REGULATION OF INFLAMMATION, LUNG INJURY AND AUTOPHAGY DURING *PSEUDOMONAS AERUGINOSA* LUNG INFECTION

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ABSTRACT:

Pseudomonas aeruginosa is an opportunistic bacterial pathogen which causes acute and chronic infections in immune compromised individuals, including cystic fibrosis (CF) patients where it represents the leading cause of morbidity and mortality. Early inflammatory responses are sufficient to clear *P. aeruginosa* infections in healthy individuals. However dysregulation of these responses in many disease states greatly increases susceptibility to infection with the bacteria. Herein we identify regulator of calcineurin-1 and calcineurin Aβ as novel negative and positive regulators of *P. aeruginosa* induced inflammation respectively. Furthermore these studies reveal substantial cross talk between pathways leading to activation of the inflammatory transcription factors NFAT and NFκB which creates biologically significant signaling redundancy following *P. aeruginosa* lung infection. Other components of innate immunity against *P. aeruginosa* are also explored; including a novel role for the evolutionarily conserved catabolic process autophagy during bacterial clearance. Combined with the recent landmark findings that autophagy is impaired in the lungs of CF patients, these findings highlight an emerging potential for 'autophagy restorative therapy' in the treatment of CF associated lung infections. Finally, a protective role for mast cells during *P. aeruginosa* induced acute lung injury is revealed. Together these findings greatly enhance our understanding of mechanisms of host defense during early innate immune responses against *P. aeruginosa* bacteria, and reveal novel opportunities for therapeutic intervention which could significantly decrease *P. aeruginosa* associated morbidity and mortality.

LIST OF ABBREVIATIONS AND SYMBOLS USED:

ALI, acute lung injury

ARDS, acute respiratory distress syndrome

ASK1, apoptosis signal regulating kinase 1

ATG, autophagy related gene

BALF, bronchoalveolar lavage fluid

BMM, bone marrow derived macrophage

BMMC, bone marrow derived mast cell

BECN1, Beclin-1

cAMP, cyclic adenosine monophosphate

CF, cystic fibrosis

CFTR, cystic fibrosis transmembrane conductance regulator

CFU, colony forming unit

Cn, calcineurin

CQ, chloroquine

COX-2, cyclooxygenase-2

CTAC, cetyltrimethylammonium chloride

DAMP, danger associated molecular pattern

DS, Down syndrome

eEF2A, eukaryotic elongation factor 2A

ELISA, enzyme linked immunosorbent assay

EMSA, electrophoretic mobility shift assay

EPS, extracellular polymeric substrate

ERK-1/2, extracellular regulated kinase 1 and 2

GFP, green fluorescent protein

GLIPR2, Gli pathogenesis-related 2,

GM-CSF, granulocyte macrophage colony stimulating factor

GSH, glutathione

HDAC, histone deacetylase

hpi, hours post infection

H&E, hematoxylin-eosin

IFN, Interferon

IKK, IκB kinase

IL, Interleukin

iNOS, inducible nitric oxide synthase

IP_3, inositol triphosphate

IRAK, interleukin-1 receptor kinase

IRF, interferon regulatory factor

JAK, Janus activated kinase

JNK, Jun N-terminal kinase

KC, keratinocyte chemoattractant

LAP, LC3 associated phagocytosis

LC3, microtubule associated protein light chain 3

LIX, LPS induced CXC chemokine

LPS, Lipopolysaccharide

LRRK2, the leucine-rich repeat kinase 2

MAC, *Mycobacterium avium* complex

MAPK, mitogen activated protein kinase

MAP2K, mitogen activated protein kinase kinase

MAP3K, mitogen activated protein kinase kinase kinase

MDR, multiple-drug resistant

MIP-2, macrophage inflammatory protein-2

mMCP-6, murine mast cell protease 6

MOI, multiplicity of infection

MPO, myeloperoxidase

MRSA, methicillin resistant *Staphylococcus aureus*

Mtb, mycobacterium tuberculosis

mTOR, mechanistic target of rapamycin

MyD88, myeloid differentiation primary-response protein 88

NAC, N-acetylcyteine

NBR-1, neighbor of BRCA gene 1

NDP52, nuclear dot protein 52 kDa

NEMO, NFκB essential modulator

NET, neutrophil extracellular trap

NFAT, nuclear factor of activated T cells

NFκB, nuclear factor kappa B

NLR, NOD-like receptor

NLRC4, NLR family CARD-domain containing protein 4

NO, nitric oxide

NOD, nucleotide-binding oligomerization domain-containing protein

NTHi, nontypeable *Haemophilus influenzae*

NTM, non-tuberculosis mycobacterium

OdDHL, N-(3-oxododecanoyl)-L-homoserine lactone

Opr, outer membrane protein

PAMP, pathogen associated molecular pattern

PBS, phosphate buffered saline

PE, phosphatidylethanolamine

PI3K, phosphatidylinositol 3 kinase

PI3P, phosphatidylinositol 3 phosphate

PLC, phospholipase C

PMN, polymorphonuclear leukocyte

PQS, *Pseudomonas* quinolone signal

PRR, pattern recognition receptor

Rap, rapamycin

RBC1CC1, RB1-inducible coiled-coil 1

RCAN1, regulator of calcineurin-1

RIP-2, receptor interacting protein-2

ROS, reactive oxygen species

shRNA, short hairpin ribonucleic acid

STAT, signal transducer and activator of transcription

SUMO, small ubiquitin-like modifier

TAK1, transforming growth factor- β activated kinase 1

TBK-1, tank-binding kinase-1

TCR, T cell receptor

TECPR1, tectonin-domain containing protein 1

TEER, transepithelial electrical resistance

TG2, tissue transglutaminase 2

TGF-β, transforming growth factor- β

TIM4, T cell immunoglobulin mucin 4

TIR, toll-interleukin 1 receptor

TIRAP, TIR adapter protein

TJ, tight junction

TLR, toll-like receptor

TGN, *trans*-Golgi network

TNF, tumor necrosis factor

TPL2, tumor progression locus 2

TRAM, TRIF related adapter protein

TRAP, TNF receptor associated factor

ULK, Unc 51-like kinase

CHAPTER 1: INTRODUCTION

1.1 *Pseudomonas aeruginosa:*

Pseudomonas aeruginosa is an environmentally ubiquitous Gram negative bacterium which commonly colonizes multiple niches both natural and artificial, such as surfaces and water supplies throughout homes, workplaces and hospitals[1, 2]. Although relatively harmless to immune competent individuals, immune dysfunction can lead to severe acute and chronic *P. aeruginosa* infections among immune compromised patients.

1.1.1 Clinical manifestations of *P. aeruginosa* infections

In hospital settings *P. aeruginosa* represents an extremely common nosocomial pathogen and is associated with severe acute infections in conditions where anatomical barriers to infection are compromised. *P. aeruginosa* is the leading cause of acute local and systemic infections in burn patients where it accounts for between 30-35% of all infections, and is associated with considerable mortality[3, 4]. Similarly, acute *P. aeruginosa* infection is the third most common cause of catheter associated urinary tract infections[5, 6] and is a leading cause of ventilator associated pneumonia[7, 8]. However the greatest threat posed by *P. aeruginosa* is to cystic fibrosis (CF) patients where chronic respiratory tract infections with the bacterium represents the leading cause of morbidity and mortality[9, 10].

P. aeruginosa infection is established in the lungs of CF patients through direct contact with carriers of the bacteria, or more commonly through colonization with wild-type planktonic strains from environmental reservoirs[11]. Bacteria that colonize the lungs early during the course of CF associated lung infection display relatively low antibiotic resistance, providing an opportunity for early therapeutic intervention[12]. However over

time the bacteria become increasingly antibiotic resistant, and establishes an intermittent, or transient infection, followed inevitably by chronic infection in the lower respiratory tract[13]. This final stage of *P. aeruginosa* infection is associated with chronic inflammation in the lungs causing tissue damage and fibrosis which leads to deteriorating lung function, and ultimately death[14].

1.1.2 Impacts of *P. aeruginosa* virulence factors on host defense

P. aeruginosa has a wide arsenal of virulence factors which it uses both to establish infection, and to evade the immune system[15]. These virulence factors can be separated into three main categories: cell surface proteins, secreted factors and exoenzymes injected into host cells via a type III secretion system. Amongst the cell surface virulence factors flagella and pili contribute both to bacterial mobility and to adhesion, facilitating initial colonization by the bacteria[16, 17]. Lipopolysaccharide (LPS) is another cell surface virulence factor that plays a role in bacterial adhesion at the site of infection[18]. The secreted polysaccharide alginate is also involved in adhesion, and contributes to antibiotic resistance and biofilm formation[19, 20]. Finally, the outer membrane protein F (OprF) can bind interferon gamma (IFNγ), decreasing the local concentration of the cytokine to inhibit the host immune response[21].

The second category of virulence factors utilized by *P. aeruginosa* are secreted virulence factors that play roles in inhibiting host immune responses. *P. aeruginosa* produce pyrocyannin during infection which induces neutrophil apoptosis and leads to impaired clearance of the invading bacteria. Production of pyrocyannin has been shown to be essential for *P. aeruginosa* virulence *in vivo*[22, 23]. Elastase is a secreted virulence factor that can destroy tight junctions between epithelial cells and facilitate bacterial

invasion[24]. Elastase is also involved in evading the immune response as it cleaves iC3b[25], CR1[25], IgG[26, 27] and FCγRIIIB[28], the receptor that binds IgG1 and IgG3. The proteolysis of these proteins effectively prevents opsinophagocytic killing of the bacteria as mediated through both complement, and IgG dependent pathways. The secreted virulence factor exotoxin A (ExoA) mediates localized cell death through inhibition of protein synthesis in surrounding cells, leading to increased bacterial virulence[29, 30]. Finally, *Pseudomonas* secretes a number of quorum sensing molecules including N-(3-oxododecanoyl)-L-homoserine lactone (OdDHL), and *Pseudomonas* quinolone signal (PQS). These factors have been identifies as skewing cytokine production towards a Th2 response. Clinically, Th2 responses are associate with poor clinical outcomes[31].

The type III secretion system is also responsible for the delivery of a number of virulence factors directly into host cells during *P. aeruginosa* infection[32]. This needle like secretion system is used to inject at least 4 compounds directly into host cells. These compounds are ExoS[33] and ExoT[34] which lead to actin skeleton rearrangement, ExoU[35] which is involved in cytotoxicity and ExoY, which increases cellular levels of cAMP[36]. Together the activity of these exotoxins has a cytotoxic effect on host cells, and increases epithelial permeability at the site of infection.

1.1.3. *P. aeruginosa* biofilm formation and antibiotic resistance

One of the major challenges in treating *P. aeruginosa* infections is that many strains of the bacteria are becoming increasingly resistant to conventional antibiotic therapy[37, 38]. Resistant strains arise both due to intrinsic resistances and due to overuse of antibiotics, eventually selecting for multiple-drug resistant (MDR) strains[39]. The severe risk that these MDR strains pose is exemplified by the Liverpool epidemic strain that

possess antibiotic resistance and increased virulence, allowing for the infection of non-immunosuppressed individuals[40, 41].

P. aeruginosa displays a wide array of intrinsic mechanisms for subverting antibiotic killing that result in a high baseline resistance to many conventional classes of antibiotics. *P. aeruginosa* has an outer membrane that is considerably less permeable than other Gram negative bacteria[42]. This decreased membrane permeability, combined with the constitutive and inducible expression of various efflux pumps greatly limits intracellular concentrations of many classes of antibiotics[43]. Finally, *P. aeruginosa* produces β-lactamase (ApmC) that degrades β-lactam based antibiotics[44].

Perhaps the most effective mechanism employed by *P. aeruginosa* to resist antibiotics is the formation of biofilms. Biofilms are highly organized three dimensional communities of bacteria encapsulated within dense extracellular polymeric substrate (EPS) composed of polysaccharide, lipid, nucleic acid and protein. Incorporation of bacteria into these structures confers substantial resistance to physical forces, including mucociliary clearance, and excludes toxic chemicals such as antibiotics[45, 46]. Formation of *P. aeruginosa* biofilms is dependent upon a conversion of bacterial phenotypes from planktonic, to a non-motile (sessile) mucoid form. Biofilm formation begins with near irreversible adhesion of the bacteria to a surface mediated by flagella, pili and cup fimbria[47]. Adherent bacteria then give rise to microcolonies where high concentrations of quorum sensing molecules orchestrate transcriptional changes leading to conversion from planktonic to mucoid phenotypes. Chief among these changes are loss of flagella[48], modification LPS lipid-A moiety[49] and O-antigen[50], as well as production of thick EPS.

Major components of EPS include one or more of three polysaccharides: alginate, Pel polysaccharide, and Psl polysaccharide[51].

1.1.4. Antimicrobial therapies for *P. aeruginosa* lung infection

Given the highly antibiotic nature of *P. aeruginosa* aggressive combinational antibiotic therapy is the current standard of care. The American Thoracic Society consensus guidelines on the management of community-acquired pneumonia in adults stipulates that when *P. aeruginosa* lung infection is suspected initial treatment should include an antipseudomonal β-lactam (pipericillin-tazobactam, cefepime, imipenem, meropenem) in combination with either ciprofloxacin, an aminoglycoside (gentamycin, streptomycin) and azithromycin, or an aminoglycoside and an antipneumococcal fluoroquinolone[52]. Cultures should be performed to confirm the presence of *P. aeruginosa,* and to determine the antibiotic susceptibilities of the strain, after which antibiotic therapies can be adjusted accordingly. Although these treatments can be effective at eradicating bacteria during the early stages of colonization in the lungs of CF patients, long term treatment results both in the selection of MDR strains of *P. aeruginosa*, and is associated with significant negative off target effects ultimately leading to poor clinical outcomes in these individuals[12].

1.2 Mechanisms of host defense during *P. aeruginosa* lung infection

P. aeruginosa does not cause severe infections in immune competent individuals due to a tightly coordinated innate immune response. Upon entering the lungs of the host, the first line of defense encountered by the invading bacteria is the respiratory epithelium. The primary function of the respiratory epithelium is to act as an anatomical

barrier preventing dissemination of the bacteria throughout the body. The lungs are also populated with resident leukocytes, including mast cells and alveolar macrophages, that act as the second line of defense both through direct killing of the bacteria, and through initiating controlled inflammatory responses. The production of inflammatory mediators by resident leukocytes, and to a lesser extent by the respiratory epithelium, leads to the recruitment of neutrophils to the site of infection that represent the third line of defense during *P. aeruginosa* infection. The recruited neutrophils are responsible for the bulk of bacterial clearance. Dysregulation of any one of these innate immune mechanisms is associated with increased susceptibility to *P. aeruginosa* infection. Each of these components is reviewed in greater detail below.

1.2.1. First line of defense: Airway Epithelium

The airway epithelium is comprised of 8 distinct epithelial cell types broadly classified as ciliated, secretory or basal[53]. The composition of the airway epithelium changes dramatically with increased branching and transitions from a pseudostratified epithelium in the large airways, to columnar and cuboidal in the small airways, and finally to an elongated monolayer of type I (ciliated) and II (secretory) pneumocytes in the alveoli[54]. The primary immunological function of the respiratory epithelium is to act as an anatomical barrier, preventing invading pathogens from entering the body. This mechanical barrier function is augmented by secretion of surfactant by secretory epithelial cells that traps invading microorganisms and prevents their adhesion to airway epithelial cells. Also the mucociliary escalator activity of ciliated epithelial cells which serves to clear the organism from the lungs[55].

The airway epithelium is impermeable to invading organisms due to the formation of tight junctions (TJs) between adjacent cells. TJs are comprised of multiple integral membrane, and peripheral membrane proteins that establish a water, solute, and pathogen impermeable barrier (barrier function) and establish apical and basolateral polarity (fence function)[56]. Amongst integral membrane TJ proteins occludin is the most widely expressed and serves as the most reliable histological marker of TJs[56]. The barrier and fence functions of the airway epithelium also play an important role in establishing chemokine gradients, leading to the selective recruitment of immune cells to the site of infection.

In addition to barrier function the airway epithelium also plays an important role in the regulation of inflammatory responses to invading pathogens. Upon *P. aeruginosa* infection the respiratory epithelium is an important source of the neutrophil chemoattractant interleukin-8 (IL-8) along with its murine analogues macrophage inflammatory protein-2 (MIP-2/CXCL-2), keratinocyte chemoattractant (KC/CXCL-1) and LPS induced CXC chemokine (LIX/CXCL5)[57], as well as IL-6[58], CCL-5 (RANTES)[58], and granulocyte macrophage colony stimulating factor (GM-CSF)[58]. Together with other cells types discussed below, these inflammatory mediators play a critical role in an effective host response to *P. aeruginosa*.

The final innate immune function of the airway epithelium during *P. aeruginosa* infection involves the direct internalization and killing of the bacteria[59-64]. Uptake of the bacteria into host epithelial cells occurs through both cystic fibrosis transmembrane conductance regulator (CFTR) dependent[61-64] and independent[60] mechanisms and plays a role in bacterial killing early in infection. In turn, *P. aeruginosa* evolved mechanisms to

disrupt endosomal trafficking and fusion[65, 66] suggesting that it may be capable of partially subverting lysosmal killing in epithelial cells. This view is consistent with emerging literature that suggests that *P. aeruginosa* can persist within host epithelial cells for extended periods of time (> 48 hours), and that this intracellular niche promotes conversion from planktonic to biofilm associated phenotypes[67].

The critical function of the respiratory epithelium during *P. aeruginosa* infection is demonstrated in CF where etiological mutations in CFTR result in the accumulation of thick mucus within the lungs, and impaired mucociliary clearance of the pathogen[68]. In addition, CFTR mutations lead to both impaired inflammatory responses to invading pathogens and chronic inflammation within the lungs leading to disruptions in epithelial barrier function[69]. Epithelial cells harboring mutations in CFTR have been shown to have both impaired internalization and impaired killing of *P. aeruginosa* bacteria[61-64]. Together these defects in innate immune function of the airway epithelium creates an environment permissive to chronic *P. aeruginosa* infection.

1.2.2 Second line of defense: resident leukocytes

Although the airway epithelium contributes to *P. aeruginosa* induced inflammation, the primary coordinators of the early phase innate immune response against the pathogen are resident leukocytes. Leukocytes contribute to host defense by direct phagocytosis and killing of the bacteria, as well as through production of inflammatory, and immunoregulatory mediators. The contributions of these cells are discussed in greater detail below.

1.2.2.1 Mast cells

Mast cells arrive at mucosal sites as immature precursor cells, and complete their differentiation once recruited to the tissue in which they become resident[70]. As a result, mast cell phenotypes are extremely plastic, and are largely determined by the biochemical nature of the tissue in which they reside[71-74]. Within the lungs mast cells are abundant and protrude into the air space where they represent 2-3% of the alveolar wall[75]. Mast cells are ideally placed to act as sentinel cells of the immune system, and are key regulators of both innate and adaptive immunity[76, 77].

In order to act as effective sentinel cells of the immune system mast cell responses need to be both diverse, in order to protect against the spectrum of pathogens routinely encountered within the airways, and specific, in order to tailor the immune response to each individual pathogen. To this end mast cells are equipped with an arsenal of pattern recognition and Fc receptors[78] that can trigger expression of numerous inflammatory and immunomodulatory factors which include preformed granules containing histamine and proteases as well as *de novo* synthesized cytokines and arachidonic acid metabolites. Furthermore, mast cells can influence surrounding cells through both the production of soluble mediators and cell to cell contact[79-81]. As a result, mast cell responses are tightly regulated and highly pathogen specific.

With respect to the role of mast cells during *P. aeruginosa* infection *in vivo* relatively little is known. Our only insights come from a recent study which employed the mast cell stabilizer cromalyn to conclude that mast cell degranulation contributes to *P. aeruginosa* derived LPS induced airway inflammation[82]. In contrast, a number of studies have examined mast cell responses to *P. aeruginosa in vitro*. These studies have demonstrated that, upon stimulation with *P. aeruginosa,* mast cells produce the pro-

inflammatory mediators IL-1α[77], IL-1β[77], IL-6[83], GM-CSF[84] and cysteinyl leukotrienes[85], along with the neutrophil chemoattractant IL-8[86], the natural killer cell chemoattractant CCL4[86] and the lymphocyte chemoattractant CCL20[84]. Mast cells also facilitate *P. aeruginosa* induced PMN transendothelial migration[77], and have been shown to directly phagocytose and kill invading bacteria[77]. However the ultimate contribution of mast cells to host defense against *P. aeruginosa* lung infection *in vivo* remains undefined.

