overnight. CFUs were enumerated, and the results were expressed as $\log_{10}$ CFU as previously described [17].

Statistical Analysis

Statistical analysis was performed using GraphPad Prism Version 5.0p (GraphPad Software, San Diego, USA). For multi-experimental group analysis, data were subjected to one-way ANOVA (analysis of variance). Rodger's method was utilized for post-hoc analysis. All data are expressed as means ± standard error of mean (SEM). The two-tailed level of significance was set to $p \leq 0.05$ for group differences.

Results

Particle size determination

The particle size is an important determinant of the fate of NPs in circulation. Smaller particles tend to mobilize in blood circulation due to rapid absorption from the site of deposition, whereas larger particles are retained at the site of administration and are cleared by reticuloendothelial system (RES). This property is particularly useful for our study due to the potential uptake of nanoparticles by antigen presenting cells (APCs), which are routinely found in the RES. The NP-HKSP prepared in this study was 234 ± 87.5 nm in size (Figure 1).

Deposition of NPs in lungs of mice following intranasal NP-HKSP administration

An initial study was performed to test the retention of NPs in lungs after intranasal administration. A comparison between the detection of empty NPs and NP-HKSP in the lungs of mice over the course of an eleven day incubation period demonstrated a significantly higher fluorescence intensity in the lungs of mice administered NP-HKSP by day 1. In contrast, minimal fluorescence was detected in the lungs of mice administered empty NPs. It has been previously suggested that 50:50 PLGA nanoparticles starts degrading to lactic acid and glycolic acid after 10-14 days. As the nile red, used in this study to visualize nanoparticles, is complexed with PLGA, tracking these nanoparticles after that time will be redundant. After this time the nanoparticle tracking may give false positive localization in other tissues based on distribution of free nile red dye. However, degradation of nanoparticles is dependent on several factors including type of drug, amount of drug and interaction of polymer-drug and nanoparticles with the host tissue. In our study, we found the antigen encapsulated nanoparticles showed longer

duration of retention, which could be due to the encapsulation of HKSP as well as interaction of nanoparticles with host tissues due to presence of immunogenic component i.e. HKSP. However, further studies needs to be done to confirm this phenomenon. The fluorescence intensity was sustained in the lungs of mice administered NP-HKSP for at least eleven days compared to the lungs of mice administered empty NPs (Figure 2).

NP-HKSP *immunization promotes potent antigen-specific IFN-γ cytokine recall response.*

Local cell mediated immune responses are essential for optimal protective adaptive immunity against *S. pneumoniae* and other pulmonary infectious diseases [19]. The current study determined how administration of NP-HKSP by intranasal route would induce an antigen-specific IFN-γ cytokine recall response by pulmonary lymphocytes. The results demonstrated a significant generation in antigen-induced IFN-γ cytokine production by lung lymphocytes isolated from mice immunized with NP-HKSP compared to the lungs of mice immunized with HKSP alone and compared to mice immunized with empty NPs. NPs encapsulated with antigen produced greater than 150 pg of IFN-γ compared to less than 90 pg produced by antigen alone (Figure 3).

NP-HKSP immunization *improves protection against pulmonary S. pneumoniae infection*

To test the efficacy of intranasal NP-based formulations as a potential strategy for vaccination against S. pneumonia infection, mice previously administered either NP-HKSP, HKSP alone or empty NPs were subsequently challenged with high doses of *S. pneumoniae* infection (LD_{80}). As shown in Figure 4, bacterial load in the lungs of mice mice previously administered NP-HKSP was 1000-fold lower than compared to HKSP-alone.

Discussion

Acquired resistance of pneumococcal bacterial strains/serotypes to available antibiotics and the ineffectiveness of current conjugate and polysaccharide vaccines warrant development of new modalities to boost self-immune defenses. Adjunctive therapies such as the use of nanoparticle delivery systems are being considered for their ability to manipulate immune responses against infectious disease [10]. The capability of these particles to slowly release its encapsulated proteins/peptides allows for more protection, making NPs a focus for vaccine development. NP technologies are emerging for their use in enhancing immunity, particularly against respiratory pathogens [19]. In our current studies we were able to establish that NP-HKSP immunization generates greater bacterial resistance compared to HKSP immunization. This is one of the first studies to identify NP technology as a promising advanced approach in prevention of *S. pneumoniae* infection, the major etiological pathogen of CAP.

Previous studies highlight the significance of nasal-pulmonary immunization in increasing the efficacy of vaccination against respiratory pathogens compared to the standard intramuscular route of immunization [19]. However, controversy still remains regarding the robustness of immunization and the potential adverse inflammatory reactions that are commonly observed in response to antigen exposures across respiratory tissues [2, 17]. In the present study, we demonstrated that NPs as a potential vehicle for vaccines antigens could produce resistance against the targeted pathogen. The PLGA based nanoparticles are biodegradable and non-immunogenic. In fact, it is one of the few US FDA approved biodegradable polymers used in nanomedicine field. Using an experimental model of *S. pneumoniae* infection, we demonstrated an increased protection in terms of reducing bacterial colonization along the respiratory tissues

compared to immunization using heat-killed cell lysate antigenic preparations alone. Also worth noting, was our inability to detect bacterial numbers in the systemic compartments of the spleen and blood of mice immunized with the NP-HKSP construct compared to immunization of HKSP alone (data not shown). This suggests that nasal delivery of antigen encapsulated in NPs not only enhances resistance across the local site of immunization, but also prevents the potential for bacterial dissemination. Thereby, potentially reducing the incidence of mortality risk associated commonly associated with *S. pneumoniae* infection observed in immune compromised individuals [25].

The necessity for improved vaccine strategies is in part associated with a lack in long-lasting antigen-specific acquired immune responses. As shown in figure 2, NP-encapsulated antigen was retained in lungs to a greater extent than empty NPs. While the precise mechanism to explain this finding is unknown, it does raise interest in defining antigen-particle interactions. One explanation could be that antigen encapsulation promotes the uptake by resident and circulating pulmonary macrophages, allowing for a prolonged local immune response. Future studies which address such intrapulmonary cellular interactions would provide important insight into future NP-based vaccine construction as immune-modulators. Recently, studies have documented inefficient generation of cell-mediated T cell responses associated with several conjugate and polysaccharide pneumococcal vaccines [3]. A hallmark of protective immunity is routinely associated with induction of IFN-γ cytokine responses as they promote the activation of macrophage responses, enabling intracellular bacterial killing [4]. In our current study, we examined the difference in antigen-mediated IFN-γ cytokine recall responses given NP-HKSP delivery system. As shown in figure 3, re-exposure of lung lymphocytes isolated from mice immunized with NP-HKSP construct produced a significantly stronger production of IFN-γ. On

the basis of this finding, one might predict that encapsulation of target pneumococcal conjugate peptide and/or polysaccharide would be advantageous in compensating for the previously reported deficiency in adaptive/memory cell-mediated immunity [3, 12, 14, 25].

In summary, this study extends the potential advantages for the use of NPs in vaccine development against infectious disease including *S. pneumonia* infection, which is a major cause of mortality rates. The results provide evidence that nasal delivery of NP-HKSP produces effective immunity. Although our current findings demonstrate protective efficacy, consideration of potential adverse effects associated with this approach will increase its promise clinically. Moreover, because PLGA-based NPs and other formulations afford the capacity to coat and/or link substrates to its surface, it is expected that future studies whereby targeting of specific immune cells will be developed thereby advancing vaccine technologies. Such cells include macrophages and dendritic cells, which are important against pneumococcal and other infectious diseases.

Figure 1.

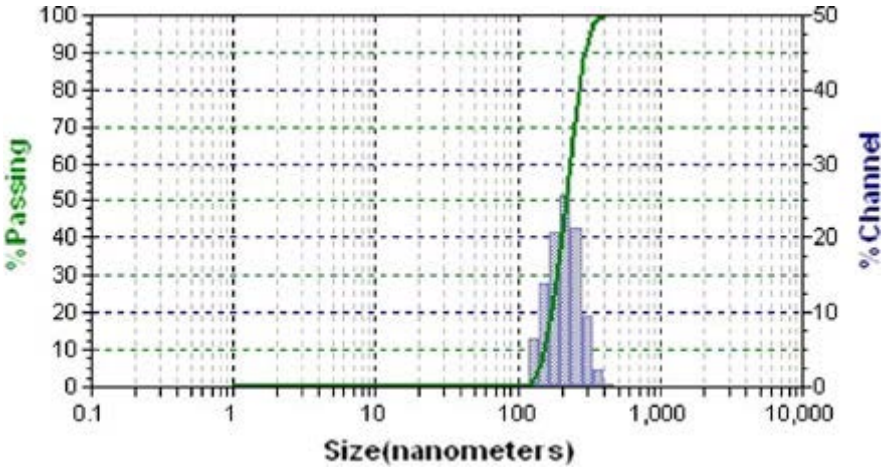

Figure 1. Particle size of HKSP encapsulated PLGA nanoparticles. The particle size of an aqueous suspension of nanoparticles measured by dynamic light scattering shows the average size and the Gaussian distribution of particles in the batch

Figure 2.

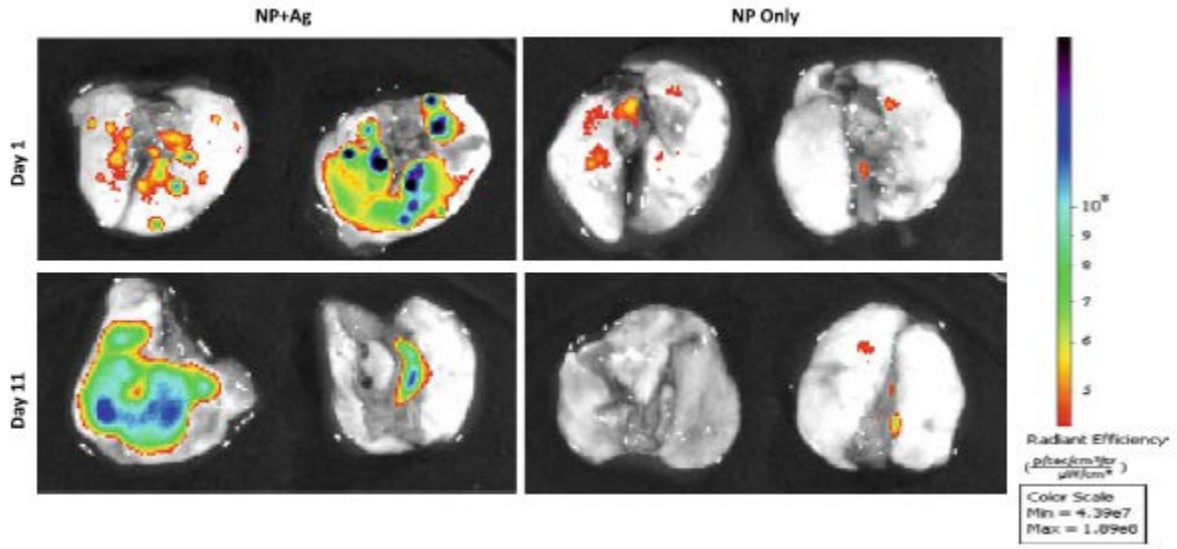

Figure 2. HKSP encapsulation sustains NP retention in lungs of antigen. Naïve mice were administered preparations of fluorescently labeled empty NP or NP encapsulated with HKSP. Deposition of NP formulations was determined on day 1 and day 11 using Lumina XR imaging system. Images are representative of five animals per group

Figure 3.

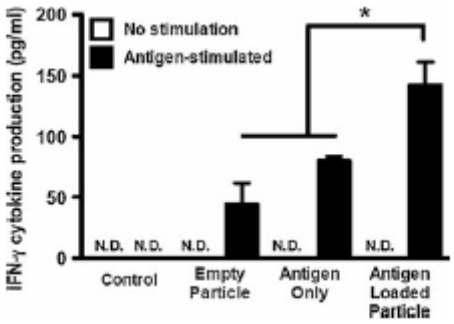

Figure 3. HKSP-encapsulated NP immunization enhances antigen-specific IFN-γ cytokine production by pulmonary leukocytes. Lung leukocytes isolated from mice previously immunized with NP-HKSP or HKSP were stimulated *in vitro* to determine their ability to secrete IFN-γ in response to HKSP stimulation. Bars represent mean (n=5) ± std. error. Asterisk * indicate significant differences ($p \leq 0.05$) between experimental groups.