Mast cells are closely associated with epithelial cells at diverse mucosal surfaces throughout the body. As a result of this close spatial relationship, and the diverse immunoregulatory roles described above, mast cells represent potent regulators of epithelial permeability *in vivo*[87]. In particular there is extensive evidence demonstrating that mast cell proteases are responsible for increasing epithelial permeability in the intestines in response to diverse stimuli, primarily through disruption of TJ proteins[88-94]. However the role of mast cells in the regulation of epithelial permeability in the lungs may be considerably different than those that reside in the intestines due to the divergent biochemical, and immunological nature of the tissues. In fact there is evidence which suggests that unlike in the intestines, mast cell proteases do not disrupt TJ proteins or induce changes in epithelial permeability in the lungs[95]. Therefore the contribution of mast cells to host defense, and epithelial permeability changes during *P. aeruginosa* infection remain poorly understood.

1.2.2.2 Alveolar macrophages

Alveolar macrophages are long lived tissue resident leukocytes that patrol the air space of the lungs[96]. They are potent phagocytes which clear debris, and clear small numbers of invading pathogens from the lungs. However their primary function during

wide spread lung infection is pathogen recognition, and coordination of the inflammatory response[97].

Unlike mast cells, the role of alveolar macrophages during *P. aeruginosa* infection *in vivo* is relatively well characterized. Depletion of alveolar macrophages results in impaired production of the critical neutrophil chemoattractants KC and MIP-2, leading to decreased neutrophil recruitment, and impaired bacterial clearance[98, 99]. Furthermore macrophages are important sources of the inflammatory cytokines IL-1β[100], IL-6[101] and TNF[101] which initiate acute phase responses[102], stimulate epithelial cells to produce neutrophil chemoattractants[100], and when dysregulated contribute to the development of sepsis[103].

1.2.3 Third line of defense: Neutrophils

Neutrophils represent a critical component of innate immunity against *P. aeruginosa*, as evidence clinically by the susceptibility of neutropenic cancer patients to severe infections with the pathogen[104]. Unlike the cell types discussed above neutrophils are not tissue resident cells, and instead need to be recruited to the site of infection. This recruitment is mediated primarily by cytokines and chemokines secreted by the tissue resident leukocytes, and epithelial cells as described above[105].

The primary role of neutrophils during *P. aeruginosa* infection is clearance of the bacteria from the lungs. Neutropenia[104, 106], dysregulation of neutrophil recruitment[106, 107] or impaired bacterial killing by neutrophils[108] results in considerable increases in bacterial burden and poor biological outcomes. Neutrophils contribute to bacterial killing through multiple mechanisms. First and foremost neutrophils are professional phagocytes which ingest and kill extracellular bacteria[105]. Neutrophils also secrete an

array a serine proteases, including neutrophil elastase which impairs *P. aeruginosa* growth and viability through degradation of the bacterial protein OprF[109]. Production of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) has also been shown to be a critical mechanism responsible for killing of internalized *P. aeruginosa* bacteria[110]. Finally, the production of neutrophil extracellular traps (NETs) contributes significantly to neutrophil mediated killing of *P. aeruginosa* bacteria[110].

Given the central role of neutrophils in direct killing of *P. aeruginosa*, it is no surprise that highly pathogenic strains of the bacteria have acquired multiple mechanisms for disrupting or subverting neutrophil mediated killing. The bacterial product pyocyanin induces neutrophil apoptosis which decreases the persistence of neutrophils in the lung and greatly increases bacterial burden *in vivo*[22, 111]. Pyocyanin has also been shown to promote NET formation leading to worsened airway inflammation[112], while acquisition of a mucoid phenotype and biofilm formation have been shown to protect *P. aeruginosa* against NET mediated killing[110]. Finally, production of quorum sensing-induced rhamnolipid by *P. aeruginosa* has also been shown to rapidly induce neutrophil necrosis which significantly impairs clearance of the bacteria[113, 114].

Although neutrophil recruitment is critical for the clearance of acute *P. aeruginosa* infection, neutrophil responses can also be damaging to host tissues. As a result, it is critical that the inflammatory cascade governing neutrophil recruitment to the lungs be tightly regulated. Dysregulation of these cascades is a contributing factor to both acute[115, 116] and chronic[117] lung injury. Neutrophil associated lung injury is mediated through multiple factors produced by neutrophils including neutrophil serine

proteases such as neutrophil elastase[118], reactive oxygen species[119] and uncontrolled pro-inflammatory cytokine production[120].

1.3 Molecular regulation of *P. aeruginosa* induced inflammation

Mounting a tightly controlled inflammatory response early in *P. aeruginosa* lung infection plays a critical role in host defense against the pathogen as impaired inflammatory responses permit colonization with the bacteria, while exaggerated inflammatory responses lead to damage to host tissues. The molecular mechanisms involved in initiating and coordinating the inflammatory response during *P. aeruginosa* infection are discussed below (Figure 1.3.1).

1.3.1 Pattern recognition receptors initiate *P. aeruginosa* induced inflammation

Pattern recognition receptors (PRRs) play a central role in the initiation of *P. aeruginosa* induced inflammation. These receptors recognize conserved molecular patterns on microorganisms which leads to the activation of pro-inflammatory transcription factors through multiple signalling pathways.

1.3.1.1 Toll-like receptor pathways

The most well studied group of PRRs which play central roles in initiating *P. aeruginosa* induced inflammation are the toll-like receptors (TLRs)[69]. TLR-4 is a sensor for lipopolysaccharide on the outer membrane of *P. aeruginosa*[121, 122]. However other *P. aeruginosa* associated patterns found on alginate[123], ExoS[124], outer membrane protein I (OprI) and slime glycoproteins[125] have also been shown to induce inflammatory signalling via this receptor. The role of TLR-4 during *P. aeruginosa* lung infection has been thoroughly examined using TLR-4-deficient mice which display significantly

impaired inflammatory cytokine production leading to impaired neutrophil recruitment and increased bacterial burden[107, 122]. TLR-2 ligands associated with *P. aeruginosa* overlap significantly with those recognized by TLR-4 and include outer membrane protein I (OprI)[126], LPS[127] and ExoS[124]. The role of TLR-2 during *P. aeruginosa* infection has been characterized using TLR-2 knockout mice which demonstrated that TLR-2 contributes to *P. aeruginosa* induced inflammation and neutrophil recruitment, but is ultimately dispensable for bacterial clearance[107, 122]. TLR-5 recognizes bacterial flagellin, a component of flagella used for bacterial motility, in order to initiate inflammatory signalling cascades[128]. TLR-5 has been proposed to play important roles in the phagocytosis of *P. aeruginosa*, as well as in the production of the inflammasome dependent cytokine IL-1β[129]. This role for TLR-5 in host defense against *P. aeruginosa* is further supported by the observation that during chronic infection the bacteria often changes to a non-motile phenotype which is associated with a loss of flagella[130, 131]. These non-motile strains of bacteria are significantly less susceptible to phagocytosis by host cells[126]. The final TLR implicated in host defense against *P. aeruginosa* is TLR-9 which recognizes intracellular unmethylated CpG motifs in bacterial DNA to induce inflammatory response, and mediate bacterial clearance[132].

Upon binding their respective ligands, TLRs can activate inflammatory responses through diverse signaling pathways in order to tailor an immune response to the specific stimuli encountered (reviewed in[69]). One TLR pathway signals through myeloid differentiation primary-response protein 88 (MyD88) that can be recruited directly by TLR-5, but requires the adapter molecule toll-interleukin 1 receptor (TIR) adapter protein (TIRAP/MAL) for recruitment to TLR-2, -4 and -9. Signaling through MyD88 promotes

phosphorylation of interleukin-1 receptor kinase (IRAK)-1 and IRAK4, which combine to activate TNF-receptor associated factor (TRAF)-6, leading to activation of the NFκB pathway, which is discussed below. TRAF-6 phosphorylation also leads to activation of any or all of the MAP kinase pathways, which are also discussed below. MyD88 dependant signaling cascades are essential for host defense against *P. aeruginosa* lung infection as evidenced by studies using MyD88 deficient mice which displayed greatly impaired early cytokine responses, neutrophil recruitment and bacterial clearance[107].

A second major pathway activated through TLR signaling involves the adapter molecule TIR domain-containing adaptor protein inducing interferon-β (TRIF) to TLR-4. Similar to MyD88, the recruitment of TRIF to TLR-4 requires an additional adapter protein, TRIF related adapter molecule (TRAM). TRIF dependant singling activates multiple pathways including the interferon regulatory factors (IRF)-1, IRF-3 and IRF-7 and NFκB via a TRAF-6 dependent, MyD88-independent mechanism. The importance of this redundancy in NFκB activation is highlighted by the fact that although MyD88 deficient mice display impaired early responses to *P. aeruginosa* infection, TRIF-dependant activation of the NFκB pathway is eventually sufficient to clear the bacteria from the lungs[133]. TRIF deficient mice also display profound deficiencies in specific inflammatory mediators resulting in delayed neutrophil recruitment and decreased bacterial clearance[134].

1.3.1.2 NOD-like receptors

Another family of PRRs involved in *P. aeruginosa* induced inflammation is the nucleotide-binding oligomerization domain-containing proteins (NOD) and NOD-like receptors (NLR). Unlike the transmembrane TLRs, NODs and NLRs are located in the

cytosol of the cells where they initiate innate immune responses against cytosolic pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs)[135]. NOD-1 has been implicated in host defense against *P. aeruginosa* where it senses cytosolic peptidoglycan to induce NFκB activity through recruitment of receptor-interacting protein 2 (RIP-2)[136, 137]. RIP-2 then mediates K63 linked polyubiquitination of NFκB essential modulator (NEMO/IKKγ), leading to activation of the IKK complex, and phosphorylation of IκB freeing NFκB for nuclear translocation[138]. NOD-1 has also been implicated in activation of AP-1[139] and IRF[140] families of transcription factors, as well as in the activation of autophagy[141]. However the contribution of NOD-1 to activation of these pathways during *P. aeruginosa* remains undefined.

Another member of the NLR family of proteins involved in *P. aeruginosa* induced inflammation is NLR family CARD-domain containing protein 4 (NLRC4) which recognizes bacterial flagella and mediates bacteria induced inflammasome activation, leading to caspase-1 activity responsible for processing and secreting IL-1β and IL-18[142-144]. Other *P. aeruginosa* PAMPs, including pili[145] and rhsT[146], have also been shown to activate the inflammasome. However the PRRs involved in these pathways remain to be identified. Biologically it appears that inflammasome activity during *P. aeruginosa* infection plays primarily a pathological role as multiple strategies for the inhibition of inflammasome activity, including deletion of NLRC4, significantly reduce *P. aeruginosa* induced lung damage, while improving bacterial clearance from the lungs[142].

1.3.2 The NFκB pathway

NFκB is widely considered a master regulator of inflammation, and is activated through diverse stimuli via signaling pathways which include, but are not restricted to the PRRs discussed above[147]. The NFκB family of transactivators is comprised of p65 (RelA), p50/p105 (NFκB1), p52/p100 (NFκB2), RelB and c-Rel which exist as hetero- or homodimers. Under resting conditions these NFκB complexes are rendered inactive and sequestered in the cytosol through interactions with a group of inhibitory proteins known as inhibitors of NFκB (IκB) of which IκBα is the predominant form[147]. Upon encountering an appropriate stimuli, information from diverse signaling pathways are integrated and lead to the activation of the IκB kinases (IKK) complex which includes IKKα, IKKβ and NEMO (IKKγ). Activation of the IKK complex promotes phosphorylation of IκBα leading to dissociation from the cytosolic NFκB complex, and targets the protein for ubiquitination and degradation via the ubiquitin proteasome system. The free NFκB complex is now able to undergo nuclear translocation and transcriptional activation.

Although NFκB plays a regulatory role in diverse biological processes including cellular proliferation and differentiation, as well as tumorigenesis, apoptosis, and tissue remodeling, the primary function of the transcription factor is initiation, and to a lesser extent the resolution of inflammatory responses. Dysregulation of the NFκB pathway underlies many human diseases and contributes to the pathogenesis of acute lung injury/acute respiratory distress syndrome (ALI/ARDS)[148, 149]. In the context of *P. aeruginosa* lung infection the activation of NFκB is essential for the production of

various inflammatory cytokines, neutrophil recruitment and bacterial clearance[107, 133, 134, 149].

1.3.3 The NFAT/Calcineurin pathway

The nuclear factor of activated T cells (NFAT) family of transcription factors arose relatively recently in evolutionary history, and can be separated into calcineurin dependent (NFATc1, NFATc2, NFATc3 and NFATc4) and calcineurin independent (NFAT5) family members[150]. Canonical activation of NFATc family members requires an increase in intracellular levels of calcium, leading to activation of the Ca^{2+}/calmodulin dependent phosphatase calcineurin (Cn) which dephosphorylates multiple residues on NFATc proteins and exposes a nuclear localization sequence, leading to nuclear import[151]. Once in the nucleus, NFATc proteins interact with nuclear binding partners (NFATn) to coordinate the expression of NFAT dependent genes, including a variety of inflammatory, and immunomodulatory cytokines[152, 153].

Calcineurin consists of both catalytic (CnA) and regulatory (CnB) subunits[154]. The catalytic subunit exists as the ubiquitously expressed alpha (CnAα) and beta (CnAβ) isoforms, as well as a gamma isoform (CnAγ) which is expressed primarily in the brain and testes. Similarly, the regulatory subunit consists of both the ubiquitously expressed CnBα and the testes specific CnBβ isoforms. In spite of their overlapping tissue expression and considerable sequence homology, CnAα and CnAβ have been found to exert biologically distinct roles *in vivo*, particularly with respect to NFAT activation where CnAβ plays a dominant role, and CnAα is largely dispensable[155-161].

Activation of the calcineurin-NFAT axis has long been known play critical roles in the acquired immune response, and it represents a frequent target of

immunosuppressive therapy among transplant recipients[162, 163]. More recently, roles for NFATc family members in bridging the innate and adaptive immune responses, as well as in the regulation of inflammatory responses have also been identified[164, 165]. Although the contribution of the Cn-NFAT axis to host defense against bacterial pathogens remains poorly understood it has been demonstrated that bacterial LPS can activate the canonical Cn-NFAT pathway through a TLR4-CD14 dependent pathway which leads to activation of phospholipase C (PLC)-γ2 and inositol triphosphate (IP_3) production promoting calcium influx[166, 167]. These findings, combined with the ability of NFAT family members to drive the production of inflammatory and immunomodulatory cytokines, strongly suggest that the Cn-NFAT axis may contribute to the coordination of bacteria induced inflammation. However the contribution of this pathway to host defense during *P. aeruginosa* infection remains entirely unknown.

1.3.4 MAP kinase pathways

The mitogen activated protein kinases (MAPKs) are a family of kinases, of which extracellular regulated kinase 1 and 2 (ERK1/2), Jun N-terminal kinase 1 (JNK1/JNK) and p38α (p38) are the most well studied in the context of innate immunity (reviewed in[168]). Activation of the MAPKs involves a signal transduction cascade beginning with the activation of MAPK kinase kinases (MAP3Ks) which phosphorylated MAPK kinases (MAP2Ks), which in turn phosphorylated and activate MAPK proteins. The primary MAP3K for p38 and JNK is TGF-β activated kinase 1 (TAK1) whose kinase activity is induced downstream of TLR signaling by TRAF6[169]. An alternative MAP3K for p38 is apoptosis signal regulating kinase 1 (ASK1) which is activated in response to ROS signaling[170]. Conversely, ERK1/2 are most commonly activated downstream TLR

signaling through the MAP3K tumor progression locus 2 (Tpl2), downstream of the IKK complex[171].

Once activated by immunological stimuli the MAPKs play diverse roles in promoting inflammation. Conditional deletion of JNK in myeloid lineage cells has revealed that the primary role of this MAPK is the generation of a pro-inflammatory M1 phenotype in macrophages[172]. ERK1/2 activity is associated with transcriptional, and post-transcriptional regulation of inflammatory cytokine production where they promote TNF, IL-1β and IL-10 production, while negatively regulating IFN-β, IL-12 and iNOS[173-175]. Activation of p38 can have both pro-inflammatory and anti-inflammatory roles. Conditional deletion of p38α in murine macrophages decreases TNF production and protects against endotoxemia, highlighting the pro-inflammatory role of the pathway[176]. However activation of p38 can also feedback to inhibit TLR signaling pathways, contributing the resolution of inflammatory signaling[177]. During *P. aeruginosa* infection numerous TLR ligands, including slime-glycoproteins, flagella and ExoS activate signaling through both the p38 and ERK1/2 MAPK pathways resulting in numerous inflammatory responses including the production of the inflammatory mediators TNF, IL-8 and cyclooxygenase COX-2 [125, 178, 179].

1.3.5 Signal transducer and activator of transcription 3

Signal transducer and activator of transcription 3 (STAT3) plays complex, and incompletely understood roles during the innate immune response. STAT3 is activated downstream of a diverse range of stimuli, many of which are associated with innate immunity including IL-6, and IFNs (reviewed in[180]). Activation of STAT3 requires sequential phosphorylation events, the first of which occurs at tyrosine 705, and is

mediated by Janus activated kinases (JAKs) recruited to the cytokine receptors upon activation[181]. Phosphorylation at this site leads to homodimerization or the formation of heterodimers between STAT3 and other STAT family members, leading to nuclear translocation. However STAT3 requires further phosphorylation at tyrosine 727 to become transcriptionally active. Tyrosine 727 phosphorylation is mediated by the MAPKs ERK1/2[182].

The STAT3 pathway was first identified as an acute phase response to the pro-inflammatory cytokine IL-6 suggesting that it is intimately involved in innate immunity[183]. STAT3 activity, particularly in tumors, has been associated with the production of a wide range of pro-inflammatory mediators[184]. However mice lacking STAT3 in various subsets of myeloid lineage cells do not display impaired inflammatory responses, but instead suffer from chronic inflammatory conditions and increased susceptibility to endotoxemia suggesting that the transcription factors acts primarily as a negative regulator of inflammation during acute inflammatory responses[185, 186]. The role of STAT3 during *P. aeruginosa* infection remains undefined.

1.3.6 Regulator of calcineurin 1

Although the effective initiation of an inflammatory response plays a critical role in host defense against *P. aeruginosa,* it is equally important that this response be tightly controlled in order to minimize damage to host tissues. One important negative regulator of inflammation is regulator of calcineurin 1 (RCAN1). RCAN1 is located within the Down syndrome (DS) critical region on chromosome 21 and is highly expressed in various tissues, including the lung. RCAN1 has recently become a protein of great interest as a candidate gene responsible for various immune deficiencies in DS patients

due to its prominent role in regulating two critical pathways that mediate inflammatory responses. The first pathway regulated by RCAN1 is the NFAT pathway. RCAN1 can interact directly with calcineurin in the cytosol in order to inhibit the phosphatase activity required for nuclear translocation of NFAT[187-189]. The second inflammatory pathway inhibited by RCAN1 the NFκB pathway. RCAN has been shown to stabilize IκB-NFκB interactions through poorly characterized calcineurin dependent[190] and calcineurin independent mechanisms[191]. Although previous work has shown that RCAN1 negatively regulates the production of various inflammatory cytokines in response to immunological stimuli, the role of the protein during *P. aeruginosa* infection remains unknown[192].

1.4 Autophagy mediated innate immunity

Macroautophagy, hereafter referred to as autophagy which literally means "self-eating", is an evolutionarily conserved catabolic process through which portions of the cytosol are sequestered and degraded within highly specialized double membrane bound vesicles termed autophagosomes. Over the past decade autophagy has emerged as key component of innate immunity against both intracellular and extracellular pathogens. The autophagy machinery, molecular regulation of autophagy in innate immunity and the numerous contributions of autophagy to host defense are discussed below.