Figure 4.

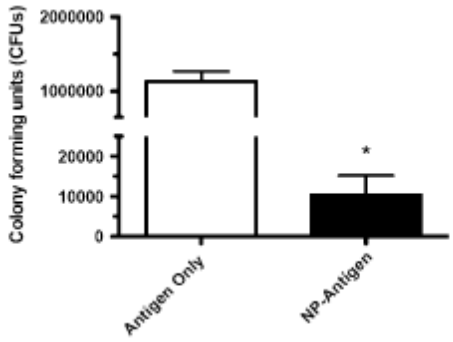

Figure 4. HKSP-encapsulated NP immunization enhances resistance against subsequent *S. pneumoniae* infection. Mice previously receiving intranasal administration of HKSP or NP-HKSP were challenged with *S. pneumoniae*. Bacterial colonization in the lungs was determined 1 day later. Bar graph represents mean (n=5) ± std. error. Asterisk * indicate significant difference ($p \leq 0.05$) between experimental groups.

REFERENCES

1. Adam A, Wright A. (2009). Mucosal perspectives in pneumococcal vaccine development: A meeting summary. *Vaccine, 28*
2. Arredouani M, Yang Z, Ning Y et al. (2006). The scavenger receptor MARCO is required for lung defense against pneumococcal pneumonia and inhaled particles. *Journal of Experimental Medicine, 177*
3. Ballester M, Nembrini C, Dhar N et al. (2011). Nanoparticle conjugation and pulmonary delivery enhance the protective efficacy of Ag85B and CpG against tuberculosis. *Vaccine, 29*
4. Bogart D, De Groot R., Hermans P. (2004). *Streptococcus pneumoniae* colonisation: The key to pneumococcal disease. *Lancet Infect Dis, 4*
5. Camacho AI, de Souza J, Sanchez-Gomez S et al. (2011). Mucosal immunization with shigella flexneri outer membrane vesicles induced protection in mice. *Vaccine, 29*
6. Cohen JM, Khandavilli S, Camberlein E et al. (2011). Protective contributions against invasive *Streptococcus pneumoniae* pneumonia of antibody and Th17-cell responses to nasopharyngeal colonisation. *Plos One, 6*
7. Derudas M, Brancale A, Naesens L et al. (2010). Application of the phosphoramidate ProTide approach to the antiviral drug ribavirin. *Bioorganic & Medicinal Chemistry, 18*(7), 2748-2755. doi: 10.1016/j.bmc.2010.02.015
8. Fattal E, Pecquet S, Couvreur P, Andremont A. (2002). Biodegradable microparticles for the mucosal delivery of antibacterial and dietary antigens. *International Journal of Pharmaceutics, 242*

9. Gelperina S, Kisich K, Iseman M, Heifets L. (2005). The potential advantages of nanoparticle drug delivery systems in chemotherapy of tuberculosis. *Am J Respir Crit Care Med, 172*
10. Gupta P, Khatri K, Goyal A, Mishra K, Vyas SP. (2007). M-cell targeted biodegradable PLGA nanoparticles for oral immunization against hepatitis B. *J Drug Target, 15*
11. Gutierro I, Hernandez R., Igartua M, Gascon A, Pedraz J. (2002). Size dependent immune response after subcutaneous, oral and intranasal administration of BSA loaded nanoparticles. *Vaccine, 21*
12. Haddadi A, Elamanchili P, Lavasanifar A, Das S. (2007). Delivery of rapamycin by PLGA nanoparticles enhances its suppressive activity on dendritic cells. *Wiley Periodicals,*
13. Handsand M, Lowman A. (2002). Biodegradable nanoparticles for drug delivery and targeting. *Curr Opin Solid State Mater Sci, 6*
14. Heuking S, Borchard G. (2012). Toll-like receptor-7 agonist decoration enhances the adjuvanticity of chitosan-DNA nanoparticles. *Journal of Pharmaceutical Sciences, 101*
15. Hvalbye, BK, Aaberge IS, Lovik M. et al. (1999). Intranasal immunization with heat-inactivated streptococcus pneumoniae protects mice against systemic pneumococcal infection. *Infection and Immunity, 67*(9), 4320-4325.
16. Keijzer C, Slutter B, van der Zee R et al. (2011). PLGA, PLGA-TMC and TMC-TPP nanoparticles differentially modulate the outcome of nasal vaccination by inducing tolerance or enhancing humoral immunity. *Plos One, 6*
17. Kim BJ, Kayembe K, Simecka JW et al. (2011). Corticotropin-releasing hormone receptor-1 and 2 activity produces divergent resistance against stress-induced pulmonary

streptococcus pneumoniae infection. *Journal of Neuroimmunology, 237*(1-2), 57-65. doi: 10.1016/j.jneuroim.2011.06.016; 10.1016/j.jneuroim.2011.06.016

18. Kruisbeek AM. (2001). Isolation of mouse mononuclear cells. *Current Protocols in Immunology / Edited by John E.Coligan ...[Et Al.], Chapter 3*, Unit 3.1. doi: 10.1002/0471142735.im0301s39; 10.1002/0471142735.im0301s39
19. Lexau C. (2005). Changing epidemiology of invasive pneumococcal disease among older adults in the era of pediatric pneumococcal conjugate vaccine. *JAMA*, (294), 2043-2051
20. Lindell DM, Morris SB, White MP et al. (2011). A novel inactivated intranasal respiratory syncytial virus vaccine promotes viral clearance without Th2 associated vaccine-enhanced disease. *Plos One, 6*
21. Marriott H, Hellewell P, Cross S et al. (2006). Decreased alveolar macrophage apoptosis is associated with increased pulmonary inflammation in a murine model of pneumococcal pneumonia. *Journal of Immunology, 177*
22. Mukerjee, A., & Vishwanatha, J. K. (2009). Formulation, characterization and evaluation of curcumin-loaded PLGA nanospheres for cancer therapy. *Anticancer Research, 29*(10), 3867-3875.
23. Ranjan, A. P., Zeglam, K., Mukerjee, A., Thamake, S., & Vishwanatha, J. K. (2011). A sustained release formulation of chitosan modified PLCL:Poloxamer blend nanoparticles loaded with optical agent for animal imaging. *Nanotechnology*, *22*(29), 295104-4484/22/29/295104. Epub 2011 Jun 21. doi: 10.1088/0957-4484/22/29/295104; 10.1088/0957-4484/22/29/295104

24. Stano A, van der Vlies AJ, Martino MM et al. (2010). PPS nanoparticles as versatile delivery system to induce systemic and broad mucosal immunity after intranasal administration. *Vaccine, 29*

25. Tan S. (2012). Phenotype and function of conventional dendritic cells are not compromised in aged mice. *Immunology and Cell Biology*, doi: 10.1038/icb.2011.104

26. Thamake, S. I., Raut, S. L., Ranjan, A. P., Gryczynski, Z., & Vishwanatha, J. K. (2011). Surface functionalization of PLGA nanoparticles by non-covalent insertion of a homo-bifunctional spacer for active targeting in cancer therapy. *Nanotechnology, 22*(3), 035101-4484/22/3/035101. Epub 2010 Dec 9. doi: 10.1088/0957-4484/22/3/035101; 10.1088/0957-4484/22/3/035101

CHAPTER IV

DISCUSSION AND FUTURE DIRECTIONS

S. pneumoniae's pathogenicity is complex. In most cases, pneumococcal species can reside within the upper respiratory airways of most hosts as an asymptomatic commensal species. Transition of *S. pneumoniae* into the lower respiratory airways and/or its transmission of invasive serotypes to susceptible hosts can cause dangerous pulmonary infection leading to pneumonia and even worse, bacteremia. As vulnerable as the respiratory tract is to local infection, behaving potentially as the conduit for systemic disease, its intimate access to the external environment provides an advantage for the delivery of therapies locally at the initial site of infection. Hence, researchers are intensely investigating the potential of respiratory vaccines and the application of pharmacological drugs to prevent and treat respiratory disease. Based on this knowledge, studies were designed to increase our understanding of the efficacy in targeting therapies to the respiratory tract focusing on the delivery of CRH. In addition, studies were designed to determine the effectiveness of targeted delivery of a nanoparticle vaccine construct to increase protection against pneumococcal infection.

Prevention and treatment are the hallmarks of reducing health risks associated with infectious disease. Since the introduction of improved pneumococcal vaccines, we have witnessed a significant reduction in the incidence of pneumonia cases in the United States and worldwide [41].

In addition, pharmaceutical advances have led to significant reductions in the number of hospital visits and mortality rates for a majority of the population [70]. However, despite improved outcomes, certain populations remain at higher risk for contracting pneumonia caused by *S. pneumoniae* and are at increased risk of death [78]. Thus, further advances in vaccine development and novel treatment modalities are necessary to address this health problem. The purpose of this project was to investigate potential therapeutic approaches that could be utilized to combat pneumococcal infection. Our ***central hypothesis*** for the entire project was that interventions utilizing novel therapeutics, administered to the respiratory tract, will aid protection in terms of prevention through vaccination and treatment by decreasing mortality caused by *S. pneumoniae*.

Antibiotics and glucocorticoids, when used in concert, have become the standard of care for severe pneumonia caused by *S. pneumoniae* to eradicate infection and decrease exaggerated inflammatory responses, particularly in cases where antibiotics fail (e.g. bacterial resistance). Increasing antibiotic resistance and the expansion in the number of pneumococcal serotypes not covered by the current vaccines that cause invasive disease raises a critical need to develop novel therapies. To date, two major obstacles pose distinct but related threats to improving health outcomes associated with pneumococcal infection. They are: (1) the lack of optimal protection through vaccination and (2) an ineffective treatment against severe pneumonia that is responsible for one of the highest mortality rates in the U.S and worldwide.

In general, adjuvant treatment has proved effective for a majority of individuals. However, recent clinical trials provide inconclusive results regarding the benefit of glucocorticoid use in terms of reducing mortality rates [46, 47]. While differences in outcomes of glucocorticoid use could be attributed to numerous variables such as co-morbidity risks, dosage and length of time of treatment, there is also a noted consensus related to common pharmacological risks associated

with its use (e.g. hyperglycemia, immune suppression, and metabolic disorders). Such findings signify a need to identify alternative adjuvant therapies with a similar mode of action without harsh side effects.

In Chapter II, studies addressed **Specific Aim 1** of the project in determining the role of intranasal administration of CRH in mediating susceptibility to *S. pneumoniae*. Here, we tested the *hypothesis* that intranasal administration of CRH during *S. pneumoniae* infection in the lung would enhance anti-inflammatory response. By defining the role of intranasal administration of CRH in mediating cellular immune and inflammatory responses against *S. pneumoniae* infection, we highlight the relevance and consequences of immunopathology that often occurs independently of bacterial clearance. We have defined the role of CRH as a suppressor of cellular immune responses potentially by control of mechanisms responsible for cellular recruitment. In addition, studies suggest that despite the important role of neutrophils in bacterial clearance, CRH's protective efficacy is enhanced in their absence. Thus, although robust innate immune responses are important for the eradication of bacterial pathogens, preventing vigorous cellular inflammatory responses that potentially compromise lung integrity is equally relevant in reducing mortality risks. In support, we have demonstrated that CRH administration or antalarmin does not affect bacterial colonization in lung compared to untreated and dexamethasone treated mice (data not shown). However, preliminary histopathological results of lung tissue samples suggest decreased leukocyte infiltrate given CRH administration compared to antalarmin and dexamethasone treatments (data not shown). Further studies which define CRH's mechanism of action including cellular targets, mediation of cytokine production, and influences on lung vascular responses will benefit its therapeutic potential.

Inflammation is absolutely required for effective bacterial clearance. Robust activation of cellular immune constituents such as neutrophils and macrophages mediated by induction of inflammatory mediators work in concert to clear infection through phagocytosis and activation of ROS. Unfortunately, this can also become a defect in the natural occurrence during severe infection. The two main conditions of *S. pneumoniae* are grounded in the theoretical functions of the immune response's role in protecting host against unwanted pathogenic invasion. One being the initiation of non-specific innate immune responses and the other being fundamental ability to protect the host from contracting disease by way of vaccination. They both rid the host of potentially harmful pneumococcal serotypes and evoke long lasting immunity against invasive pneumococcal serotypes. Such principles have been the hallmark of advancing vaccines and adjuvant therapies to reduce the incidence and mortality associated with *S. pneumoniae* infection.