1.4.1 The autophagy machinery

Autophagy is carried out by a large group of autophagy-related (ATG) genes. Autophagy begins with the *de novo* synthesis of a cup shaped autophagosome precursor called a phagophore or isolation membrane, the formation of which is mediated by a phosphatidylinositol 3 phosphate (PI3P) rich endoplasmic reticulum associated structure

called the omegasome[193, 194]. This phagophore continues to expand, and displays a distinct inward curvature that eventually leads to the formation of a double membrane bound spherical compartment, the autophagosome, in which a portion of the cytosol is now sequestered. This autophagosome then goes on to fuse with late endosomes (termed amphisomes) and lysosomes (termed autolysosomes) leading to the eventual degradation of the cytosolic contents[195, 196] (Figure 1.4.1).

1.4.1.1 Initiation: the ULK1/2 complex

In mammals initiation of autophagy is mediated through the initiation complex which is composed of an Unc-51-like kinase (ULK)(either ULK-1 or ULK-2), ATG13, RB1-inducible coiled-coil 1 (RBC1CC1, also known as FIP200) and ATG101 (reviewed in[197, 198]). The composition of this initiation complex does not change in response to autophagy inducing stimuli. Instead, under steady state conditions the mechanistic target of rapamycin (mTOR) complex interacts with this initiation complex, leading to phosphorylation of ULK proteins, and ATG13. Dissociation of mTOR in response to autophagy inducing stimuli results in dephosphorylation of these proteins, and activation of the complex which leads to recruitment of downstream ATG proteins and autophagosome formation[199, 200].

1.4.1.2 Nucleation: Class III PI3K complex

In order for nucleation of the phagophore to occur the PI3P rich omegasome must first be created. The generation of PI3P, which is essential for autophagy[201], is mediated by an active class III PI3K complex which is composed of Beclin-1 (BECN1/ATG6), VPS14 (PI3KR4/p150), ATG14, AMBRA-1 and the PI3K VPS34 (PI3K3C3) (reviewed in[198, 202]). An alternative form of the complex also participates in the autophagy pathway

in which ATG14 and AMBRA-1 are replaced with UVRAG and BIF-1 (SH3GRB1)[203]. The PI3K activity of these complexes is regulated at the level of complex formation. The anti-apoptotic protein Bcl-2 is a negative regulator of class 3 PI3K through physical interactions with BECN1, which prevents the protein's incorporation into an active PI3K complex[204, 205]. Conversely, binding of AMBRA-1 and BIF-1 positively regulate the ATG-14 and UVRAG PI3K complexes respectively[206, 207]. A number of autophagy related PI3P binding proteins have been identified including WIPI-1[208] and WIPI-2[209], although the ultimate role of these proteins during autophagy remains poorly defined.

1.4.1.3 Elongation: ubiquitin like conjugation pathways and ATG9

Elongation of the phagophore is mediated through two ubiquitin like conjugation pathways. The first of these pathways covalently links the ubiquitin like protein ATG12 to ATG5 with ATG7 acting as the E1 activating enzyme and ATG10 acting as the E2 conjugating enzyme (reviewed in[210]). ATG5-ATG12 then interacts no-covalently with ATG16L1 which then forms ATG5-ATG12-ATG16L1 dimers which associate with the phagophore and drive elongation through participation in a second ubiquitin like conjugation pathway[211].

This other ubiquitin like conjugation pathway mediates processing of microtubule associated protein light chain 3 (LC3) (reviewed in[210]). In this pathway the serine protease ATG4 acts as the E1 activating enzyme by cleaving the C-terminus of LC3, exposing a glycine residue. LC3 is then transferred to ATG3 through the activity of ATG7, which acts as the E2 conjugating enzyme, and finally is covalently linked to phosphatidylethanolamine (PE) through the activity of the ATG5-ATG12-ATG16L1 complex which is generated as described above. The PE-conjugated form of LC3 is

referred to as LC3-II, while the unprocessed cytosolic form of the protein is referred to as LC3-I, and monitoring LC3-I to LC3-II conversion is commonly used to measure autophagy experimentally[212]. Although LC3 processing is essential for autophagy, the precise role of the protein in autophagosome formation remains unclear.

The final player in phagophore elongation and autophagosome formation is ATG9. Under steady state conditions ATG9 localizes to the *trans*-Golgi network (TGN) and the endosomal pathway[213]. However in response to autophagy inducing stimuli, ATG9 cycles between the elongating phagophore and the TGN, and is thought to play a role in directing membrane components required for autophagosome formation[213]. The recruitment of ATG9 to the phagophore is mediated in part by the ULK-1 initiation complex[213].

1.4.2 Xenophagy

Xenophagy refers to the selective recruitment and degradation of intracellular pathogens through the autophagy pathway. Although the molecular machinery involved in xenophagy does not differ from that of the canonical autophagy pathway, there is the added requirement of adapter proteins that recognize invading organisms, and recruit them to phagophores for incorporation into autophagosomes (reviewed in[214]). Generally, these proteins recruit pathogens to the autophagosome through binding both to molecular patterns on the surface of the microbe, as well as various autophagosome associated proteins.

The first group of adapter proteins include: p62 (SQSTM1)[215], the nuclear dot protein 52 kDa (NDP52)-tank-binding kinase-1 (TBK-1) complex[216], neighbour of BRCA gene 1 (NBR1)[217], and optineurin[218]. These adapter protein play non-redundant

roles in recruiting ubiquitinated pathogens to the autophagy pathway through interactions with both bacteria associated ubiquitin, and autophagosome associated LC3 through ubiquitin binding motifs, and LCE interacting domains respectively[219]. Ubiquitination of cytosolic pathogens represents a pivotal step for their recruitment to the autophagosome. However ubiquitination targets, the nature of the ubiquitin chains, and the ubiquitin ligases involved in coating these pathogens remain poorly understood (reviewed in[220]). The adapter protein tectonin-domain containing protein 1 (TECPR1) also mediates selective authophagy of *Shigella flexneri* through a similar mechanism which involves binding to the bacterial protein VirG, and the autophagosome associated proteins ATG5 and WIPI2[221].

Xenophagy has also been implicated in the clearance of damaged vesicular compartments, including bacteria containing endosomes and lysosomes. When membrane damage occurs in these compartments, certain glycans become aberrantly exposed on the outer membrane, which can be bound by a family of lectins known as Galectins. In addition to its ability to interact with LC3 and ubiquitin, NDP52 can also bind to one of these Galectin family members, Galectin-8, which targets the damaged vesicle to the autophagy pathway for degradation[222].

Known substrates for xenophagy include *Mycobacterium tuberculosis*[223], *Salmonella enterica*[216, 218, 222], *Shigella flexneri* [224], *Lysteria monocytogenese*[225] and *Legionella pneumophelia*[226]. The importance of xenophagy is underscored by the observation that pathogens targeted for degradation via xenophagy have evolved diverse mechanisms for evading or subverting the autophagy pathway (reviewed in[227]).

1.4.3 LC3 associated phagocytosis

In addition to driving xenophagy, many of the components of the autophagy pathway also play a role in a process called LC3 associated phagocytosis (LAP) which is morphologically and biochemically distinct from autophagy[228]. LAP involves the recruitment of LC3-II to the membranes of phagosomes containing ligands for TLR-2[228], TLR-4[228], TLR-9[228] or T cell immunoglobulin mucin 4 (TIM-4)[229]. Recruitment of LC3 to these phagosomes is dependent upon ATG5, ATG7 and the BECN-1 PI3K complex, but is independent of autophagosome formation resulting in a single membrane bound, LC3-positive phagosome[228]. This recruitment of LC3 to phagosome greatly enhances lysosomal fusion kinetics[228] and plays a critical role in the killing of phagocytosed bacteria[230] and the removal of apoptotic cells[229, 231].

1.4.4 Regulation of inflammation by autophagy

In addition to the roles in direct pathogen clearance by xenophagy and LAP, autophagy also impacts numerous other aspect of innate immunity. ATG proteins have been shown to dynamically interact with components of the type I IFN pathway leading to net feedback inhibition of IFN production[232]. Autophagy also plays an important role in clearing inflammasome agonists from the cytosol which increases the threshold for inflammasome activation responsible for IL-1β and IL-18 production[233]. Aberrant activation of inflammasomes stemming from mutations in the autophagy related protein ATG16L1 is through to be an underlying mechanism involved in Crohn's disease[234]. Dysregulation of autophagy has also been shown to lead to significant production of IL-1α through a ROS and calpain-1 dependent mechanism stimulated through accumulation of damaged mitochondria within the cytosol[235].

1.4.5 Regulation of autophagy by immunological stimuli

Autophagy is regulated by numerous immunological stimuli through diverse molecular mechanisms (reviewed in[236]). Cellular amino acid starvation is often associated with bacterial and viral infection and acts as a potent danger signal to induce autophagy in host cells through mTOR dependent pathways[237]. Furthermore, activation of many PRRs leads to upregulation of autophagy, including many of those receptors involved in host defense against *P. aeruginosa* which were discussed previously[236, 238]. Cytokines are also potent regulators of autophagy where inflammatory T_H1-associated cytokines such as IFNγ[239] and IL-1β[240] promote autophagy, while T_H2-assocaited cytokines such as IL-4 and IL-13 supress autophagy[241]. Finally, autophagy is regulated through local production of ROS[242].

1.4.6 Defective autophagy in cystic fibrosis

CF is caused by mutations in CFTR which disrupt anion channel activity, as well as cellular distribution of the resultant protein. Oxidative stress has emerged as a feature of CF airways because etiologic mutations in CFTR increase the levels of intracellular reactive oxygen species (ROS)[243]. One consequence of increased ROS in airways epithelial cells is enhanced activity of tissue transglutaminase 2 (TG2), a calcium-dependent enzyme that creates intra- or intermolecular covalent bonds between proteins[244]. In cells bearing mutations in CFTR TG2 undergoes ROS-dependent small ubiquitin like-modifier (SUMO)ylation which greatly enhances stability and activity of the protein[244, 245]. This aberrant TG2 activity crosslinks target proteins and results in aggresome formation. Among targets of TG2-mediated cross-linking substrates is the essential autophagy protein BECN-1 which becomes sequestered in histone de HDAC6-, p62- and ubiquitin-containing cytoplasmic aggresomes as a result of aberrant TG2

activity. BECN-1 sequestration in aggresomes results in the dislodgement of class III PI3K complexes from the endoplasmic reticulum, thereby inhibiting autophagy[246]. The contribution of impaired autophagy in disease progression, and opportunistic infections in CF patients remains poorly understood

1.5 Objectives

The objectives of the current study is to examine multiple facets of the innate immune response against *P. aeruginosa* with the aim of understanding how various disease states contribute to susceptibility to infection with the bacteria, and the identification of novel opportunities for therapeutic intervention. Studies focus on three main areas: the role of autophagy in host defense against *P. aeruginosa* and the potential of autophagy based antimicrobial strategies; the contribution of the RCAN1-calcineurin axis to *P. aeruginosa* induced inflammation; the role of mast cells during *P. aeruginosa* induced lung injury.

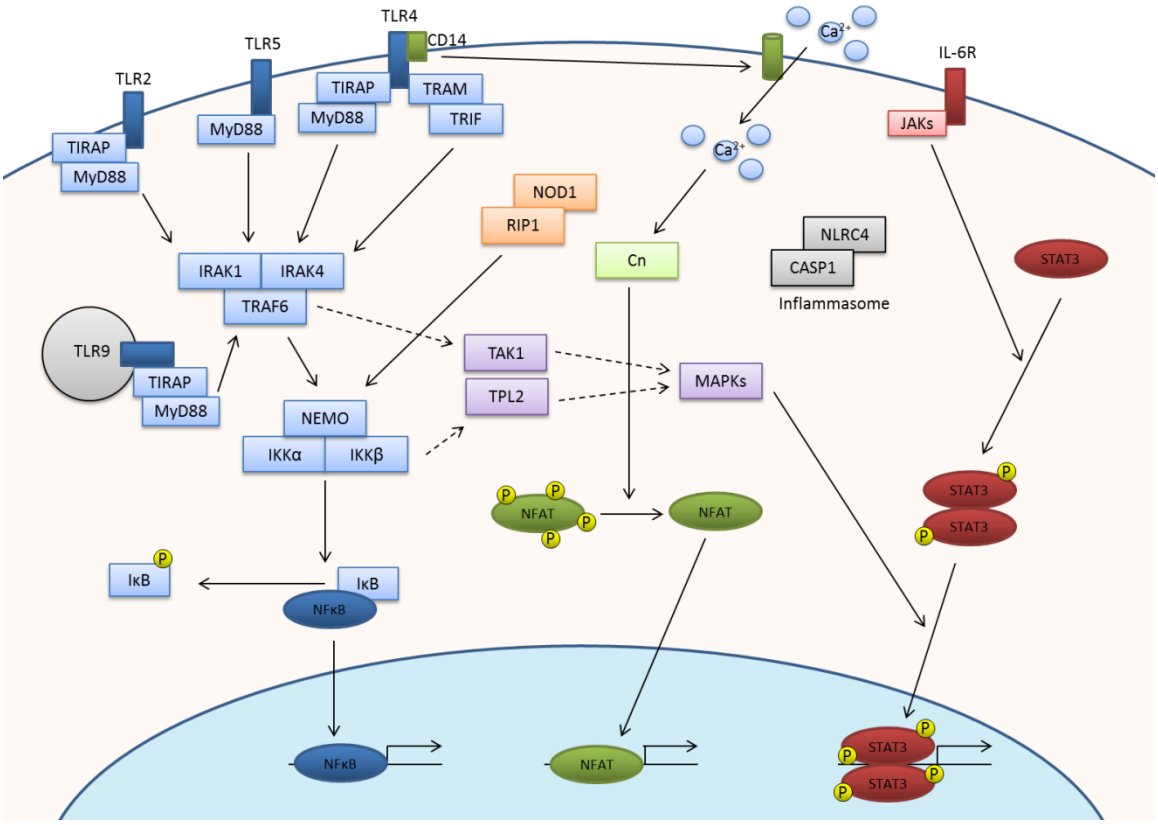

Figure 1.3.1: Pattern recognition receptor signaling pathways coordinate inflammatory responses during *P. aeruginosa* infection. Innate immune signaling in response to *P. aeruginosa* is triggered by a number of PRRs including TLRs which lead to activation of the TRAF6 through both MyD88 and TRIF dependent mechanisms. TRAF6 goes on to active the IKK complex which phosphorylates IκB and frees NFκB for nuclear translocation and transcriptional activity. NOD1 can promote NFκB activity through the IKK complex as well. Similarly NLRC4 is also activated during *P. aeruginosa* infection which leads to activation of the NLRC4 inflammasome responsible for processing of pro-IL-1β and pro-IL-18 into their active forms. Engagement of TLR signaling activates the MAP3Ks TAK1 and TPL2, leading to activation of MAPK cascades. TLR signaling can also lead to activation of the calcineurin-NFAT pathway through TLR4 associated CD14 which induces an influx of extracellular calcium leading to activation of calcineurin and dephosphorylation of NFAT which exposes a nuclear localization sequence. Finally autocrine and paracrine production of IL-6 activates a variety of JAKs through the IL-6 receptor leading to phosphorylation and dimerization of STAT3. An additional phosphorylation mediated by the MAPKs ERK1/2 is required for transcriptional activity of the STAT3 complex.

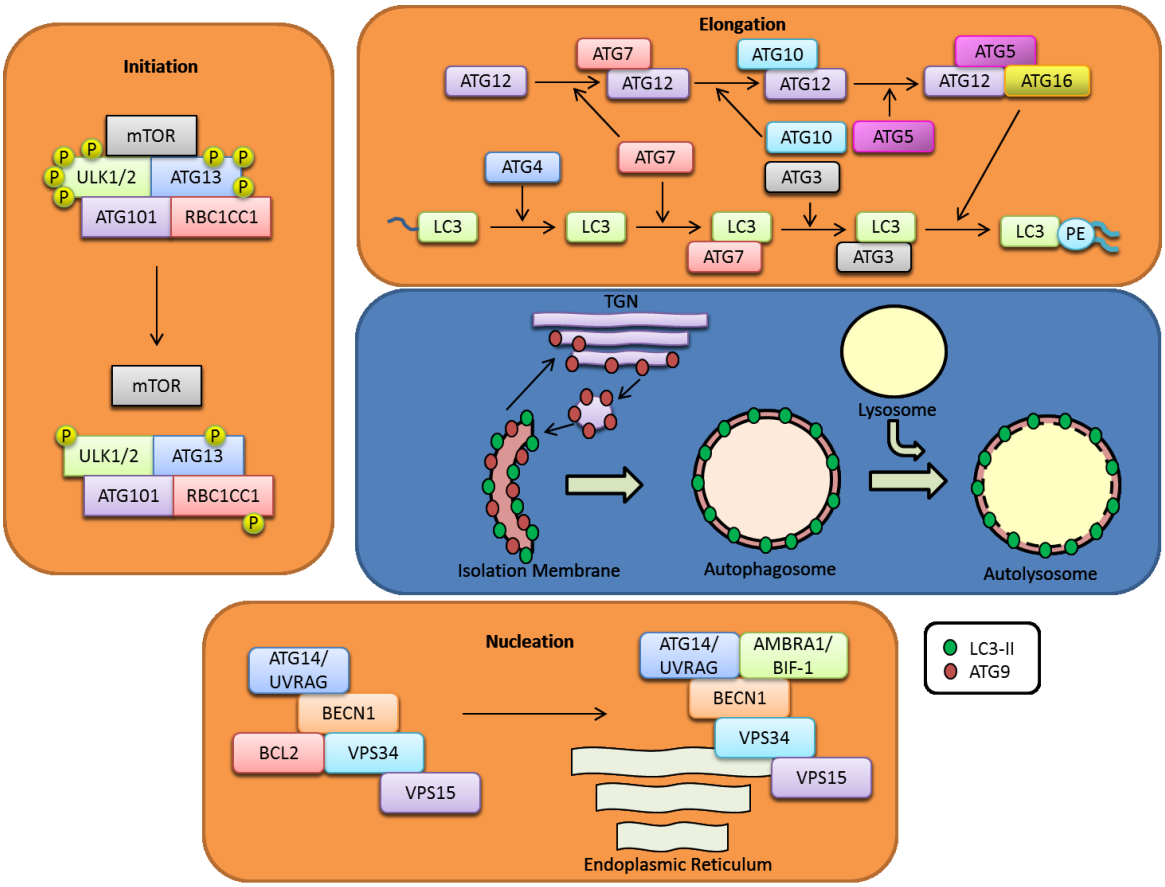

Figure 1.4.1: The autophagy machinery. Autophagy is suppressed by mTOR which, when active, interacts with ULK1/2 initiation complex, leading to phosphorylation of ULK1/2 and ATG13. When appropriate stimuli are encountered mTOR is inactivated, leading to dephosphorylation of the ULK1/2 complex, and initiation of autophagy. The formation of the autophagosome begins with the nucleation of an isolation membrane which is dependent on the activity of the BECN1 PI3K complex. Under steady state conditions the BECN1 complex is inhibited by interactions with BCL2. However upon induction of autophagy BECN1 dissociates from BCL2 and forms an active PI3K complex at the omegasome on the endoplasmic reticulum with the activators AMBRA1 or BIF1. Elongation of the isolation membrane is mediated through two ubiquitin like conjugation pathways leading to the conversion of cytosolic LC3-I to PE conjugated, autophagosome associated LC3-II. Elongation is also dependent upon the activity of ATG9 which shuttles between the TGN and the expanding isolation membrane. Once the formation of the autophagosome is complete it fuses with the lysosomal pathway, leading to the formation of an autolysosome, and degradation of the autophagosomal contents.

CHAPTER 2: AUTOPHAGY ENHANCES BACTERIAL CLEARANCE DURING *P. AERUGINOSA* LUNG INFECTION

Autophagy enhances bacterial clearance during *P. aeruginosa* lung infection

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Robert D. Junkins: Designed and executed experiments and analyzed data for all figures and tables. Wrote and edited manuscript.

Anne Shen: Contributed to establishment of stable cell lines. Repeated Western blots for figure 2.1.4A.

Kiril Rosen: Provided LC3-GFP construct and provided technical assistance while establishing LC3-GFP assay, and when analyzing TEM images.

Craig McCormick: Provided LC3-mCherry construct, contributed to study design, participated in manuscript editing and revisions.