In theory, inducing local immune responses across the respiratory tract would be optimal against pneumonia caused by *S. pneumoniae* and other respiratory pathogens. However, more studies that test the efficacy of intranasal vaccination against *S. pneumoniae* is necessary because of a lack of information related to its potential benefit. In this study, we tested the efficacy of a preventative technique to pneumococcal infection utilizing a targeted nanoparticle vaccination approach. We hypothesized that local vaccination across the respiratory tract would increase the induction of protective immunity against *S. pneumoniae* infection. Thus, **Specific Aim 2** was to determine whether retention of intranasally administrated PLGA nanoparticles encapsulated with *S. pneumoniae*-whole antigen would increase protection against *S. pneumoniae*. We determined that the NP affected cytokine production and bacterial clearance. Our results validated our proposed method of cellular uptake of our NPs and their presentation to adaptive immune cells. In addition, we demonstrated that our NPs, once presented to the adaptive immune cells, created

a memory response against pneumococcal infection. Overall, we developed a preventative, therapeutic approach that could be promising towards the progression of this field. With these novel approaches addressing clinical prevention and treatment, we will be able to focus our future studies on techniques utilizing both CRH and NPs.

The greatest advantage of this project was the ability to track where the NPs were deposited in the lung following intranasal administration. This allowed us to not only confirm that our vaccination was deposited at its intended location, but we were also able to determine in a time dependent fashion how long it resided there. With advantages, projects also have limitations. Limitations do exist in this model specifically with the route of administration. Though intranasal administration is common, polymeric NPs have not been administered in human hosts. Though our ultimate goal for this project was to find more suitable therapeutic approaches for pneumococcal infection, further studies are needed to demonstrate suitability for human hosts. To translate into human use, we would need to construct an intranasal vaccine using an inactivated portion of the antigen to prevent the potential of infecting human hosts. This would mimic vaccines that are currently in use clinically. Though we have found a promising therapeutic approach utilizing NP as a delivery system, many future studies will need to take place before we translate it to human usage.

Our future studies will expound upon the results of our work in both chapter II (CRH and treatment therapy) and chapter III (NPs and preventative therapy). In chapter II we established that when CRH is present, the effect of neutrophils, if any, is masked. To clearly illustrate the activity of CRH our first study will be to perform a comprehensive analysis of the cellular immune and inflammatory response (flow cytometry, cytokines, function of cells in presence/absence of CRH. Our next set of studies will incorporate the use of antibiotics,

specifically penicillin and streptomycin +/- CRH and antagonist to confirm that the mechanism we have observed is related to CRH-R1. Similar to the studies in chapter II, these studies will analyze bacterial burden, survival outcomes, and immunological responses during infection. Our last set of experiments will involve the PLGA NPs, used in chapter III, where we will utilize specific antigens from the bacteria instead of total crude antigen as we have demonstrated. These studies will begin with the administration of the NP vaccine and upon challenging these animals they will be assessed for survival, bacterial clearance, and cellular response following this therapy. Our eventual goal is to find a therapeutic approach that will combat pneumococcal infection. By combining our usage of CRH and NP manipulation, we will reach our goal of a single therapeutic treatment for populations not covered through current vaccine strategies.

References

1. Abdel-Mottaleb, M. M., Neumann, D., & Lamprecht, A. (2011). Lipid nanocapsules for dermal application: A comparative study of lipid-based versus polymer-based nanocarriers. *European Journal of Pharmaceutics and Biopharmaceutics : Official Journal of Arbeitsgemeinschaft Fur Pharmazeutische Verfahrenstechnik e.V, 79*(1), 36-42.
2. Adcock IM, Caramori G, Kirkham PA. Strategies for improving the efficacy and therapeutic ratio of glucocorticoids. Curr Opin Pharmacol. 2012 Mar 23.
3. Agnihotri, S. A., & Aminabhavi, T. M. (2006). Novel interpenetrating network chitosan-poly(ethylene oxide-g-acrylamide) hydrogel microspheres for the controlled release of capecitabine. *International Journal of Pharmaceutics,324*(2), 103-115.
4. Ai, J., Biazar, E., Jafarpour, M., Montazeri, M., Majdi, A., Aminifard, S., et al. (2011). Nanotoxicology and nanoparticle safety in biomedical designs. *International Journal of Nanomedicine, 6*, 1117-1127.
5. Aliberti, S., & Kaye, K. S. (2013). The changing microbiologic epidemiology of community-acquired pneumonia. Postgraduate Medicine, 125(6), 31-42.
6. Almagro P, Cabrera FJ, Diez J, Boixeda R, Ortiz B, Murio C, Soriano JB; Working Group on COPD of the Spanish Society of Internal Medicine. Co morbidities and short-term prognosis in patients hospitalized for acute exacerbation of COPD. The ESMI study. Chest. 2012 Apr 5.
7. AlonsoDeVelasco, E., Verheul, A. F., Verhoef, J., & Snippe, H. (1995). Streptococcus pneumoniae: Virulence factors, pathogenesis, and vaccines. *Microbiological Reviews, 59*(4), 591-603.

8. Anton, P.M., et al., *Corticotropin-releasing hormone (CRH) requirement in Clostridium difficile toxin A-mediated intestinal inflammation.* Proc Natl Acad Sci U S A, 2004. 101(22): p. 8503-8.
9. Bawage SS, Tiwari PM, Pillai S, Dennis V, Singh SR (2013). Recent Advance in Diagnosis, Prevention, and Treatment of Human Respiratory Syncytial Virus. *Advances in Virology*, December.
10. Belocerkovskaja, J. G., Romanovskih, A. G., & Styrt, E. A. (2016). Pneumococcal vaccine in adults reduces the risk of infections caused by streptococcus pneumoniae. Klinicheskaia Meditsina, 94(1), 61-66.
11. Birjandi, S., & Witte, P. (2011). Why are the elderly so susceptible to pneumonia? *Expert Review of Respiratory Medicine, 5*(5), 593-595. doi:10.1586/ers.11.50; 10.1586/ers.11.50
12. Boyce, W.T., et al., *Psychobiologic reactivity to stress and childhood respiratory illnesses: results of two prospective studies.* Psychosom Med, 1995. 57(5): p. 411-22.
13. Bozkurt, A., Ghandour, S., Okboy, N., Oner, S., Arbak, S., Coskun, T., & Yegen, B. C. (2001). Inflammatory response to cold injury in remote organs is reduced by corticotropin-releasing factor. *Regulatory Peptides, 99*(2-3), 131-139.
14. Cao, J., Boucher, W., Kempuraj, D., Donelan, J. M., & Theoharides, T. C. (2006). Acute stress and intravesical corticotropin-releasing hormone induces mast cell dependent vascular endothelial growth factor release from mouse bladder explants. *The Journal of Urology, 176*(3), 1208-1213.
15. Cao, J., C.L. Cetrulo, and T.C. Theoharides, *Corticotropin-releasing hormone induces vascular endothelial growth factor release from human mast cells via the cAMP/protein*

kinase A/p38 mitogen-activated protein kinase pathway. Mol Pharmacol, 2006. 69(3): p. 998-1006