Tong-Jun Lin: Oversaw study design and data analysis, provided funding and participated in manuscript editing and revisions

2.1. Abstract:

Pseudomonas aeruginosa is an opportunistic bacterial pathogen which is the leading cause of morbidity and mortality among cystic fibrosis patients. Although *P. aeruginosa* is primarily considered an extracellular pathogen, recent reports have demonstrated that throughout the course of infection the bacterium acquires the ability to enter and reside within host cells. Normally intracellular pathogens are cleared through a process called autophagy which sequesters and degrades portions of the cytosol, including invading bacteria. However the role of autophagy in host defense against *P. aeruginosa in vivo* remains unknown. Understanding the role of autophagy during *P. aeruginosa* infection is of particular importance as mutations leading to cystic fibrosis have recently been shown to cause a blockade in the autophagy pathway, which could increase susceptibility to infection. Here we demonstrate that *P. aeruginosa* induces autophagy in mast cells, which have been recognized as sentinels in the host defense against bacterial infection. We further demonstrate that inhibition of autophagy through pharmacological means or protein knockdown inhibits clearance of intracellular *P. aeruginosa in vitro*, while pharmacologic induction of autophagy significantly increased bacterial clearance. Finally we find that pharmacological manipulation of autophagy *in vivo* effectively regulates bacterial clearance of *P. aeruginosa* from the lung. Together our results demonstrate that autophagy is required for an effective immune response against *P. aeruginosa* infection *in vivo*, and suggest that pharmacological interventions targeting the autophagy pathway could have considerable therapeutic potential in the treatment of *P. aeruginosa* lung infection.

2.2 Introduction:

Pseudomonas aeruginosa is an environmentally ubiquitous Gram negative bacterial pathogen which is a leading cause of morbidity and mortality among CF patients and the immunocompromised[247]. In healthy individuals *P. aeruginosa* infection triggers strong inflammatory responses, mediated largely through TLR signaling pathways, which lead to neutrophil recruitment and effective clearance of the bacteria[69]. The coordination of these early host responses to the pathogen are largely mediated by resident immune cells in the airway such as mast cells or alveolar macrophages[82, 98, 99]. Mast cells are recognized as sentinel cells of the immune system in the respiratory tract where they represent up to 2% of the alveolar wall and protrude into the airspace of the lung where they are ideally placed to be first responders to invading pathogens[75]. Upon encountering pathogens mast cells not only produce various cytokines to coordinate further immune responses[76, 77], but also act as phagocytes, internalizing and killing invading organisms[248]. Active interactions between mast cells and *P. aeruginosa* have been observed[77, 84, 249].

CF patients almost invariably become chronically infected with *P. aeruginosa.* Lung infection with *P. aeruginosa* correlates clinically with decreased lung function and impaired survival[250]. Many factors contribute to the increased susceptibility to *P. aeruginosa* infection observed in CF patients. Mutations in the CFTR which cause CF lead to osmotic dysregulation resulting the accumulation of thick mucus at the surface of epithelial cells which impairs the clearance of pathogens from the lungs[251]. Furthermore CFTR mutations have been shown to dysregulate TLR signaling and surface expression leading to impaired and prolonged inflammatory responses to the pathogen[69]. However,

recently a novel effect of mutations to the CFTR has been identified which leads to dysregulation of an evolutionarily conserved catabolic process called macroautophagy, which is hereafter referred to as autophagy[246]. Mutations to the CFTR have been shown to lead to upregulation of ROS production, and enhanced tissue transglutaminase activity that combine to drive the crosslinking and inactivation of the beclin-1 PI3K complex which represents a central component of the autophagy pathway[246].

Autophagy is an evolutionarily conserved catabolic process through which portions of the cytosol are sequestered and degraded within highly specialized double membrane bound vesicles termed autophagosomes. Over the past decade autophagy has emerged as a central component of the innate and adaptive immune responses where it plays roles in antigen presentation including cross-presentation, direct and indirect killing of intracellular and extracellular pathogens, generation of bactericidal peptides and the regulation of inflammatory responses[252-254]. Autophagy has been implicated in *P. aeruginosa* infection in cultured macrophages *in vitro*[255]. However, the biological significance of autophagy in *P. aeruginosa* infection *in vivo* and its role in mast cell-*P. aeruginosa* interactions remain undefined.

One of the greatest challenges in the treatment of *P. aeruginosa* infection is the highly antibiotic resistant nature of the bacteria[256]. The recent emergence of multi-drug resistant *P. aeruginosa* strains leading to increased morbidity and mortality in susceptible populations highlights the need for novel therapeutic strategies for the treatment of *P. aeruginosa* infections[257-259]. Recently it has been proposed that *P. aeruginosa* bacteria have the ability to reside within host cells where they can evade host immune cells, and that the development of intracellular infections may represent a mechanism contributing

to antibiotic resistance[67, 260]. Given the well characterized central role of autophagy in the clearance of intracellular pathogens[261], and the observation that autophagy is impaired in the airways of cystic fibrosis patients, we set out to examine the role of autophagy in host defense against *P. aeruginosa in vivo*, and explored the therapeutic potential of pharmacological manipulation of the autophagy pathway during *P. aeruginosa* lung infection. Our results demonstrate that *P. aeruginosa* infection induces autophagy in mast cells which are abundant in the airways where they play a central role in host defense against *P. aeruginosa*[76, 82], as well as in bronchial epithelial cells which have been proposed to act as a reservoir of intracellular bacteria during chronic *P. aeruginosa* infection[59-61, 67, 262]. We further demonstrated that inhibition of the autophagy pathway significantly impairs clearance of *P. aeruginosa* from mast cells and human bronchial epithelial cells, while induction of the process enhances bacterial killing. Finally we demonstrate that pharmacological manipulation of the pathway effectively regulates bacterial clearance *in vivo*. Thus, induction of autophagy could represent a novel therapeutic approach for the treatment of *P. aeruginosa* infection.

2.3 Materials and Methods:

Ethics statement: All animal protocols were approved by the University Committee on Laboratory Animals, Dalhousie University, in accordance with the guidelines of the Canadian Council on Animal Care. Animals were housed in specific pathogen free facilities, and anesthetized with ketamine to minimize suffering during relevant procedures.

Animals: C57/BL6 mice were purchased from Charles River Laboratories (Wilmington, MA) and used between the ages of 8 - 10 weeks.

Antibodies: Antibodies for actin (sc1616), GFP (sc8334), mouse mast cell tryptase (mMCP6) (sc32473), rabbit anti-goat IgG HRPO (sc2768), donkey anti-mouse IgG (sc2314), goat anti-rabbit IgG HRPO (sc2004) and biotin conjugated mouse anti-goat IgG (sc2489) were purchased from Santa Cruz Biotechnology (Dallas, TX). An antibody for LC3 (clone 2G6) was purchased from Nano Tools (San Diego, CA). Antibodies for Atg5 (#2630) and Atg7 (#2631) were purchased from Cell Signaling Technology (Danvers, MA).

Bacterial internalization and killing: Bacterial internalization and killing was measured as previously described[77, 263]. Briefly, 1×10^5 wild-type HMC-1 5C6 cells, or 1×10^4 16HBEo$^-$ or CFBE14o$^-$ cells were left untreated or pretreated for 1 hour with 20 μM chloroquine diphosphate salt (Simga Aldrich, St. Louis, MO) or 2 μM rapamycin (LC Laboratories, Woburn, MA). Alternatively, untreated HMC-1 5C6 cells stably expressing Atg5 or Atg7 shRNA were used. Cells were infected in 100 μL of serum free IMDM media with *P. aeruginosa* strain 8821 at a 1:20 MOI for 3 hours. Extracellular bacteria were then killed with cell impermeable antibiotics (50 U/mL each of penicillin and streptomycin (Life Technologies, Burlington, ON), 200 μg/mL gentamycin (Life Technologies), 100 μg/mL ceftazidime hydrate (Sigma Aldrich) and 100 μg/mL piperacillin sodium salt (Sigma Aldrich)) for 10 minutes (for the study of bacterial internalization) or 3 hours (for the study of bacterial killing). Cells were then washed in

PBS, and lysed in 100 μL PBS containing 0.2% Triton X-100 (Sigma Aldrich) and a serial dilution of 10 μL of lysate was streaked in duplicate on LB agar plates, and incubated overnight at 37 ^{o}C. CFUs were counted the next day. Samples containing no cells were used to identify background CFUs which were subtracted from samples at each time point.

Bacterial preparation: The laboratory strains of *P. aeruginosa* PAK and PAO.1 were gifts from Dr. J. Boyd (Institute for Marine Bioscience, National Research Council, Halifax, NS) and Dr. D. Wozniak (Ohio State University, Columbus, OHIO, USA) respectively. *P. aeruginosa* strain 8821, a mucoid strain isolated from a cystic fibrosis patient, was a gift from Dr A. Chakrabarty (University of Illinois, Chicago, IL, USA)[264]. *P. aeruginosa* strains were cultured as described previously[134]. Briefly, suspension cultures were grown until reaching the early stationery phase. Bacteria were washed in phosphate buffer and resuspended in 0.9% saline for *in vivo* experiments or PBS for *in vitro* assays. Mice were infected as described below. *Escherichia coli* stain DH5α was purchased from Life Technologies (Burlington, ON) and cultured according to the suppliers instructions.

Cell Culture: Mouse bone marrow-derived mast cells (BMMCs) were cultured from C57/BL6 mice as previously described[265]. Mast cells were confirmed by toluidine blue staining and following 5–6 weeks in culture, mast cell purity was > 98%.

Human mast cells HMC-1 5C6 were a kind gift from Dr. Dr. B. M. Henz (Virchow Klinikum, Berlin)[266] and were maintained in IMDM supplemented with 10%

FBS and 50 U/mL each of penicillin and streptomycin in a 5% CO_2-humidified atmosphere at 37°C. Culture medium was further supplemented with 500 µg/mL G418 for the maintenance of HMC-1 5C6 cell stably expressing LC3-GFP, and 10 µg/mL puromycin for the maintenance of HMC-1 5C6 cells stably expressing NT, Atg5 or Atg7 shRNA. All experiments were carried out in the absence of selection.

Highly purified cord blood-derived mast cells (CBMC) (> 95% purity) were obtained by long term culture of cord blood progenitor cells as previously described[248]. The percentage of mast cells in the cultures was determined by toluidine blue staining of cytocentrifuged samples. Mature mast cells after more than 8 weeks in culture were identified by their morphological features and the presence of metachromatic granules, at which time they were used for this study.

The human epithelial cell line 16HBEo$^-$ and the stable CFTR ΔF508 homozygous cell line CFBE14o$^-$ were gifts from Dr. D. Gruenert (University of California, San Francisco) and have been described previously[267, 268]. Cells were maintained in MEM supplemented with 10% FBS and 50 U/mL each of penicillin and streptomycin in a 5% CO_2-humidified atmosphere at 37°C.

Cytokine production: Concentrations of IL-1β, TNF, IL-6, MIP2 and RANTES in BALF and lung supernatants was determined by ELISA as described previously using antibody pairs from R&D Systems (Minneapolis, MN, USA)[77].

LC3-GFP assay: Two million HMC-1 5C6 cells stably expressing LC3-GFP were left untreated or treated as indicated. Cells were then fixed at various time points in 4%

paraformaldehyde and cytocentrifuged onto glass slides for 5 minutes at 200 rpm for examination by fluorescence microscopy (Nikon Eclipse E600; Nikon, Tokyo, Japan) and by confocal laser scanning microscopy (Zeiss LSM510, Zeiss, Toronto, Ontario, Canada). Alternatively 16HBEo$^-$ cells were grown to confluence on poly-L-lysine coated glass coverslips and transiently transfected with LC3-GFP using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. Cells were then left untreated, or treated as indicated and fixed in 4% paraformaldehyde and examined as described above. At least one hundred cells per slide were counted and cells containing 5 or more GFP punctuations were considered LC3 positive.

Lung infection with *P. aeruginosa* and collection of lung and bronchioalveolar lavage fluid (BALF): Mice were infected with 1×10^9 CFU *P. aeruginosa* intranasally for 24 hours. BALF was then obtained by lavaging the lung with 3× 1mL PBS containing 100 μg/mL soybean trypsin inhibitor (Sigma Aldrich). The lung tissues were obtained for detection of cytokines, myeloperoxidase (MPO) and bacterial colony-forming units (CFU) counting.

Lung tissues were homogenized in 50 mM HEPES buffer (4 μL/1 mg lung) containing 100 μg/mL soybean trypsin inhibitor. For counting bacterial CFU, 10 μL of the homogenate was plated onto an agar dish and incubated for 24 h at 37°C. The homogenate was centrifuged at 4°C for 30 min at 18000g. The supernatant was stored at −80°C for later cytokine analysis. The pellet was resuspended and homogenized in 0.5% cetyltrimethylammonium chloride (CTAC) (4 μL/mg lung) and centrifuged as above. The cleared extract was used for MPO assay.

BALF (10 μL) was plated on an agar dish and incubated for 24 h for CFU counting. For detection of cytokines and MPO activity, BALF was centrifuged at 480g for 5 minutes at 4°C. The supernatants were used for cytokine analysis. The pellets were resuspended in 1mL NH_4Cl (0.15M) and centrifuged as before to lyse red blood cells. The supernatants were discarded and the pellets were resuspended in 0.5% CTAC (250 μL/mouse) then centrifuged. The cleared extracts were used for MPO assay.

For survival studies, mice were infected with 1×10^9 CFU *P. aeruginosa* strain 8821 as described above. Mice were then monitored for 10 days, and moribund animals were euthanized.

Mast cell nucleofection and generation of stable cell lines: HMC-1 5C6 cell were nucleofected with LC3-GFP, LC3-mCherry, NT shRNA, Atg5 shRNA or Atg7 shRNA as previously described[269]. Briefly HMC-1 5C6 cells were resuspended at 4×10^6 cells/transfection in Amaxa nucleofector solution and electroporated with 8 μg DNA using Amaxa Nucleofector II Device program U-023 (Lonza, Basel, Switzerland).

HMC-1 5C6 cells stably expressing LC3-GFP were generated by culturing nucleofected cells under 500 μg/mL G418 (Invitrogen) selection for 4 weeks starting 24 hours after nucleofection. Cells were then sorted for GFP^{mid} expressing cells using a BD FACSAria1 (BD Biosciences, Mississauga, Ontario), and maintained under G418 selection. HMC-1 5C6 cells stably expressing NT, Atg5 or Atg7 shRNA were generated by culturing nucleofected cells under 10 μg/mL puromycin selection for 4 weeks starting 24 hours after nucleofection. Knockdown was confirmed by Western blot analysis as described below.

Mast cell tryptase (mMCP6) solid phase ELISA

Mice were left untreated, or infected with *P. aeruginosa* strain 8821 as described above. Lung tissue homogenates, BALF supernatants and serum proteins were absorbed onto a high binding Nunc MaxiSorp 96 well plate (Thermo Fischer Scientific, Rochester, NY) overnight at 4^{o}C. Plates were washed 4 times with 200 µL 0.01% Tween-20 in PBS then blocked for 2 hours at room temperature in 2% BSA in PBS. Plates were washed 4 times with 200 µL 0.01% Tween-20 in PBS then anti-mouse tyrptase antibody diluted 1:50 in 0.2% BSA, 0.05% Tween-20 in PBS was added and incubated overnight at 4^{o}C. Plates were washed 4 times with 200 µL 0.01% Tween-20 in PBS then biotin conjugated mouse anti-goat IgG diluted 1:100 in 0.2% BSA, 0.05% Tween-20 in PBS was added and incubated at room temperature for 2 hours. Plates were washed 5 times with 200 µL 0.01% Tween-20 in PBS then ELISAs were developed using the Invitrogen ELISA amplification system (Life Technologies) according to the manufacturer's directions.

MPO assay: The MPO assay was used to determine the infiltration of neutrophils into the lungs of the mice as described previously[270]. Briefly, samples in duplicate (75 µL) were mixed with equal volumes of the substrate (3,3′,5,5′-tetramethyl-benzidine dihydrochloride, 3 mM; Resorcinol, 120 µM; and H_2O_2, 2.2 mM) for 2 minutes. The reaction was stopped by adding 150 µL of 2 M H_2SO_4. The optical density was measured at 450nm.

Pharmacological manipulation of autophagy in vivo: Autophagy was inhibited *in vivo* through the administration of chloroquine diphosphate salt (Sigma Aldrich, St. Louis MO) intraperitoneally in PBS at a dose of 60 mg/kg/day for 3 days prior to infection as described previously[271]. Autophagy was induced *in vivo* through the administration of rapamycin (LC Laboratories, Woburn, MA) in diluent (5.2% Tween 80, 5.2% polyethylene glycol 400 in sterile water) at a dose of 10 mg/kg, 24 hours prior to infection as described previously[272].

Plasmids: LC3-GFP was generated as previously described[273] and was generously donated by Dr. T. Yoshimori (Osaka University, Japan). LC3-mCherry was generated as previously described[274] and was generously donated by Dr. T. Johansen (University of Tromso, Norway). Non-targeting, Atg5 and Atg7 specific shRNA were generated as previously described[275].

Statistics: Data are presented as mean ± SEM of the indicated number of experiments. Statistical significance between treatment groups was determined using ANOVA and post-hoc Tukey's honest significance multiple comparisons test, or when there were only two groups are being compared, by an unpaired t-test. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA).

Transmission Electron Microscopy: Untreated and *P. aeruginosa* treated mast cells were fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated with ethanol, and embedded in epoxy resin for thin sectioning, followed by standard staining

in uranium and lead salts, as described previously[276]. Thin sections were observed in a JEOL JEM-1230 transmission electron microscope equipped with a Hamamatsu ORCA-HR high-resolution (2,000 by 2,000 pixels) digital camera, and images were saved as TIFF files. Autophagosomes were identified based on the appearance of their characteristic double membrane, and heterogeneous contents (Reviewed in[277]). For area analysis electron micrographs were saved as TIFF images so that 1 pixel is representative of 1 unit area of the cell. The area of the cytoplasm was then defined using Photoshop (Adobe, San Jose, CA) by subtracting the number of pixels contained within the nuclear envelope from the number of pixels contained within the plasma membrane. The number of pixels in each cell contained within autophagosomes was then determined and expressed as a percentage of the number of pixels contained within the cytoplasm of the cell.

Western Blot and Scanning Densitometry: Cells lysates (15 – 40 μg) were subjected to electrophoresis in 10% or 12% SDS-polyacrylamide gels. Gels were transferred to polyvinylidene difluoride membrane, blotted with primary and secondary antibodies as indicated, and detected by an enhanced chemiluminescence detection system (Western Lightning Plus-ECL; PerkinElmer, Waltham, MA). Scanning densitometry analysis was performed using Scion Image (Scion Corporation, Frederick, MD).

2.4 Results:

P. aeruginosa *induces autophagy in mast cells*: Mast cells are important sentinel cells of the immune system, playing a critical role in sensing invading pathogens and

coordinating the appropriate immune response against *P. aeruginosa*[76, 82]. Due to the high density of the cells along the airways, and their phagocyte capacity, mast cells also represent the first line of defense against pathogens within the respiratory tract. Our lab has extensively studied the roles of mast cells during host defense against *P. aeruginosa*. However the role of autophagy in mast cells in the context of host defense, as well as the biological role of autophagy during *P. aeruginosa* infections remains unknown. In order to examine the role of autophagy in mast cells during *P. aeruginosa* infection, bone marrow derived mast cells (BMMCs) were cultured from C57/BL6 mice. These cells were then infected with *P. aeruginosa* strain 8821 at an MOI of 1:100. Whole cell lysates were prepared at various time points as indicated, and subjected to western blot analysis for microtubule associated protein light chain 3 (LC3) and actin loading control. Upon the induction of autophagy the cytoplasmic form of LC3 (LC3-I) becomes cleaved and conjugated to phophotidylethanolamine (PE) through a ubiquitin like conjugation pathway. This PE conjugated form of the protein (LC3-II) becomes and remains associated with autophagosomal membrane throughout the maturation cycle of the vesicle. The conversion of cytosolic LC3-I to autophagosome associated LC3-II is diagnostic of autophagy and can be tracked by Western blot analysis[278]. In untreated cells, the PE conjugated LC3-II form of the protein predominated within the cells, consistent with a previously describe role for LC3 in mast cell granule formation[279]. However while the cytoplasmic LC3-I levels remained unchanged upon stimulation with *P. aeruginosa,* the levels of LC3-II accumulated well above basal levels, peaking around 18 hours post infection (hpi), indicating an induction of autophagy (Figure 2.1A and 2.1B).

Autophagy can also be monitored using a construct consisting of LC3 conjugated to GFP. Upon the induction of autophagy, LC3-GFP becomes redistributed from a diffuse cytosolic distribution to distinct GFP positive puncta representing autophagosomes. In order to monitor autophagy following *P. aeruginosa* infection using this technique, the HMC-1 5C6 human mast cell line was stably transfected with an LC3-GFP construct. These cells were then infected with *P. aeruginosa* strain 8821 at a 1:100 MOI and fixed at various time points post-infection for examination by fluorescence microscopy. The untreated cells displayed primarily a diffuse cytosolic distribution of LC3-GFP prior to exposure to *P. aeruginosa*. However upon stimulation with the bacteria for 18 hours the fluorescence became largely localized to discrete GFP positive puncta indicative of autophagosomes (Figure 2.1 C). In order to further quantify the induction of autophagy, the percentage of cells at each time point which displayed greater than 5 discrete GFP puncta was assessed (Figure 2.1 D). A time dependent increase in GFP puncta positive cells was observed, which peaked 18 hpi, and was significantly increased compared to untreated cells between the 12 and 24 hpi time points.

HMC-1 5C6 cells stably expressing LC3-GFP were further used to assess flux through the autophagy pathway. As autophagosomes mature fluorescently inactive GFP is cleaved from LC3-II and accumulates in the cell. The appearance of this free GFP can be tracked as a measure of flux through the autophagy pathway[280-282]. LC3-GFP expressing HMC-1 5C6 cells were left untreated (NT) or infected with *P. aeruginosa* at an MOI of 1:100. Lysates were prepared at the time points indicated, and were subjected to Western blot analysis for free GFP (Figure 2.1 E). No free GFP was detected in untreated cells. However free GFP protein began to appear in the cells as soon as 12 hpi,

and continued to increase at longer time points. These data demonstrate that autophagy is induced in mast cells following *P. aeruginosa* infection, and infection with the bacteria promotes flux through the pathway.

We further characterized the dose response of autophagy induced by *P. aeruginosa* strain 8821, PAK and PAO.1 as well as *E. coli* stain DH5α. A dose-dependent increase in LC3-GFP puncta formation in HMC-1 5C6 cells was observed in response to all strains of bacteria suggesting that the induction of autophagy in mast cells may be a generalized host response to *P. aeruginosa* and other gram negative bacteria (Figure 2.2).

As previous reports have suggested that LC3 becomes incorporated into the membranes of mast cell granules[279], we set out to ensure the LC3 positive structures we observed were not mast cell granules. Because the fixation conditions required for staining mast cell granules are acidic, the HMC-1 5C6 LC3-GFP cell line could not be used to examine colocalization of LC3 positive puncta with mast cell granules as GFP has a pKa of 6.0 and is not fluorescent under acidic conditions[283, 284]. In order to visualize LC3 localization under these conditions HMC-1 5C6 cells were transiently transfected with LC3-mCherry, which retains fluorescence under acidic pH[284]. LC3-mCherry expressing cells were then fixed and stained for mast cell granules using toluidine blue and subjected to fluorescent and light microscopy (Figure 2.3 A). Consistent with previous reports, the HMC-1 5C6 cells were not well granulated, with only 21% of cells containing granules[285]. Of the granulated cells there was on average only 7 granules per cell. The number of mast cell granules and the number of LC3-mCherry positive mast cell granules were counted in 100 cells containing at least one

granule, and one LC3-mCherry positive puncta (Figure 2.3 B). Colocalization between LC3 and mast cell granules was very rarely observed. Furthermore, there was no correlation between the number of LC3-mCherry positive puncta, and the number of mast cell granules in a given cell (Figure 2.3 C). Together these results indicate that LC3 positive structures in HMC-1 5C6 cells are not mast cell granules.

P. aeruginosa ***induces autophagy in primary human and mouse mast cells and becomes incorporated into autophagosomes.*** We next set out to examine the ultrastructural characteristics of autophagosomes in primary human and murine mast cells. To address this question we infected primary human cord blood derived mast cells (CBMCs) and primary mouse BMMCs with *P. aeruginosa* strain 8821 at an MOI of 1:100. Eight hours later cells were fixed and processed for TEM viewing (Figure 2.4 A and 2.4 B). Highly vesicularized double membrane bound vesicles characteristic of autophagosomes were observed in both untreated (NT) and *P. aeruginosa* (Ps.a) treated cells. The percentage of cytosol contained within autophagosomes, as well as the number of autophagosomes per cross-section was significantly increased in *P. aeruginosa* treated cells (Figure 2.4 C and 2.4 D). Furthermore, *P. aeruginosa* bacteria were repeatedly seen inside the cell contained within double membrane bound vesicles (insert), although these observations were infrequent. Together these results demonstrate that autophagy is induced by *P. aeruginosa* in primary human and mouse mast cells, and that the bacteria can become incorporated into autophagosomes *in vitro*.

Autophagy contributes to bacterial killing by mast cells in vitro following P. aeruginosa infection: Having observed bacteria inside autophagosome like structures, we next set out to examine the impact of autophagy on bacterial killing in mast cells following *P. aeruginosa* infection. In order to examine the role of autophagy in bacterial killing HMC-1 5C6 cell lines stably expressing non-targeting shRNA (HMC-1 NT shRNA) or shRNA directed against the essential autophagy genes Atg5 (HMC-1 Atg5 shRNA) or Atg7 (HMC-1 Atg7 shRNA) were generated. Knockdown was confirmed by western blot analysis (Figure 2.5 A). Knockdown efficiency for Atg5 and Atg7 shRNA were determined to be 76% and 86% respectively as determined by scanning densitometry.

In order to assess the role of autophagy in the killing of internalized bacteria wild-type HMC-1 cells were left untreated (NT), or pretreated for one hour with the autophagy inhibitor chloroquine, or the autophagy inducer rapamycin. These cells, along with HMC-1 cells expressing non-targeting (NT), or Atg5 or Atg7 specific shRNA, were left uninfected or infected for 3 hours at a 1:20 MOI with *P. aeruginosa* strain 8821. Cells were then treated with cell impermeable antibiotics for 3 hours to kill extracellular bacteria. The effect of each treatment on autophagy was assessed by LC3 Western blot analysis (Figure 2.5 B) and using an LC3-GFP assay (Figures 2.5C and D). Autophagy was induced following *P. aeruginosa* infection, and a further accumulation of autophagosome associated LC3-II was observed when autophagic flux was blocked by chloroquine, or when autophagy was pharmacologically induced with rapamycin. Knockdown of Atg5 and Atg7 significantly decreased autophagy compared to NT shRNA and wild-type controls. These treatments were then used to assess the impact of autophagy on bacterial internalization and killing in cultured mast cells. No significant

differences were observed when cells were treated with cell impermeable antibiotics for 10 minutes indicating that genetic or pharmacological manipulation of the autophagy pathway did not affect internalization of the bacteria (Figure 2.5 E). Importantly, in the bacterial killing assay where cells were treated with cell impermeable antibiotics for 3 hours, manipulation of the autophagy pathway was observed to differentially regulate the killing of internalized bacteria (Figure 2.5 F). Inhibition of autophagy through pharmacological and genetic means resulted in significantly decreased bacterial killing compared to untreated, and non-targeting shRNA controls. Conversely, induction of autophagy with rapamycin was found to significantly increase bacterial killing following *P. aeruginosa* infection. These results suggest that autophagy contributes to the killing of internalized bacteria in mast cells, and pharmacological manipulation of the pathway can enhance clearance of intracellular *P. aeruginosa.*

Pharmacological manipulation of autophagy modulates the clearance of* P. aeruginosa *from bronchial epithelial cells. Given the ability of *P. aeruginosa* to enter and reside within bronchial epithelial cells[59-61, 67, 262], we set out to examine the role of autophagy in these cells during *P. aeruginosa* infection. The human bronchial epithelial cell line 16HBEo$^-$ was left untreated or was infected with *P. aeruginosa* strain 8821 at MOIs of 1:1, 1:10 and 1:100. Eighteen hours post infection lysates were collected and subjected to Western blot analysis for LC3 and actin (Figure 2.6 A). Alternatively, 16HBEo$^-$ cells transiently transfected with LC3-GFP were treated as above, and fixed for examination by confocal microscopy (Figure 2.6 B and C). Autophagy was found to be induced by *P.*

aeruginosa in 16HBEo- cells in a dose dependent manner indicating that the induction of autophagy within the airways by *P. aeruginosa* is not restricted to mast cells.

We next assessed whether manipulation of autophagy in these cells could modulate clearance of intracellular bacterial as was observed in mast cells. Human bronchial epithelial cells (16HBEo$^-$ cells) were either transiently transfected with LC3-GFP for confocal microscopy or were left untransfected for Western blot analysis. Cells were then either left untreated or were pre-treated for one hour with chloroquine to inhibit autophagic flux, or rapamycin to induce autophagy. Cells were left uninfected or infected with *P. aeruginosa* at an MOI of 1:20 for 3 hours after which cell impermeable antibiotics were added for an additional 3 hours. Finally cells were either fixed for confocal microscopy (Figure 2.6 D and E) or lysed for Western blot analysis (Figure 2.6 F). Treatment with either chloroquine or rapamycin did not affect the internalization of *P. aeruginosa* (Figure 2.6 G). As was observed in mast cells, chloroquine treatment inhibited bacterial killing (with increased number of intracellular bacteria). In contrast, treatment with rapamycin promoted bacterial killing (with reduced bacterial numbers in 16HBEo$^-$ epithelial cells) (Figure 2.6 H).

In order to assess the therapeutic potential of these treatments in a model of CF, internalization and killing of *P. aeruginosa* under the above conditions was further compared in normal human 16HBEo$^-$ bronchial epithelial cells, and CFTR ΔF508 homozygous CFBE14o$^-$ epithelial cells. Consistent with previous reports, cells harboring CFTR ΔF508 mutations displayed decreased internalization of *P. aeruginosa* (Figure 2.7 A)[61]. Interestingly, CFBE14o$^-$ cells also displayed a marked impairment in their ability to kill internalized bacteria. Pharmacological manipulation of autophagy differentially

regulated killing of internalized bacteria in these cells. Induction of autophagy with rapamycin promoted bacterial killing (Figure 2.7 B). These results suggest that pharmacological manipulation of autophagy not only enhances clearance of *P. aeruginosa* by normal epithelial cells, but can also restore bacterial clearance from epithelial cells harboring mutations in CFTR.

Pharmacological manipulation of autophagy differentially regulates bacterial clearance in vivo following* P. aeruginosa *infection. Having identified that pharmacological manipulation of autophagy regulates killing of *P. aeruginosa* bacteria by mast cells and bronchial epithelial cells *in vitro* we next set out to study the therapeutic potential of pharmacological manipulation of autophagy *in vivo* during *P. aeruginosa* lung infection. However since the contribution of mast cells during *P. aeruginosa* infection *in vivo* is incompletely understood, we first examined whether mast cells are activated during *P. aeruginosa* infection *in vivo*. The mast cell specific protease mMCP-6 was used as mast cell activation marker *in vivo*. The level of mMCP-6 in the serum, BALF and lung was measured in mice 24 hours after infection with *P. aeruginosa* strain 8821 (1×10^9 CFU/mouse). *P. aeruginosa* infection increased levels of mMCP-6 in the lungs and serum (Figure 2.8). These data suggest that mast cells are activated during *P. aeruginosa* lung infection.

In order to assess the impact of inhibition of autophagy on *P. aeruginosa* lung infection the lysotropic base chloroquine was used. *In vitro* experiments demonstrated that chloroquine did not impact mast cell numbers or viability during *P. aeruginosa* infection (Figure 2.9). Mice were pretreated with CQ by intraperitoneal injection at a

dose of 60 mg/kg/day for 3 days prior to infection, or received an equivalent volume of PBS. Mice were then challenged intranasally with *P. aeruginosa* strain 8821 or a saline control. Twenty four hours later mice were sacrificed and lung tissue and bronchioalveolar lavage fluid (BALF) was collected. Bacterial burden was assessed by counting CFUs in serial dilutions of lung homogenates (Figure 2.10 A) and BALF (Figure 2.10 B). CQ treatment significantly increased bacterial load following *P. aeruginosa* infection in both the lungs and the BALF. Animal survival was also assessed for 10 days post infection with 1×10^9 CFU *P. aeruginosa* strain 8821. While no mortality was observed in animals treated with PBS control, 40% mortality was observed in the chloroquine treated mice ($n = 15$ mice/group, $p < 0.05$) (Figure 2.10 E). Thus, treatment with CQ reduced the clearance of *P. aeruginosa* from the lung and impaired animal survival.

Given that neutrophil recruitment to the site of infection contributes to the clearance of *P. aeruginosa*, we assessed neutrophil accumulation in the lungs and BALF of CQ and saline treated mice through assaying the activity of the neutrophil specific enzyme myeloperoxidase (MPO). MPO activity was unaffected by CQ treatment both in the lungs and the BALF (Figure 2.11 A). The autophagy pathway has also been proposed to play a role in regulating inflammatory cytokine production [286]. In order to determine whether the differences in bacterial clearance observed in chloroquine treated mice were associated with dysregulation of cytokine responses, the levels of various inflammatory cytokines were assessed in the lungs and BALF of *P. aeruginosa* and saline treated mice pretreated with PBS or chloroquine (Table 2.1). No significant differences were observed in the levels of any of the cytokines assayed suggesting that manipulation of the

autophagy pathway did not impact host inflammatory responses. Together these data suggest that the defect in bacterial clearance in CQ treated mice is associated not with coordination of the immune response, but instead with impaired bacterial killing.

Having demonstrated that pharmacological disruption of the autophagy pathway impairs host defense against *P. aeruginosa*, we next set out to test the therapeutic potential of pharmacological induction of the pathway. One of the best studied pharmacological inducers of autophagy is rapamycin, which promotes autophagy through inhibition of the mammalian target of rapamycin (mTOR), a master regulator of the autophagy pathway[287, 288]. As with chloroquine, rapamycin treatment did not impact mast cell numbers or viability during *P. aeruginosa* infection *in vitro* (Figure 2.9). Mice were pretreated with rapamycin at a dose of 10 mg/kg, or an equivalent volume of diluent both at the time of infection, and one day prior. Mice were infected intranasally with 1×10^9 CFU/mouse with *P. aeruginosa*. Twenty four hours later mice were sacrificed and lung tissue and BALF were collected. Bacterial burden was assessed by counting CFUs in serial dilutions of lung homogenates (Figure 2.10 C) and BALF (Figure 2.10 D). We found that rapamycin significantly reduced the bacterial load in both the lungs and the BALF of rapamycin treated mice compared to diluent treated control mice. To examine whether pharmacological modulation of autophagy affects animal survival, mice were pretreated with rapamycin or diluent and intranasally infected with *P. aeruginosa* strain 8821. Animal survival was observed for 7 days. *P. aeruginosa* did not cause mortality in mice treated rapamycin or diluent (Figure 2.10 E).

We next assessed cytokine production (Table 2.2) and neutrophil infiltration (Figure 2.11 B) in the lungs and BALF of rapamycin and diluent treated mice.

Consistent with the reported anti-inflammatory roles of rapamycin, treatment of mice with rapamycin showed significantly reduced levels of almost every inflammatory cytokine tested[289]. As a result, neutrophil recruitment into the lungs and BALF of rapamycin treated animals was also impaired. Together these data suggest that in spite of the impaired inflammatory responses observed in rapamycin treated animals, the accompanying induction of autophagy was able to enhance bacterial clearance above the levels observed in diluent treated animals, supporting a critical role for autophagy in the clearance of *P. aeruginosa* bacteria *in vivo*.

2.5 Discussion:

P. aeruginosa infection remains the number one cause of morbidity and mortality among cystic fibrosis patients who almost invariably become chronically infected with the bacteria [250]. Autophagy represents an evolutionarily conserved mechanism for the clearance of intracellular pathogens, and recent reports have shown the pathway to be dysregulated in the lungs of cystic fibrosis patients[246]. In the present study we examined the contribution of autophagy to the clearance of the cystic fibrosis pathogen *P. aeruginosa*. We found that *P. aeruginosa* induces autophagy in mast cells, which play an important role as sentinel cells during *P. aeruginosa* lung infection. Furthermore bacteria were observed inside autophagosomes, and pharmacological or genetic manipulation of the pathway modulated clearance of internalized bacteria *in vitro*. Similarly pharmacological modulation of autophagy also increased clearance of *P. aeruginosa* from human epithelial cells. Induction of autophagy using rapamcyin was also able to correct defects in the clearance of intracellular bacteria observed in epithelial cells

harboring CFTR ΔF508 mutations. Finally, pharmacological manipulation of the autophagy pathway effectively regulated bacterial clearance from the lungs of infected mice *in vivo*. Together these findings suggest that autophagy is induced in mast cells and epithelial cells in response to *P. aeruginosa.* Pharmacological manipulation of autophagy has considerable therapeutic potential for the treatment of *P. aeruginosa* lung infection.

The emergence of multi-antibiotic resistant strains of *P. aeruginosa* represents a very real threat to the life expectancy and quality of life in cystic fibrosis patients[257-259]. One possible contributing factor to the antibiotic resistant nature of *P. aeruginosa* is the observation that the bacteria has the ability infect host cells, where it can survive for long periods of times within the cytosol, sheltered from cell impermeable antibiotics[59-61, 67, 262]. Normally, intracellular pathogens are targeted for degradation through the autophagy pathway[252]. However, the autophagy pathway has been shown to be impaired in cystic fibrosis patients by mutations in CFTR which lead to dysregulation of the beclin-1 PI3K complex[246]. Given our observations that pharmacological manipulation of the autophagy pathway *in vivo* effectively regulates the clearance of a strain of *P. aeruginosa* isolated from a cystic fibrosis patient, and that pharmacological induction of autophagy corrects defects in the clearance of intracellular bacteria from CF epithelial cells, it is likely that impaired autophagy in cystic fibrosis patients contributes to colonization with the bacteria. As a result, therapies targeted at restoring or enhancing normal autophagy in the lungs of cystic fibrosis patients could significantly enhance clearance of the bacteria from lungs. Therapies aimed at restoring normal autophagy in cystic fibrosis patients have recently been reviewed[290], and treatment of mice harboring mutations in the CFTR with the autophagy inducer rapamycin has been shown to enhance clearance of the cystic

fibrosis pathogen *Burkhoderia cepacia* from the lungs[272]. Together with our data, these findings demonstrate the potential of autophagy based therapy in treating common infections in cystic fibrosis patients. Promisingly, autophagy based therapies could serve as an excellent complement to conventional antibiotic therapies. While antibiotic therapies can target extracellular populations of bacteria, the induction of autophagy has the ability to target intracellular bacteria. Targeting both populations could not only enhance killing of the bacteria, but also eradicate a pool of bacteria contributing to chronic infections, and the development of antibiotic resistance[67]. Furthermore, restoring normal autophagy in mice harboring mutations in CFTR also resolves chronic inflammation observed in the lungs[291], suggesting that as well as resolving *P. aeruginosa* infection, combining antibiotic therapy with autophagy therapy could help resolve damaging inflammation associated with deteriorating lung function in cystic fibrosis patients[292, 293].