16. Cao, J., Papadopoulou, N., Kempuraj, D., Boucher, W. S., Sugimoto, K., Cetrulo, C. L., & Theoharides, T. C. (2005). Human mast cells express corticotropin-releasing hormone (CRH) receptors and CRH leads to selective secretion of vascular endothelial growth factor. *Journal of Immunology (Baltimore, Md.: 1950), 174*(12), 7665-7675.
17. Cassiere, H. A., & Niederman, M. S. (1998). Community-acquired pneumonia. *Disease-a-Month : DM, 44*(11), 613-675.
18. Chaniotou, Z., Giannogonas, P., Theoharis, S., Teli, T., Gay, J., Savidge, T., . . . Karalis, K. P. (2010). Corticotropin-releasing factor regulates TLR4 expression in the colon and protects mice from colitis. *Gastroenterology, 139*(6), 2083-2092.
19. Chomoucka, J., Drbohlavova, J., Huska, D., Adam, V., Kizek, R., & Hubalek, J. (2010). Magnetic nanoparticles and targeted drug delivering. *Pharmacological Research, 62*(2), 144-149.
20. Cohen, J. M., Khandavilli, S., Camberlein, E., Hyams, C., Baxendale, H. E., & Brown, J. S. (2011). Protective contributions against invasive streptococcus pneumoniae pneumonia of antibody and Th17-cell responses to nasopharyngeal colonisation. *PloS One, 6*(10), e25558.
21. Crofford, L.J., et al., *Local secretion of corticotropin-releasing hormone in the joints of Lewis rats with inflammatory arthritis.* J Clin Invest, 1992. 90(6): p. 2555-64.
22. De Pascale G, Bello G, Antonelli M., Steroids in severe pneumonia: a literature review. Minerva Anestesiol. 2011 Sep;77(9):902-10.

23. Deville S, Arous JB, Bertrand F, Borisov V, Dupuis L (2012). Efficacy of intranasal and spray delivery of adjuvanted live vaccine against infectious bronchitis virus in experimentally infected poultry. *Procedia in Vaccinology,* v6, p85-92.

24. Ellis JA, Gow SP, Waldner CL, Shields S, Wappel S, Bowers A, Lacoste S, Xu Z, Ball E (2016). Comparative efficacy of intranasal and oral vaccines against *Bordetella bronchiseptica* in dogs. *The Veterinary Journal,* v. 212, p71-77.

25. Frank U, Tacconelli E (2012). Classification of the Antibiotics. *The Daschner Guide to In-Hospital Antibiotic Therapy European Standards*, 300 p20.

26. Frei CR, Bell AM, Traugott KA, Jaso TC, Daniels KR, Mortensen EM, Restrepo MI, Oramasionwu CU, Ruiz AD, Mylchreest WR, Sikirica V, Raut MR, Fisher A, Schein JR. A clinical pathway for community-acquired pneumonia: an observational cohort study. BMC Infect Dis. 2011 Jul 6;11:188.

27. Gay J, Kokkotou E, O'Brien M, Pothoulakis C, Karalis KP., Corticotropin-releasing hormone deficiency is associated with reduced local inflammation in a mouse model of experimental colitis. Endocrinology. 2008 Jul;149(7):3403-9. Epub 2008 Apr 10

28. Garibaldi RA. (1985). Epidemiology of community-acquired respiratory tract infections in adults. Incidence, etiology, and impact. *The American Journal of Medicine*, June28;78(6B):32-7.

29. Gonzales, X. F., Deshmukh, A., Pulse, M., Johnson, K., & Jones, H. P. (2008). Stress-induced differences in primary and secondary resistance against bacterial sepsis corresponds with diverse corticotropin releasing hormone receptor expression by pulmonary CD11c+ MHC II+ and CD11c- MHC II+ APCs. *Brain, Behavior, and Immunity, 22*(4), 552-564.

30. Gonzalez-Rey, E., Fernandez-Martin, A., Chorny, A., & Delgado, M. (2006). Therapeutic effect of urocortin and adrenomedullin in a murine model of crohn's disease. *Gut, 55*(6), 824-832.

31. Gould K (2016). Antibiotics: from prehistory to the present day. *Journal of Antimicrobial Chemotherapy*, 71:572-575.

32. Gysling, K., et al., *Corticotropin-releasing hormone and urocortin: redundant or distinctive functions?* Brain Res Brain Res Rev, 2004. 47(1-3): p. 116-25.

33. Holmkvist AD, Friberg A, Nilsson UJ, Schouenborg J. Hydrophobic ion pairing of a minocycline/Ca^{2+}/AOT complex for preparation of drug-loaded PLGA nanoparticles with improved sustained release. *International Journal of Pharmaceutics*, Volume 499, Issues 1-2 29 February 2016, p351-357.

34. Ivy, A. S., Rex, C. S., Chen, Y., Dube, C., Maras, P. M., Grigoriadis, D. E., . . . Baram, T. Z. (2010). Hippocampal dysfunction and cognitive impairments provoked by chronic early-life stress involve excessive activation of CRH receptors. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 30*(39), 13005-13015.

35. Jabir NR, Tabrez S, Ashraf GM, Shakil S, Damanhouri GA, Kamal MA (2012). Nanotechnology-based approaches in anticancer research. *International Journal of Medicine*,7, p4391-4408.

36. Jain, R., et al., *Corticotropin-releasing factor modulates the immune response to stress in the rat.* Endocrinology, 1991. 128(3): p. 1329-36.

37. Jessop, D.S., M.S. Harbuz, and S.L. Lightman, *CRH in chronic inflammatory stress.* Peptides, 2001. 22(5): p. 803-7.

38. Jinno S, Jacobs MR. Pneumonia Due to Drug-Resistant Streptococcus pneumonia, Curr Infect Dis Rep. 2012 Jun;14(3):292-9

39. Jose RJ, Periselneris JN, Brown JS (2015). Community-acquired pneumonia. *Current Opinion in Pulmonary Medicine,* p212-218.

40. Joubert MK, Hokom M, Eakin C, Zhou L, Deshpande M, Baker MP, Goletz TJ, Kerwin BA, Chirmule N, Narhi LO, Jawa V. Highly aggregated antibody therapeutics can enhance the in vitro innate and late-stage T-cell immune responses, J Biol Chem. 2012 May 14.

41. Kadioglu, A., Weiser, J., Paton, J., & Andrew, P. (2008). The role of streptococcus pneumoniae virulance factors in host respiratory colonization and disease. *Nat Rev Microbiol, 6*

42. Karalis, K., Sano, H., Redwine, J., Listwak, S., Wilder, R. L., & Chrousos, G. P. (1991). Autocrine or paracrine inflammatory actions of corticotropin-releasing hormone in vivo. *Science (New York, N.Y.), 254*(5030), 421-423.

43. Khan MN, Pichichero ME (2014). The host immune dynamics of pneumococcal colonization: Implications for novel vaccine development. *Human Vaccines & Immunotherapeutics,* volume 10, p3688-3699.

44. Kim, B. J., Kayembe, K., Simecka, J. W., Pulse, M., & Jones, H. P. (2011). Corticotropin-releasing hormone receptor-1 and 2 activity produces divergent resistance against stress-induced pulmonary streptococcus pneumoniae infection. *Journal of Neuroimmunology, 237*(1-2), 57-65.

45. Kozakiewicz L, Phuah J, Flynn J, Chan J (2013). The Role of B Cells and Humoral Immunity in *Mycobacterium tuberculosis* Infection. *Advances in Experimental Medicine and Biology*, 783, p225-250.
46. Krüger S, Welte T. Biomarkers in community-acquired pneumonia, Expert Rev Respir Med. 2012 Apr;6(2):203-14.
47. Kumar A. Optimizing antimicrobial therapy in sepsis and septic shock, Crit Care Nurs Clin North Am. 2011 Mar;23(1):79-97.
48. Kumari, A., Yadav, S. K., & Yadav, S. C. (2010). Biodegradable polymeric nanoparticles based drug delivery systems. *Colloids and Surfaces.B, Biointerfaces, 75*(1), 1-18.
49. Lee N, Hui DS. Dexamethasone in community-acquired pneumonia, Lancet. 2011 Sep 10;378(9795):979-80; author reply 981.
50. Leung DY, Bloom JW: Update on glucocorticoid action and resistance. J Allergy Clin Immunol 2003, 111:3-22. Brain Res Rev, 2004. 47(1-3): p. 116-25.
51. Li, Y., Hill, A., Beitelshees, M., Shao, S., Lovell, J. F., Davidson, B. A., et al. (2016). Directed vaccination against pneumococcal disease. Proceedings of the National Academy of Sciences of the United States of America, 113(25), 6898-6903.
52. Ma XC, Chen LT, Oliver J, Horvath E, Phelps CP. Cytokine and adrenal axis responses to endotoxin. Brain Res. 2000 Apr 7;861(1):135-42.
53. Malley, R., Trzcinski, K., Srivastava, A., Thompson, C. M., Anderson, P. W., & Lipsitch, M. (2005). CD4+ T cells mediate antibody-independent acquired immunity to pneumococcal colonization. *Proceedings of the National Academy of Sciences of the United States of America, 102*(13), 4848-4853.

54. Meijvis SC, Hardeman H, Remmelts HH, Heijligenberg R, Rijkers GT, van Velzen-Blad H, Voorn GP, van de Garde EM, Endeman H, Grutters JC, Bos WJ, Biesma DH. Dexamethasone and length of hospital stay in patients with community-acquired pneumonia: a randomised, double-blind, placebo-controlled trial, Lancet. 2011 Jun 11;377(9782):2023-30. Epub 2011 Jun 1.

55. Mudshinge, S. R., Deore, A. B., Patil, S., & Bhalgat, C. M. (2011). Nanoparticles: Emerging carriers for drug delivery. Saudi Pharmaceutical Journal : SPJ : The Official Publication of the Saudi Pharmaceutical Society, 19(3), 129-141.

56. Muglia, L. J., Jacobson, L., Weninger, S. C., Karalis, K. P., Jeong, K., & Majzoub, J. A. (2001). The physiology of corticotropin-releasing hormone deficiency in mice. *Peptides, 22*(5), 725-731.

57. Murphy SL, Kochanek D, Xu J, Aria E (2015). Mortality in the United States, 2014. *Centers for Disease Control and Prevention, National Center for Health Statistics Data Brief,* No. 229, December.

58. O'Kane, M., Murphy, E. P., & Kirby, B. (2006). The role of corticotropin-releasing hormone in immune-mediated cutaneous inflammatory disease. *Experimental Dermatology, 15*(3), 143-153.

59. Peng, X. L., Xu, X. J., Small, M., Fu, X., & Jin, Z. (2016). Prevention of infectious diseases by public vaccination and individual protection. *Journal of Mathematical Biology.*

60. Perrin MH, Sutton SW, Cervini LA, Rivier JE, Vale WW. Comparison of an agonist, urocortin, and an antagonist, astressin, as radioligands for characterization of corticotropin-releasing factor receptors, J Pharmacol Exp Ther. 1999 Feb;288(2):729-34.

61. Ponnappan S, Ponnappan U (2011). Aging and Immune Function: Molecular Mechanisms to Interventions. *Antioxidants & Redox Signaling*, 14(8), p1551-1585.

62. Potter, E., Behan, D. P., Linton, E. A., Lowry, P. J., Sawchenko, P. E., & Vale, W. W. (1992). The central distribution of a corticotropin-releasing factor (CRF)-binding protein predicts multiple sites and modes of interaction with CRF. *Proceedings of the National Academy of Sciences of the United States of America*, *89*(9), 4192-4196.