Therapeutic manipulation of the autophagy pathway has become a topic of considerable interest over the past decade due to implication of the pathway in a wide range of pathologies. However, despite the recent interest in autophagy modulators, the inducers of autophagy currently available surfer from considerable undesirable off target effects, raising the need for development of novel, specific autophagy inducers. Current autophagy inducers can be grouped into three kinds of compounds: i) mTOR inhibitors, ii) modulators of calcium dependent signaling or iii) IP_3 inhibitors [293]. Each of pathways targets plays integral roles in multiple cell pathways making them poor therapeutic targets for the specific induction of autophagy. In the current study, the mTOR inhibitor rapamycin was used to induce autophagy. In addition to inducing autophagy, rapamycin

also has well characterized immunosuppressive effects[289]. Accordingly, decreased inflammatory cytokine production was observed in our model which led to impaired neutrophil recruitment to the site of infection. These defects were likely independent of the effects of rapamycin on autophagy as inhibition of the pathway did not impact inflammatory cytokine production. Similarly, chloroquine, the autophagy inhibitor used in this study, is a lysotoropic base which raises the pH of lysosomes, preventing degradation through the autophagy pathway. However inhibition of classical phagocytic killing by chloroquine cannot be ruled out. It is important to note that in spite of the immunosuppressive effects of rapamycin which led to decreased neutrophil recruitment to the lungs of infected mice, enhanced bacterial clearance was observed following rapamycin treatment, reinforcing the contribution of autophagy to bacterial killing in our model. The development of specific autophagy modulators, as well as therapeutic strategies for restoring normal autophagy in cystic fibrosis patients will be an essential step towards improved treatment of *P. aeruginosa* infection. Excitingly, recent work has identified a peptide fragment from beclin-1 which strongly and specifically induces autophagy, and was able to decrease mortality associated with viral pathogens *in vivo*[294]. Such targeted therapeutic strategies for the modulation of autophagy have considerable clinical potential for the effective control and irradiation of *P. aeruginosa* lung infections.

Intracellular pathogens have developed a wide range of mechanisms for subverting or usurping the host autophagy pathway to promote their pathogenicity and survival[227, 295]. Given the novel observations that autophagy contributes to the clearance of *P. aeruginosa in vivo*, it raises the possibility that the bacteria have evolved similar mechanisms to avoid killing through the autophagy pathway. *P. aeruginosa* bacteria

employ various mechanisms which skew the host immune response towards a Th2 phenotype[21, 31]. It has long been thought that a Th2 like response is favorable in the clearance of extracellular infections, while a Th1 response favors killing of intracellular infections. However, in the case of *P. aeruginosa* infections a Th2-like response is in fact correlated with poorer lung function and clinical outcomes compared to the minority of patients which mount a Th1-like response, underscoring the importance of the intracellular phase of *P. aeruginosa* infection[296-298]. Interestingly, Th2 cytokines have been demonstrated to suppress autophagy, suggesting that the Th2 responses driven by *P. aeruginosa* could promote the bacteria's intracellular survival by preventing activation of the autophagy pathway. In addition, *P. aeruginosa* secretes exotoxin A which shuts down host protein synthesis through inhibiting eukaryotic elongation factor 2A (eEF2A) which greatly enhances the virulence of the bacteria[299]. However disruption of the eEF2A signaling pathways has also been shown to impair activation of autophagy[300]. Thus, *P. aeruginosa* may employ multiple mechanisms for disrupting the autophagy pathway, contributing to enhanced bacterial survival and pathogenicity.

Given the increasing prevalence of multi-antibiotic resistant strains of *P. aeruginosa* bacteria, novel therapeutic approaches for the treatment of chronic infections in individuals with cystic fibrosis will be essential to ensure the continued health of these patients in the coming years. Here we demonstrate that *P. aeruginosa* induces autophagy in mast cells, and that the pathway contributes to the killing of internalized bacteria *in vitro*. We further demonstrate that therapeutic intervention aimed at inducing autophagy with rapamycin correlates with decreased bacterial loads following *P. aeruginosa* lung infection *in vivo,* in spite of decreased inflammatory responses and neutrophil infiltration

caused by rapamycin treatment. This work provides the first evidence that the pharmacological manipulation of the autophagy pathway has considerable therapeutic potential in the treatment of *P. aeruginosa* infections *in vivo*.

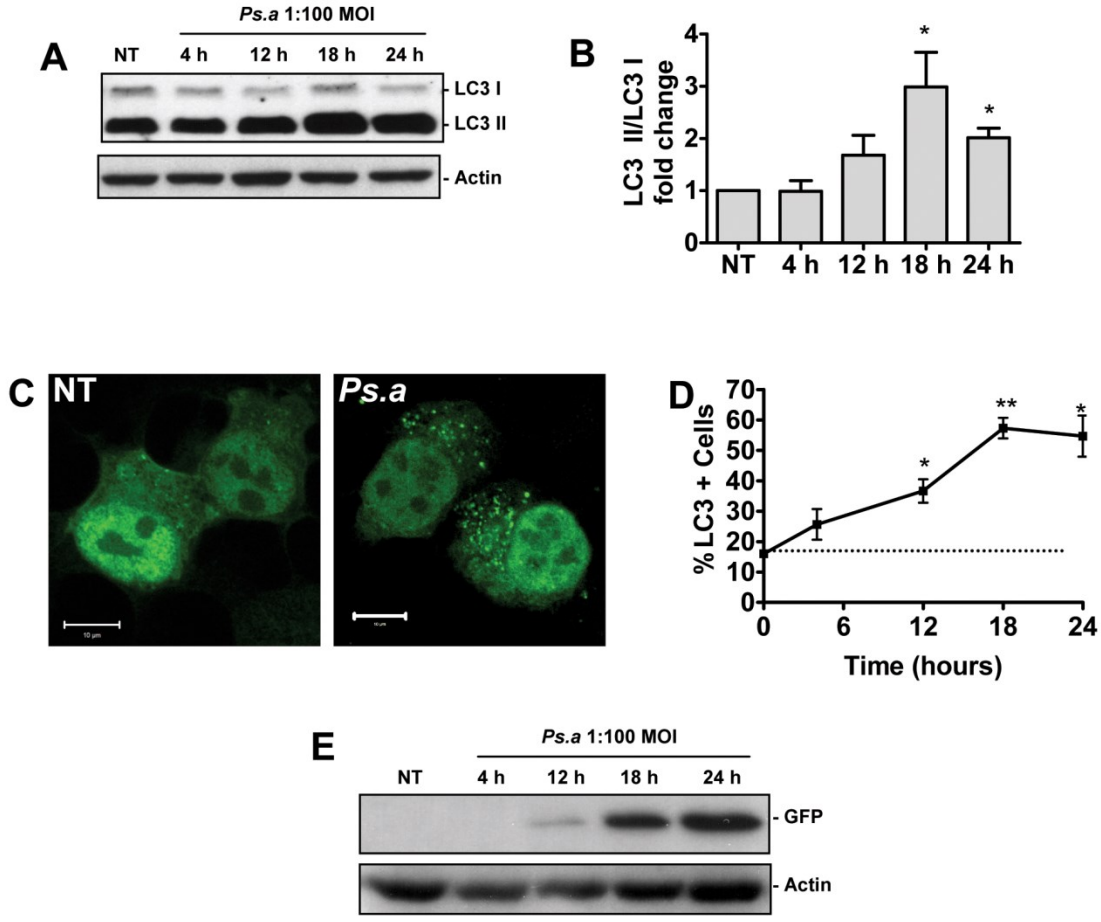

Figure 2.1: *P. aeruginosa* induces autophagy in mast cells. Primary mouse BMMCs were left untreated (NT) or infected with *P. aeruginosa* strain 8821 at an MOI of 1:100. Lysates were collected at the time points indicated and subjected to Western blot analysis for LC3 and actin loading control (A). The fold change in the ratio of LC3-I to LC3-II normalized to actin was determined by scanning densitometry (B) (n = 3 ± SEM, *p < 0.05). HMC-1 5C6 human mast cell line stably expressing LC3-GFP reporter were left untreated (NT) or infected with *P. aeruginosa* at an MOI of 1:100 for 18 hours (*Ps.a*) before being fixed and examined by laser scanning confocal microscopy (C). HMC-1 5C6 cells stably expressing LC3-GFP were left untreated (NT) or infected with *P. aeruginosa* strain 8821 at an MOI of 1:100. At the indicated time points cells were fixed for the study by confocal microscopy, and the percentage of cells displaying 5 or more GFP puncta was assessed (D). (n=3 ± SEM, *p < 0.05, **p < 0.01). Lysates were also prepared from HMC-1 5C6 cells stably expressing LC3-GFP and Western blot analysis for free GFP and actin loading control was performed (E).

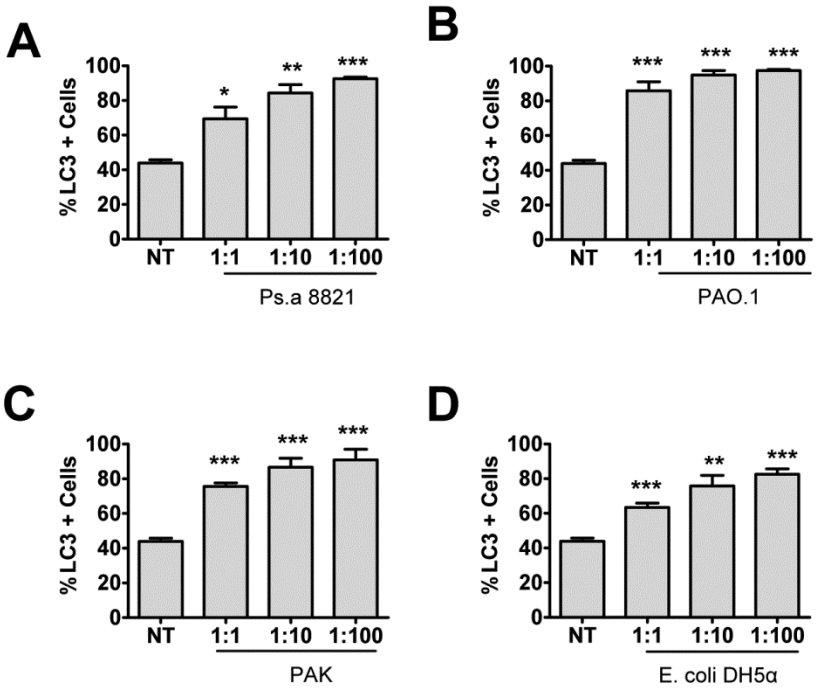

Figure 2.2: Strain and dose effects of *P. aeruginosa* and *E. coli* on autophagy in mast cells: Wild-type or LC3-GFP expressing HMC-1 5C6 cells were left untreated or infected at a 1:1, 1:10 and 1:100 MOI with *P. aeruginosa* strains 8821 (A, E), PAO.1 (B, F), PAK (C, G) or *E. coli* strain DH5α (D, H). Cells were incubated for 18 hours at 37^{o}C then lysed for Western blot analysis of LC3 and actin (A-D) or fixed and examined by fluorescence microscopy for the percentage of cells containing greater than 5 LC3-GFP puncta (E-H) (n = 3 ± SEM, *p < 0.05, **p < 0.01, ***p < 0.005).

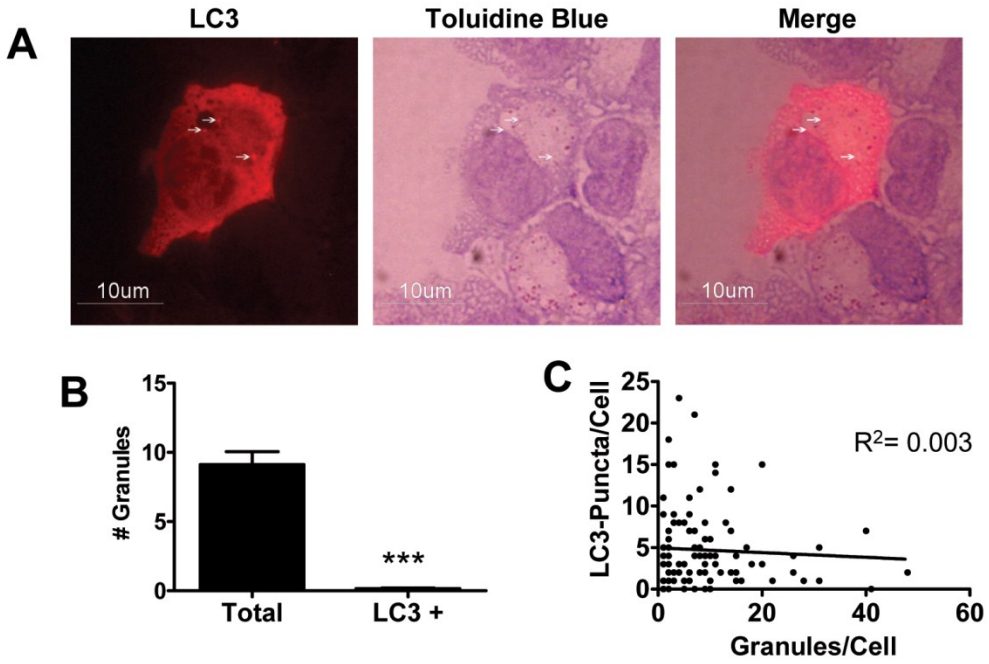

Figure 2.3: LC3 does not colocalize with granules in HMC-1 cells: HMC-1 5C6 cells were transiently transfected with LC3-GFP-mCherry then fixed and stained with toluidine blue 48 hours later. Cells were examined by fluorescence and light microscopy then LC3-mCherry positive puncta, and toluidine blue positive granules were identified. Colocalization of LC3 puncta (indicated with arrows) with mast cell granules was assessed (A). The average number of total granules and LC3 positive granules per cell was determined (B) (n = 100 ± SEM, ***$p < 0.005$). The correlation between the number of granules and the number of LC3 positive puncta in each cell was also assessed (C) (n = 100).

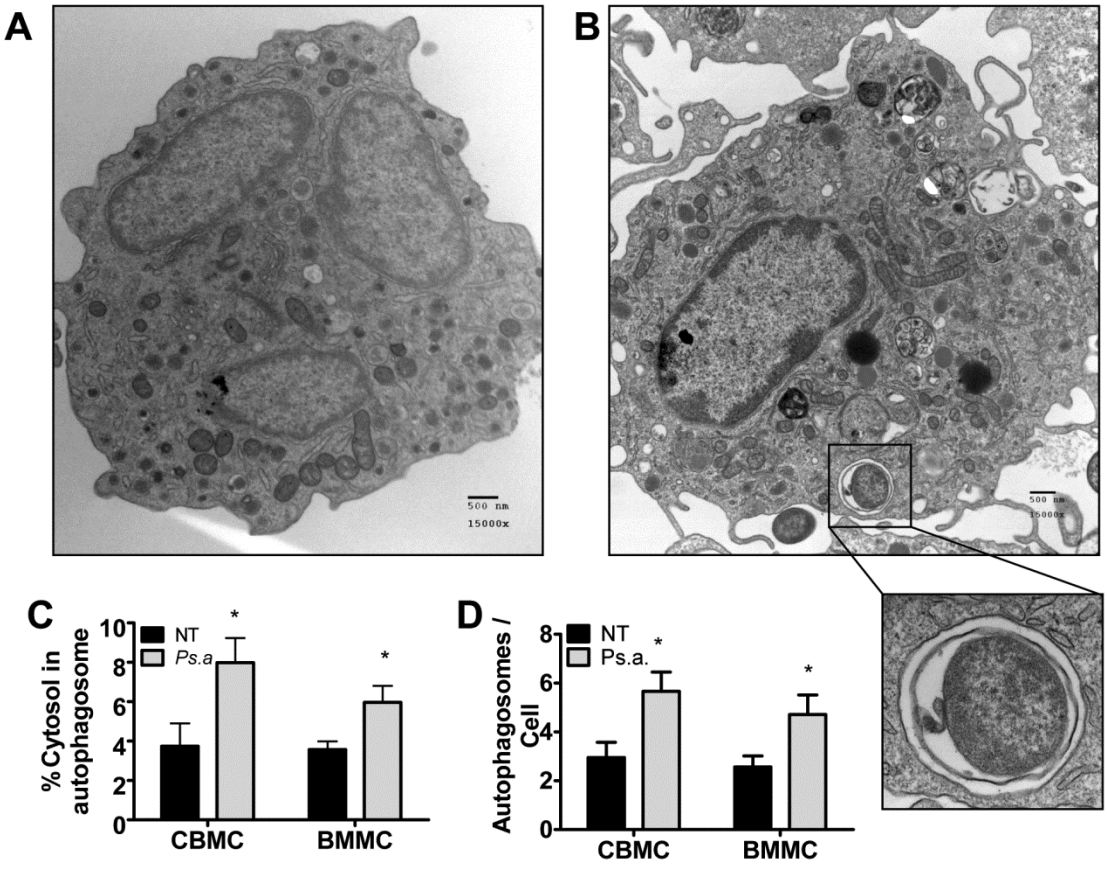

Figure 2.4: *P. aeruginosa* induces autophagy in primary human and mouse mast cells, and becomes incorporated into autophagosomes. Primary human cord blood derived mast cells (CBMCs) and mouse bone marrow-derived mast cells (BMMCs) were left untreated (NT) or infected with *P. aeruginosa* strain 8821 at an MOI of 1:100 for 8 hours. Then cells were fixed and processed for transmission electron microscopic study. Representative images of untreated (A) and *P. aeruginosa* treated (B) CBMCs are shown. *P. aeruginosa* could clearly be seen inside double membrane bound vesicles inside the mast cells (insert). The percentage of cytosol encompassed within autophagosomes (C) and the number of autophagosomes per cross section (D) in CBMCs and BMMCs was calculated by area analysis of cross sections containing a portion of the cell nucleus. (n = 21 ± SEM, *$p < 0.05$)

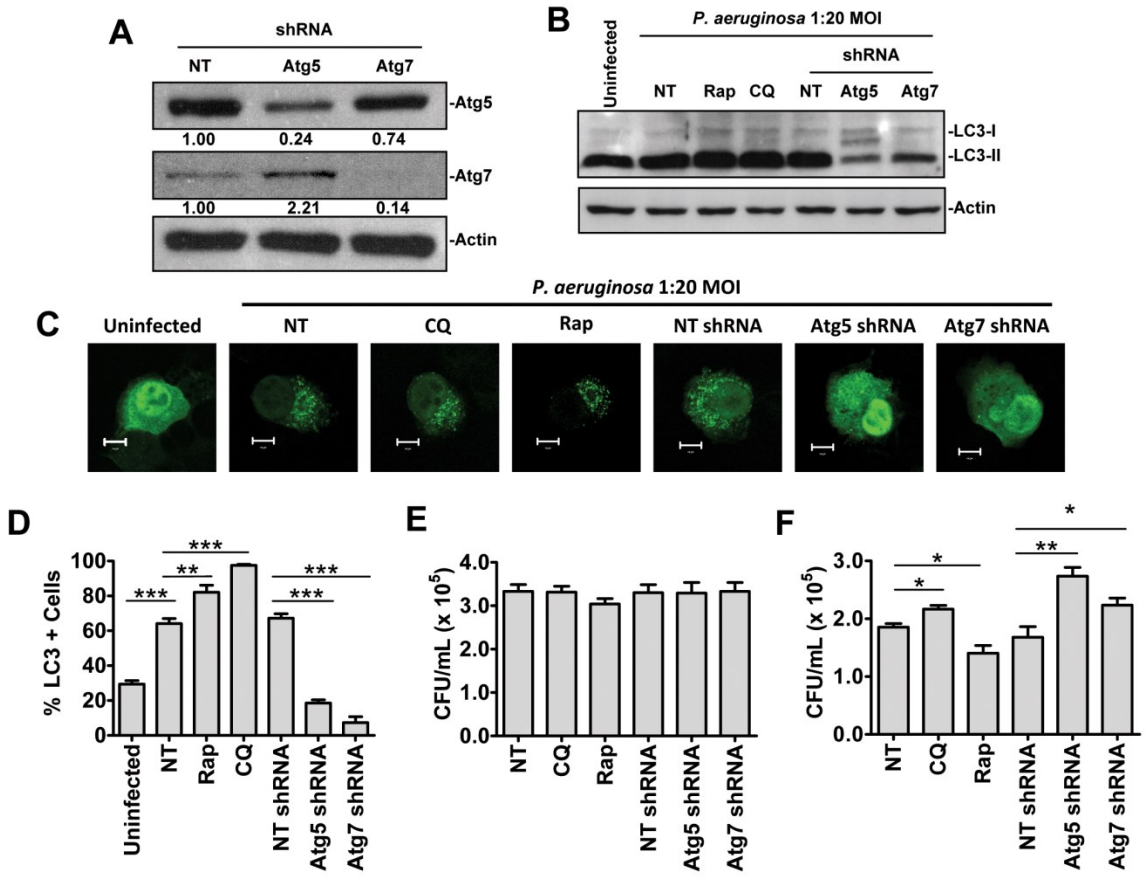

Figure 2.5: Autophagy contributes to bacterial killing by mast cells *in vitro* following *P. aeruginosa* infection. HMC-1 5C6 cells stably expressing non-targeting (NT) shRNA or shRNA specific for the autophagy genes Atg5 or Atg7 were subjected to western blot analysis to confirm protein knockdown (A). Numbers below each band represent fold change vs. NT shRNA group and normalized to actin as determined by scanning densitometry. Wild-type HMC-1 5C6 cells were left untreated (NT) or pretreated for one hour with 20 μM chloroquine (CQ) or 2 μM rapamycin (Rap). These cells, along with HMC-1 5C6 cells stably expressing NT, Atg5 or Atg7 specific shRNA were left uninfected, or infected at a 1:20 MOI with *P. aeruginosa* strain 8821 for 3 hours. Cell impermeable antibiotics were added to kill extracellular bacteria for the times indicated. Cells were then lysed and subjected to Western blot analysis for the autophagy marker LC3 and acting loading control (B). Alternatively HMC1-5C6 cells expressing LC3-GFP and treated as above were monitored by laser scanning confocal microscopy (C and D) (n = 3 ± SEM, **p < 0.01, ***p < 0.005). Lysates were prepared from HMC-1 5C6 cells treated as described above, and incubated in the presence of cell impermeable antibiotic for 10 minutes to determine bacterial internalization (E) or 3 hours to examine bacterial killing (F). Serial dilutions of these lysates were streaked on LB agar plates and CFU/mL lysate was determined (n = 6 ± SEM, *p < 0.05, **p < 0.01).

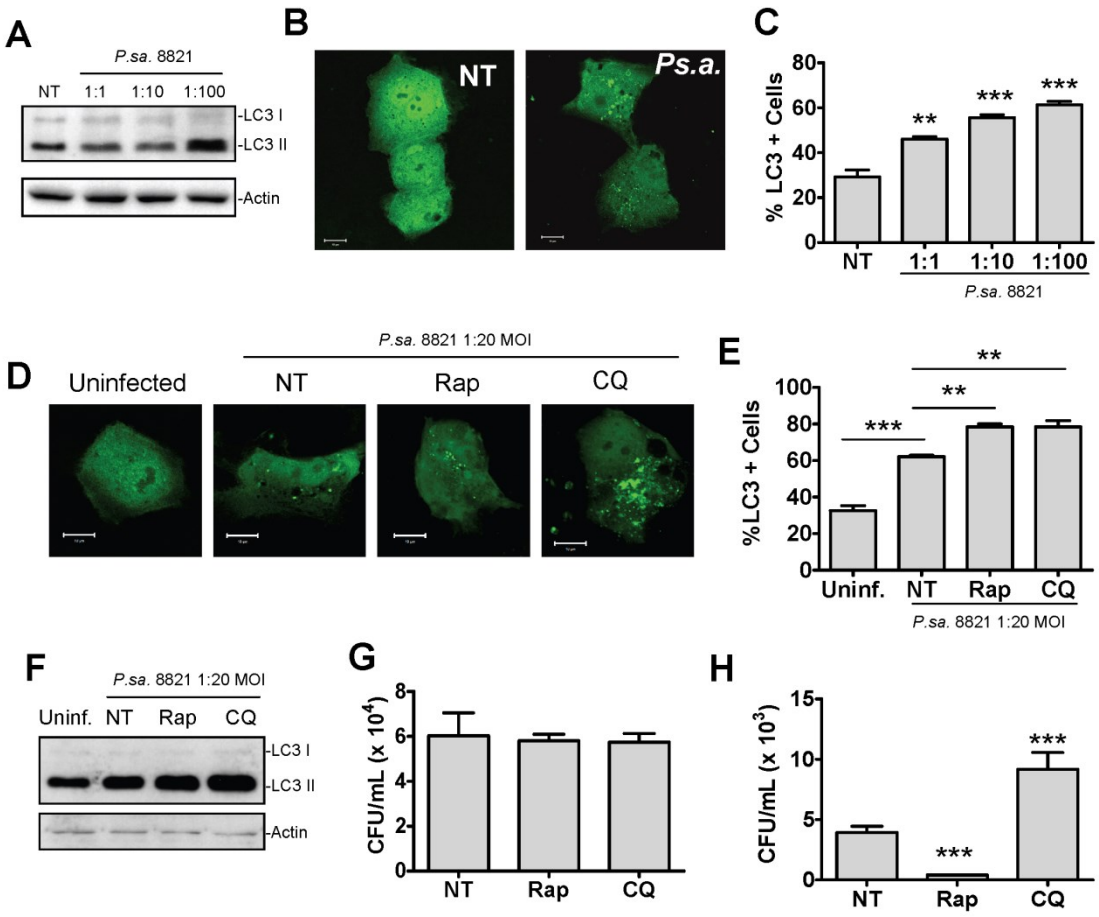

Figure 2.6: Autophagy enhances clearance of intracellular *P. aeruginosa* from normal human bronchial epithelial cells. The human bronchial epithelial cell line 16HBEo$^-$ was left uninfected, or infected with *P. aeruginosa* at an MOI of 1:1, 1:10 and 1:100. Lysates were collected 18 hours post infection and subjected to Western blot analysis for LC3 and actin (A). Alternatively, cells were transiently transfected with LC3-GFP two days prior to infection then treated as described above. Eighteen hours post infection cells were fixed and examined by laser scanning confocal microscopy (B) and the percentage of cells containing greater than 5 LC3-GFP puncta was determined (C) (n = 3 ± SEM, **p < 0.01, ***p < 0.005). Untransfected, or LC3-GFP expressing 16HBEo$^-$ cells were left untreated, or pretreated for one hour with rapamycin (2 μM) or chloroquine (20 μM) then infected at a 1:20 MOI with *P. aeruginosa* strain 8821. Cell impermeable antibiotics were added 3 hours later, then after an additional 3 hour incubation cells were either fixed for evaluation of autophagy by confocal microscopy (D and E) (n = 3 ± SEM, *p < 0.05, **p < 0.01, ***p < 0.005) or lysed and analyzed by Western blot analysis for LC3 and actin (F). Alternatively, cells were lysed 10 minutes (G) or 3 hours (H) after the addition of cell impermeable antibiotics and streaked on LB agar plates for the determination of intracellular CFUs (n = 5 ± SEM, ***p < 0.005). The 10 minute and 3 hour time points represent bacterial internalization and killing respectively.

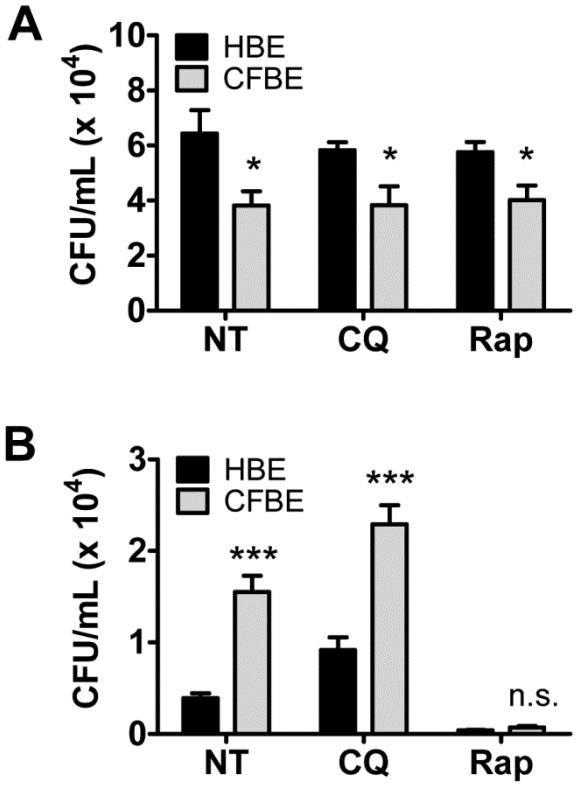

Figure 2.7: Induction of autophagy restores bacterial clearance in CF epithelial cells: Ten thousand 16HBE14o- (HBE) normal human bronchial epithelial cells or CFBE41o- (CFBE) homozygous CFTR ΔF508 cystic fibrosis bronchial epithelial cells were left untreated (NT) or pretreated for one hour with 20 μM chloroquine (CQ) or 2μM rapamycin (Rap). Cells were then infected at a 1:20 MOI with *P. aeruginosa* strain 8821 for 3 hours. Cell impermeable antibiotics were added for 10 minutes (A) or 3 hours (B) then serial dilutions of cell lysates were streaked to assess intracellular CFUs. The 10 minute and 3 hour time points represent bacterial internalization and bacterial killing respectively.

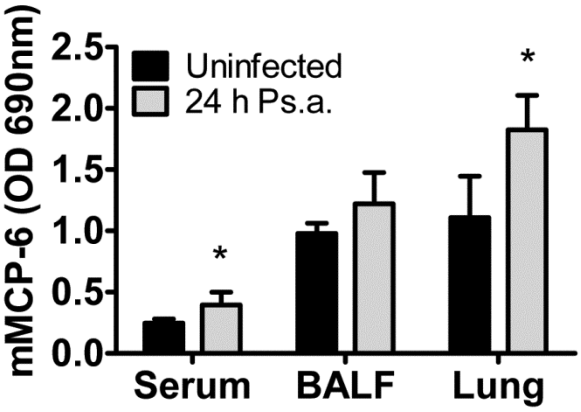

Figure 2.8: Mast cells contribute to host defense against *P. aeruginosa*: C57BL/6 mice were left uninfected, or infected intranasally with 109 *P. aeruginosa* strain 8821. Twenty four hours later serum, BALF and lung tissue was collected and the relative concentrations of the mast cell specific protease mMCP6 were determined by solid phase ELISA (n=6 ± SEM, *$p < 0.05$)

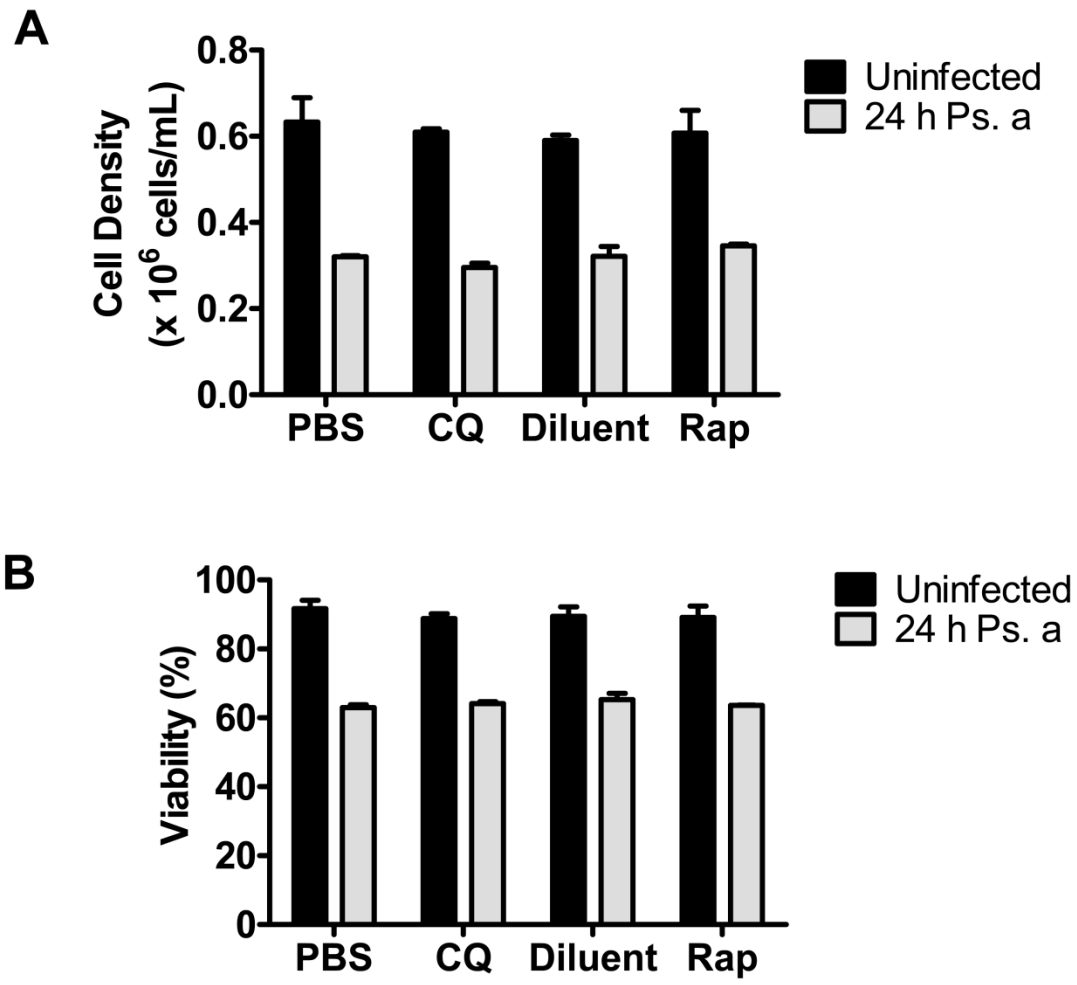

Figure 2.9: Pharmacological manipulation does not impact mast cell survival following *P. aeruginosa* infection: BMMCs from C57BL/6 mice were pretreated for 24 hours with 20 μM chloroquine (CQ), 2 μM rapamycin (RAP), or an equivilant volume of PBS or rapamycin diluent. Cell were then left uninfected, or infected with *P. aeruginosa* strain 8821 at an MOI of 1:10 for for 24 hours after which cell density (A) and viability (B) was determined by trypan blue staining (n= 3 ± SEM)

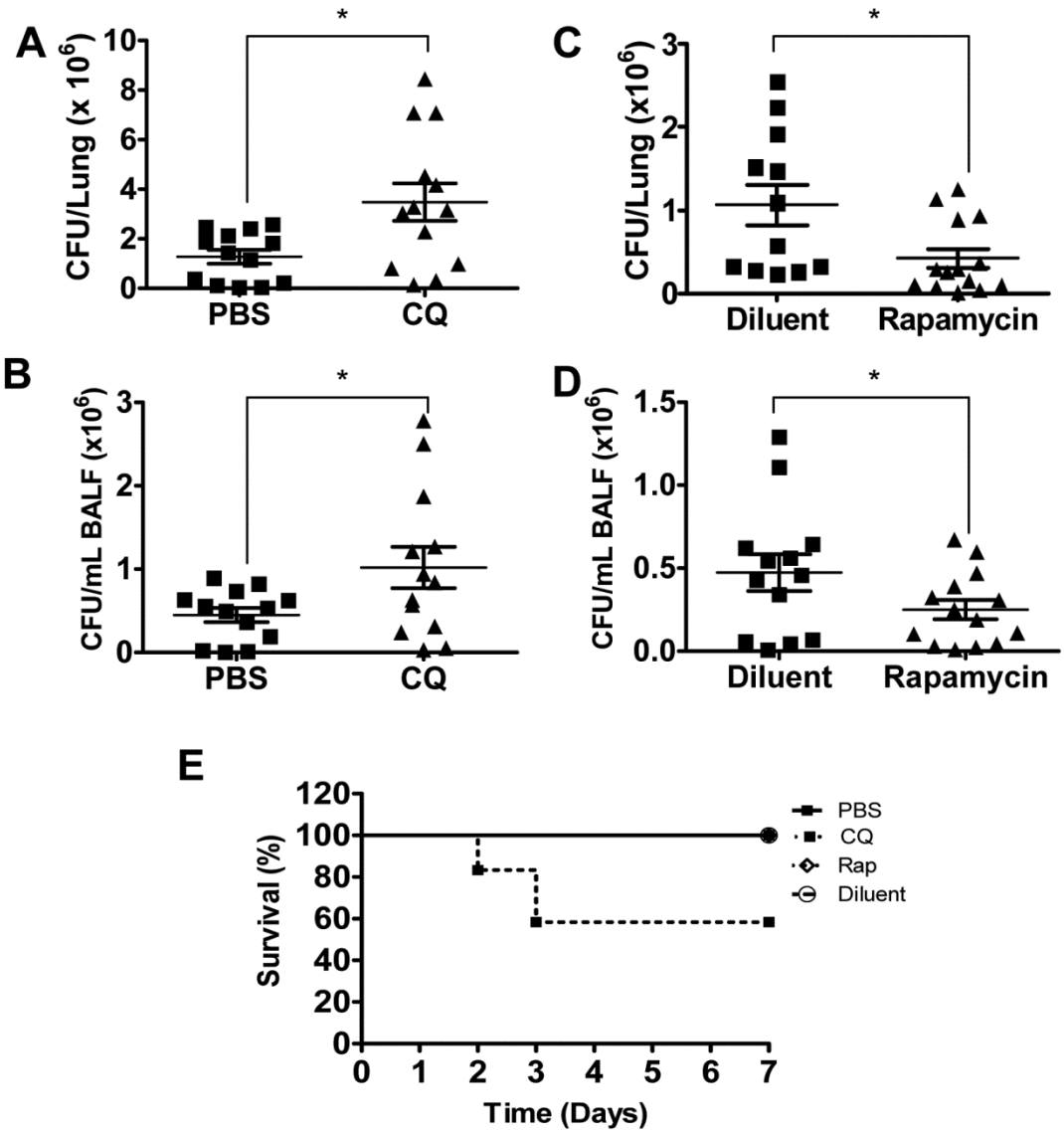

Figure 2.10: Pharmacological manipulation of autophagy modulates *P. aeruginosa* clearance *in vivo*. C57BL/6 mice were treated with intraperitoneal injection of PBS or 60 mg/kg/day chloroquine (CQ) in PBS for 3 days (A-B, E) or with intraperitoneal injection of diluent or 10 mg/kg/day rapamycin for 1 day (C-E), then infected intranasally with 10^9 CFU/mouse *P. aeruginosa* strain 8821. Twenty four hours later mice were sacrificed and CFU was counted in the Lung (A, C) and BALF (B, D). (n = 13-15 ± SEM,*$p < 0.05$). Alternatively animal survival was monitored for 10 days post infection (n = 15 ± SEM, *$p < 0.05$)

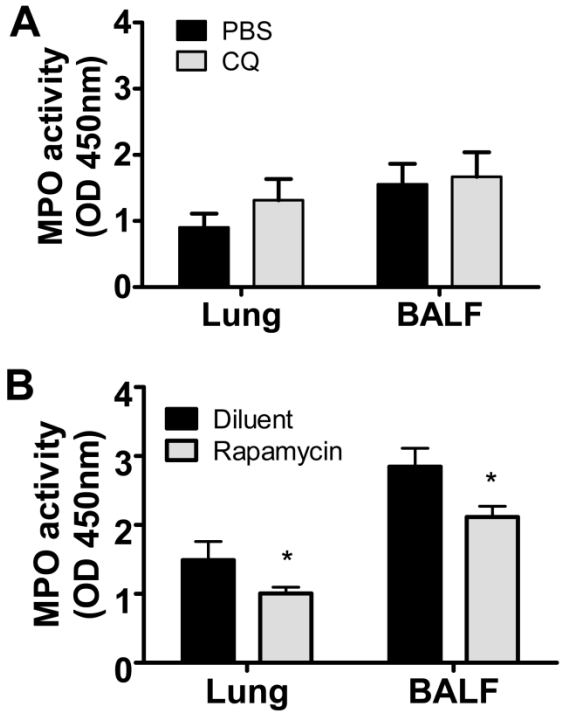

Figure 2.11: Rapamycin but not chloroquine impairs neutrophil infiltration into the lungs and BALF during *P. aeruginosa* lung infection. C57BL/6 mice were treated with intraparteneal injections of PBS or 60 mg/kg/day chloroquine (CQ) in PBS for 3 days (A) or with intraparteneal injections of diluent or 10 mg/kg/day rapamycin for 1 day (B), then infected intranasally with 109 CFU/mouse *P. aeruginosa* strain 8821. Twenty four hours later mice were sacrificed and lung tissue and BALF was assayed for the activity of the neutrophil specific enzyme MPO (n=13-15 ± SEM, *$p < 0.05$).

Table 2.1: Chloroquine treatment does not affect inflammatory cytokine production following *P. aeruginosa* lung infection.