63. Radulovic, M., et al., *Corticotropin-releasing factor receptor 1 in mouse spleen: expression after immune stimulation and identification of receptor-bearing cells.* J Immunol, 1999. 162(5): p. 3013-21.

64. Refojo D, Holsboer F, CRH signaling, Molecular specificity for drug targeting in the CNS, 2009, Ann N Y Acad Sci; Oct 1179; 106-19.

65. Rhen, T., & Cidlowski, J. A. (2005). Antiinflammatory action of glucocorticoids--new mechanisms for old drugs. *The New England Journal of Medicine*, *353*(16), 1711-1723.

66. Rodgers, G. L., & Klugman, K. P. (2011). The future of pneumococcal disease prevention. *Vaccine*, *29 Suppl 3*, C43-8.

67. Sasayama, D., Hori, H., Iijima, Y., Teraishi, T., Hattori, K., Ota, M., . . . Kunugi, H. (2011). Modulation of cortisol responses to the DEX/CRH test by polymorphisms of the interleukin-1beta gene in healthy adults. *Behavioral and Brain Functions : BBF*, *7*, 23-9081-7-23.

68. Sauce D, Larsen M, Fastenackels S, Duperrier A, Keller M, Grubeck-Loebenstein B, Ferrand C, Debre P, Sidi D, Appay V (2009). Evidence of premature immune aging in patients thymectomized during early childhood. *The Journal of Clinical Investigation*, 119(10), p3070-3078.

69. Schuster, K. M., Macleod, J. B., Fernandez, J. B., Kumar, M., & Barquist, E. S. (2012). Adrenocorticotropic hormone and cortisol response to corticotropin releasing hormone in the critically ill-a novel assessment of the hypothalamic-pituitary-adrenal axis. *American Journal of Surgery, 203*(2), 205-210.

70. Sethi S, Murphy TF, Infection in the pathogenesis and course of chronic obstructive pulmonary disease., N Engl J Med. 2008 Nov 27;359(22):2355-65.

71. S., Silverman, E. S., Palmer, L. J., Lazarus, R., Silverman, E. K., et al. (2004). Corticosteroid pharmacogenetics: Association of sequence variants in CRHR1 with improved lung function in asthmatics treated with inhaled corticosteroids. *Human Molecular Genetics, 13*(13), 1353-1359.

72. Silverman, E.S., et al., *Corticotropin-releasing hormone deficiency increases allergen-induced airway inflammation in a mouse model of asthma.* J Allergy Clin Immunol, 2004. 114(4): p. 747-54.

73. Skrupky, L. P., Kerby, P. W., & Hotchkiss, R. S. (2011). Advances in the management of sepsis and the understanding of key immunologic defects. *Anesthesiology, 115*(6), 1349-1362.

74. Southworth T, Metryka A, Lea S, Farrow S, Plumb J, Singh D.IFNγ synergistically enhances LPS signalling in alveolar macrophages from COPD patients and controls by corticosteroid resistant STAT1 activation. Br J Pharmacol. 2012 Feb 21.

75. Starfield, B., Shi, L., & Macinko, J. (2005). Contribution of primary care to health systems and health. The Milbank Quarterly, 83(3), 457-502.

76. Sternberg EM, Licinio J. Overview of neuroimmune stress interactions. Implications for susceptibility to inflammatory disease, Ann N Y Acad Sci. 1995 Dec 29;771:364-71.

77. Sun, K., et al., Analysis of murine genetic predisposition to pneumoccocoal infection reveals a critical role of alveolar macrophages in maitnaining the sterility of thelower respriatro tract, Infect Immun. 2011 May;79(5):1842-7.

78. Tali-Maamar, H., Laliam, R., Bentchouala, C., Touati, D., Sababou, K., Azrou, S., . . . Rahal, K. (2012). Serotyping and antibiotic susceptibility of streptococcus pneumoniae strains isolated in algeria from 2001 to 2010. *Medecine Et Maladies Infectieuses, 42*(2), 59-65.

79. Tsatsanis, C., et al., *Corticotropin releasing factor receptor 1 (CRF1) and CRF2 agonists exert an anti-inflammatory effect during the early phase of inflammation suppressing LPS-induced TNF-alpha release from macrophages via induction of COX-2 and PGE2.* J Cell Physiol, 2007. 210(3): p. 774-83.

80. Vlahos R, Wark PA, Anderson GP, Bozinovski S.Glucocorticosteroids Differentially Regulate MMP-9 and Neutrophil Elastase in COPD. PLoS One. 2012;7(3):e33277. Epub 2012 Mar 7.

81. Wang, W., Zhang, X., Yang, L., Liu, D., Liu, G., & Zhou, J. (2012). Lipopolysaccharide upregulates the expression of corticotropin-releasing hormone via MAP kinase pathway in rat peritoneal macrophages. *Molecular and Cellular Biochemistry, 361*(1-2), 1-7.

82. Webster, E. L., Torpy, D. J., Elenkov, I. J., & Chrousos, G. P. (1998). Corticotropin-releasing hormone and inflammation. *Annals of the New York Academy of Sciences, 840*, 21-32.

83. Wilczewska, A. Z., Niemirowicz, K., Markiewicz, K. H., & Car, H. (2012). Nanoparticles as drug delivery systems. Pharmacological Reports : PR, 64(5), 1020-1037.

84. Woods, R. J., David, J., Baigent, S., Gibbins, J., & Lowry, P. J. (1996). Elevated levels of corticotrophin-releasing factor binding protein in the blood of patients suffering from arthritis and septicaemia and the presence of novel ligands in synovial fluid. *British Journal of Rheumatology, 35*(2), 120-124.

85. Wunderink RG, Adjunctive therapy in community-acquired pneumonia. Semin Respir Crit Care Med. 2009 Apr;30(2):146-53. Epub 2009 Mar 18.

86. Zhu, H., Wang, J., Li, J., & Li, S. (2011). Corticotropin-releasing factor family and its receptors: Pro-inflammatory or anti-inflammatory targets in the periphery? *Inflammation Research : Official Journal of the European Histamine Research Society ...[Et Al.], 60*(8), 715-721.

87. Zmijewski, M. A., & Slominski, A. T. (2010). Emerging role of alternative splicing of CRF1 receptor in CRF signaling. *Acta Biochimica Polonica, 57*(1), 1-13.