		Uninfected[A]		24 h *P. aeruginosa*[B]	
		PBS	Chloroquine	PBS	Chloroquine
Lung					
	IL-6	113.8 ± 10.8	103.7 ± 7.6	449.2 ± 60.7	601.9 ± 121.1
	RANTES	160.9 ± 15.2	127.1 ± 8.9	8301.2 ± 1514.2	9363.1 ± 1500.1
	IL-1β	208.9 ± 20.9	168.5 ± 25.2	501.0 ± 24.3	526.8 ± 8.9
	TNF	156.3 ± 11.9	135.4 ± 18.9	721.7 ± 72.9	750.9 ± 57.9
	MIP-2	313.9 ± 19.3	333.9 ± 20.8	512.0 ± 34.8	558.1 ± 49.4
BALF					
	IL-6	151.3 ± 10.8	155.4 ± 11.7	356.8 ± 78.5	304.7 ± 26.9
	RANTES	2114.8 ± 463.2	2451 ± 441.1	4882.5 ± 888.0	6029.1 ± 10.20.1
	IL-1β	523.4 ± 36.2	427.4 ± 40.9	4125.4 ± 493.5	4237.3 ± 1288.1
	TNF	156.3 ± 11.9	142.4 ± 9.4	245.8 ± 21.6	245.1 ± 24.5
	MIP-2	381.1 ±15.7	256.0 ± 18.5	683.7 ± 32.0	710.6 ±25.5

[A]Data are the mean ± SEM of 5 mice per group (pg/mL)

[B]Data are the mean ± SEM of 13-14 mice per group (pg/mL)

Table 2.1: C57BL/6 mice were treated with intraperitoneal injections of PBS or 60 mg/kg/day chloroquine (CQ) in PBS for 3 days, and then left uninfected, or infected intranasally with 10^9 CFU/mouse *P. aeruginosa* strain 8821. Twenty four hours later mice were sacrificed and lung tissue and BALF was collected and assayed for concentrations of indicated cytokines via ELISA.

Table 2.2: Rapamycin impairs inflammatory cytokine responses following *P. aeruginosa* lung infection

		Uninfected[A]		24 h *P. aeruginosa*[B]	
		Diluent	Rapamycin	Diluent	Rapamycin
Lung					
	IL-6	113.8 ± 10.8	103.7 ± 7.6	203.4 ± 17.2	180.9 ± 26.5
	RANTES	127.1 ± 8.9	100.9 ± 20.3	1969.4 ± 162.8	1231.9 ± 159.3**
	IL-1β	208.8 ± 20.9	168.5 ± 25.2	2307.3 ±297.0	1309.9 ± 135.3***
	TNF	120.3 ± 4.8	88.9 ± 6.9*	201.8 ± 16.0	134.0 ± 10.5***
	MIP-2	333.9 ± 20.8	168.5 ± 25.17**	1520.3 ± 117.1	1012.9 ± 173.4***
BALF					
	IL-6	151.3 ± 10.8	155.4 ± 11.7	1633.5 ±138.2	1127.4 ± 165.4*
	RANTES	914.8 ± 159.3	971.6 ± 101.7	2726.3 ± 197.2	2250.7 ± 203.7
	IL-1β	128.9 ± 5.01	80.4 ± 11.2**	194.7 ± 29.9	159.6 ± 13.1
	TNF	120.3 ± 4.7	88.8 ± 6.9**	1283.8 ± 231.6	451.7 ± 55.3**
	MIP-2	333.9 ± 20.7	168.5 ± 25.2***	826.1 ± 182.9	346.7 ± 71.1**

[A]Data are the mean ± SEM of 5 mice per group (pg/mL)

[B]Data are the mean ± SEM of 18-19 mice per group (pg/mL)

*p<0.05, **p<0.01, ***p<0.001

Table 2.2: C57BL/6 mice were treated with intraperitoneal injections of diluent or 10 mg/kg/day rapamycin for 1 day, and then left uninfected, or infected intranasally with 10^9 CFU/mouse *P. aeruginosa* strain 8821. Twenty four hours later mice were sacrificed and lung tissue and BALF was collected and assayed for concentrations of indicated cytokines via ELISA.

CHAPTER 3: REGULATOR OF CALCINEURIN-1 SUPRESSESS INFLAMMATION DURING RESPIRATORY TRACT INFECTIONS

Regulator of calcineurin-1 suppresses inflammation during respiratory tract infections

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Author contributions:

Robert D. Junkins: Designed and executed experiments and analyzed data for all figures and tables. Wrote and edited manuscript.

Adam J. MacNeil: Performed replicates for Figure 3.1.4 A and B, and provided technical assistance with EMSA assays.

Zhegnli Wu: Assisted with experimental designed, and interpretation of data.

Craig McCormick: Contributed to manuscript editing and revisions.

Tong-Jun Lin: Oversaw study design and data analysis, provided funding and participated in manuscript editing and revisions.

3.1 Abstract:

Respiratory tract infection with *Pseudomonas aeruginosa* is a common cause of hospitalization in immune compromised individuals. However, the molecular mechanisms involved in the immune response to *P. aeruginosa* lung infection remain incompletely defined. Here we demonstrate that the regulator of calcineurin 1 (RCAN1) is a central negative regulator of inflammation in a mouse model of acute bacterial pneumonia using the opportunistic bacterial pathogen *P. aeruginosa*. RCAN1-deficient mice display greatly increased mortality following *P. aeruginosa* lung infection despite enhanced neutrophil recruitment and bacterial clearance. This mortality is associated with higher systemic levels of pro-inflammatory cytokines in RCAN1-deficient animals. These aberrant inflammatory responses coincide with increased transcriptional activity of pro-inflammatory RCAN1-target proteins NFAT and NF-κB. In addition, we reveal a novel regulatory role for RCAN1 in the ERK/STAT3 pathway both *in vitro* and *in vivo,* suggesting that aberrant STAT3 activity may significantly contribute to delayed resolution of inflammatory responses in our model. Together, these findings demonstrate that RCAN1 is a potent negative regulator of inflammation during respiratory tract infections.

3.2 Introduction:

Down syndrome (DS) is caused by trisomy of chromosome 21 and represents single most common chromosomal anomaly in live born infants, occurring with an incidence of one in every 600 to 900 live births[301-303]. Along with severe mental and anatomical abnormalities, DS is associated with a wide range of immunodeficiencies[304]. Included are deficiencies in adaptive immunity such as lymphopenia[305], impaired antibody response to immunization[306, 307] as well as decreased levels of IgA[308]. Deficits in innate immunity have also been identified primarily involving impaired neutrophil chemotaxis [309, 310].

In spite of severe anatomical and mental defects associated with the condition the most common health complication associated with DS is recurrent and persistent respiratory tract infections[311, 312], among which the greatest cause of hospital admission are bacterial and viral pneumonias[313, 314]. Although there have been multiple efforts to characterize immunodeficencies in DS patients, the molecular and genetic mechanisms which underlie many of these defects remain poorly defined. Regulator of calcineurin 1 (*RCAN1*) is a DS-associated gene which has recently emerged as an attractive candidate for mediating some of the immunodeficiencies associated with this syndrome.

The human *RCAN1* gene was previously known as *DSCR1* (Down syndrome critical region 1)[315], *Adapt78*[316], myocyte-enriched calcineurin interacting protein 1 (*MCIP1*)[189], *calcipressin 1*[317], and calcineurin binding protein 1 (*CBP1*)[187]. *RCAN1* is located within the DS critical region on chromosome 21 and is highly expressed in various tissues, including heart, lung, kidney, brain, muscle, liver, and testis[189, 318, 319]. The *RCAN1* gene consists of seven exons, of which exons 1 to 4 can be alternatively

transcribed or spliced to produce different mRNA isoforms[320]. Deletion of exons 5 and 6 from the mouse *Rcan1* gene leads to deficiency of the RCAN1 protein and produces mice that are viable and fertile providing a useful tool for the study of RCAN1 function[321].

RCAN1 has recently become a protein of great interest as a candidate gene responsible for various immunodeficiencies in DS patients due mainly to its prominent role in regulating two critical pathways that mediate inflammatory responses. The first pathway mediated by RCAN1 is the nuclear factor of activated T cells (NFAT). RCAN1 can interact directly with calcineurin in the cytosol in order to inhibit the phosphatase activity of the protein required for the dephosphorylation and nuclear translocation of NFAT[187-189]. NFAT activation has been implicated in the function of a wide variety of immune cells including T cells[322], B cells[323], mast cells[324], natural killer cells[325] and macrophages[326] where it induces various cytokines including IL-2[322], IL-3[327], IL-4[328], IL-5[329], IL-6[330], TNF[331] and GM-CSF[332], among others.

The second inflammatory pathway mediated by RCAN1 is the NF-κB pathway. NF-κB is a major regulator of inflammation as it induces expression of a plethora of inflammatory cytokines including IL-1β[333], IL-6[334], TNF[335] and IL-8[336] in humans, as well as the murine neutrophil chemoattractant chemokines macrophage inflammatory protein 2 (MIP-2/CXCL2)[337], keratinocyte chemoattractant (KC/CXCL1)[338] and lipopolysaccharide induced CXC chemokine (LIX/CXCL5)[339]. RCAN1 has been proposed to play negative regulatory roles in the NF-κB pathway through both calcineurin dependent[190] and calcineurin independent mechanisms[191], both of which lead to enhanced stability of a family of NF-κB inhibitory molecules known as IκB.

Previous work has focused on the role of RCAN1 in the production of various inflammatory cytokines in response to immunological stimuli[340]. However the biological implications of *RCAN1* during infection remain poorly understood. To examine the role of the RCAN1 during the innate immune response, we use a lung infection model of acute bacterial pneumonia that employs the opportunistic bacterial pathogen *Pseudomonas aeruginosa,* which is associated with high levels of morbidity and mortality among cystic fibrosis patients and the immunocompromised individuals[9]. We find that RCAN1-deficiency results in greatly increased mortality following *P. aeruginosa* infection despite enhancement of neutrophil recruitment and bacterial clearance from the lungs. The increased mortality was associated with an inappropriate early cytokine response following infection with *P. aeruginosa* mediated through increased activation of the pro-inflammatory transcription factors NF-κB and NFAT. Unexpectedly, we further identified a novel regulatory role for RCAN1 in a third inflammatory pathway leading to the activation of STAT3. These data confirm that RCAN1 is a central negative regulator of inflammatory cytokine production, and trisomy of the gene warrants further investigation as a contributing factor to immune dysfunction in DS patients.

3.3 Materials and Methods

Animals

The RCAN1 gene was targeted for deletion by standard homologous recombination in embryonic stem cells (Sv129 strain), followed by generation of chimeric mice, which were subsequently bred to pass the targeted allele into the germline in the C57BL/6

genetic background, as described elsewhere[321]. These mice were originally provided by Dr. Jeffery Molkentin (Cincinnati Children's Hospital Medical Center, University of Cincinnati, Cincinnati, OH). C57/BL6 mice were purchased from Charles River Laboratories and bred in the same facility as RCAN1 deficient mice were used as wild-type controls. All animal protocols were approved by the University Committee on Laboratory Animals, Dalhousie University, in accordance with guidelines of the Canadian Council on Animal Care.

Animal survival and antibiotic therapy studies

For survival experiments the laboratory strain of *P. aeruginosa* PAK (a gift from Dr. J Boyd (Institute of Marine Bioscience, National Research Council, Halifax, NS, Canada)) was used. Mice were intranasally infected with 1 LD_{50} of *P. aeruginosa* (2.25 x 10^7 CFU/mouse). For antibiotic therapy studies mice were treated as previously described[341]. Briefly, animals were administered 200 mg/kg ceftazidime (Sigma-Aldrich, St. Louis, MO) subcutaneously every 8 hours, starting 2 hours post-infection in order to achieve serum levels of ceftazidime similar to those observed clinically[341, 342]. Animals were monitored daily for 10 days and the weight, body temperatures and disease scores were measured. Serum was collected 24 hours prior to infection then 3, 6 and 12 hours post infection to monitor systemic inflammation. Moribund animals were sacrificed according to the guidelines of the Canadian Council on Animal Care.

Antibodies

Antibodies for actin (sc1616), rabbit anti-goat IgG HRPO (sc2768) and goat anti-rabbit IgG HRPO (sc2004) were purchased from Santa Cruz Biotechnology (Dallas, TX). Antibodies for phospho-IκB (#2859), total IκB (#9242), phosphor-STAT3 serine 727 (#9134), phospho-STAT3 tyrosine 705 (#9145), total STAT3 (#9132), phospho-ERK (#9101) and total ERK (#9102) were purchased from Cell Signalling Technology (Danvers, MA). An antibody for RCAN1 (Ab6315c) was purchased from Abgent (San Diego, CA).

Bacterial preparation

P. aeruginosa were cultured as described previously[134]. Briefly, suspension cultures were grown until reaching the early stationery phase. Bacteria were washed in phosphate buffer and resuspended in saline for *in vivo* experiments or PBS for *in vitro* assays. Mice were infected as described below.

Cytokine production

Concentrations of IL-1β, TNF, IL-6, MIP2, TGF-β, LIX and KC the lungs, BALF, serum and culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) as described previously using antibody pairs from R&D Systems (Minneapolis, MN)[77].

Lung infection with P. aeruginosa and collection of lung and bronchoalveolar lavage fluid (BALF)

For bacterial clearance experiments *P. aeruginosa* strain 8821 (a gift from Dr A. Chakrabarty, University of Illinois, Chicago, IL), a mucoid strain isolated from a cystic fibrosis patient was used[264]. Mice were infected with 10^9 CFU of *P. aeruginosa* intranasally for 24 hour time points, and 10^7 CFU for 4 hour infections. After 4 or 24 hours, BALF was obtained by lavaging the lung with 3x 1 mL phosphate buffer saline containing soybean trypsin inhibitor (100 μg/mL). The lung tissue, spleens and blood were obtained for detection of cytokines, myeloperoxidase (MPO) and bacterial colony-forming units (CFU) counting.

Lung tissue and spleens were homogenized in 50 mM HEPES buffer (4 μL/1 mg lung) containing soybean trypsin inhibitor (100 μg/mL). For counting bacterial CFU, 10 μL of the homogenate, or blood was plated onto an agar dish and incubated for 24 h at 37°C. The lung homogenate was centrifuged at 4°C for 30 min at 18000g. The supernatant was stored at −80°C for later cytokine analysis. The pellet was resuspended and homogenized in 0.5% cetyltrimethylammonium chloride (CTAC) (4 μL/mg lung) and centrifuged as above. The cleared extract was used for MPO assay.

BALF (10 μL) was plated on an agar dish and incubated for 24 h for CFU counting. For detection of cytokines and MPO activity, BALF was centrifuged at 480g for 5 minutes at 4°C. The supernatants were used for cytokine analysis. The pellets were resuspended in 1 mL NH_4Cl (0.15M) and centrifuged as before to lyse red blood cells. The supernatants were discarded and the pellets were resuspended in 0.5% cetyltrimethylammonium chloride (CTAC) (250 μL/mouse) then centrifuged. The cleared extracts were used for MPO assay.

Myeloperoxidase (MPO) assay

The MPO assay was used to determine the infiltration of neutrophils into the lungs of the mice as described previously.[270] Briefly, samples in duplicate (75 µL) were mixed with equal volumes of the substrate (3,3′,5,5′-tetramethyl-benzidine dihydrochloride, 3 mM; Resorcinol, 120 µM; and H_2O_2, 2.2 mM) for 2 minutes. The reaction was stopped by adding 150 µL of 2 M H_2SO_4. The optical density was measured at 450 nm.

Nuclear extract preparation and electrophoresis mobility shift assay (EMSA)

Nuclear protein extracts were obtained using a nuclear extract kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. EMSA was performed as previously described [343]. Briefly probe labeling was accomplished by treatment with T4 kinase (Life Technologies Inc, Burlington, ON) in the presence of [^{32}P] adenosine triphosphate (Perkin Elmer, Waltham, MA). Labeled oligonucleotides were purified on a Sephadex G-25M column (GE healthcare, Pittsburgh, PA). Ten micrograms of nuclear protein was added to a 10 µL volume of binding buffer supplemented with 1µg of poly-(dI-dC) (GE healthcare, Pittsburgh, PA) for 15 min. Labeled double-stranded oligonucleotide was added to each reaction mixture, which was incubated at room temperature for 30 min and separated by electrophoresis on a 6% polyacrylamide gel in 0.5X Tris-boric acid-EDTA buffer. Gels were vacuum-dried and subjected to autoradiography. The following synthesized double-stranded oligonucleotides were used: NFAT binding consensus sequence on mouse IL-13 promoter 5′-AAGGTGTTTCCCCAAGCCTTTCCC-3′ (Sigma-Aldrich, St. Louis, MO), STAT3 consensus sequence on the IL-6 promoter 5’ GATCCTTCTGGGAATTCCTAGATC-3’

(Santa Cruz, Dallas, TX) and NF-κB consensus sequence on the IL-6 promoter 5′-AGTTGAGGGGACTTTCCCAGGC-3 (Promega, Madison, WI).

Statistics

Data are presented as mean ± S.E. of the indicated number of experiments. Statistical significance was determined by assessing means with an unpaired t test. Differences were considered significant at *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

Western Blot and Scanning Densitometry

Cells lysates (15 - 20 μg) were subjected to electrophoresis in 10% SDS-polyacrylamide gels. Gels were transferred to polyvinylidene difluoride membrane, blotted with primary and secondary antibodies as indicated, and detected by an enhanced chemiluminescence detection system (Western Lightning Plus-ECL; Perkin Elmer, Waltham, MA). Scanning densitometry was performed using Scion Image (Scion Corporation, Frederick, MD).

3.4 Results:

RCAN1-deficient mice display increased mortality despite enhanced bacterial clearance and neutrophil infiltration in vivo. Previous reports have implicated the Down syndrome critical region gene *RCAN1* as a negative regulator of various inflammatory pathways. However, the biological impact of RCAN1 during bacterial pneumonia remains unclear. In order to address this question we used a *P. aeruginosa* model of acute bacterial pneumonia in wild type (+/+) and RCAN1-deficient mice (-/-) in order to assess the impact of the protein on mortality and bacterial clearance *in vivo*.

Wild-type and RCAN1-deficient mice were infected with 1 LD_{50} of the laboratory strain of *P. aeruginosa* PAK. Animal mortality was then monitored for 10 days post infection (Figure 3.1 A). Unexpectedly, while 50% of wild-type animals succumbed to the infection, primarily between the 24 and 48 hours post infection (hpi), 100% of the RCAN1-deficient mice died within the first 18 hpi, suggesting that RCAN1 plays a critical role in host defense against *P. aeruginosa* lung infection. To examine whether antibiotic therapy affects RCAN1-regulated response in *P. aeruginosa* infection, mice were treated with a concentration of the antibiotic ceftazidime which yields serum concentrations similar to those achieved clinically[341, 342]. While antibiotic therapy was able to increase survival in wild-type animals, it was unable to rescue RCAN1-deficient mice which all succumbed to the infection in the first 18 hpi.

In order to further characterize the role of RCAN1 in host defense against *P. aeruginosa,* the impact of RCAN1 on bacterial burden was assessed in wild-type and RCAN1-deficient animals. To address this question, mice were infected intranasally with *P. aeruginosa* strain 8821 (isolated from the lungs of a cystic fibrosis patient[264]), as this strain is associated with lower mortality *in vivo*. Bacterial burden was assessed in the lung and bronchoalveolar lavage fluid (BALF) of wild-type and RCAN1-deficient mice at 4 and 24 hpi. No differences were observed at 4 hpi, likely due to the high bacterial burden, and time required for the recruitment and phagocytosis of the bacteria by neutrophils (data not shown). Contrary to expectation there was significantly more bacteria detected in the lungs of wild-type mice compared to RCAN1-deficient animals 24 hpi (Figure 3.1B). A similar trend was observed in the BALF of RCAN1-deficient animals but it did not reach statistical significance (Figure 3.1C). In order to determine

whether RCAN1 deficiency affects bacterial dissemination, the bacterial burden in the spleen and blood was assessed. The levels of bacteria in both the serum and spleen were decreased in RCAN1-deficient mice (Figure 3.1 D and E). These results suggest that the decreased bacterial load in the lungs was not a result of impaired containment of the infection. The bacterial burden in the RCAN1 deficient mice is lower than that in wild-type mice.

As the clearance of *P. aeruginosa* from the lungs is largely dependent upon the recruitment of neutrophils to the site of infection[344], we next set out to measure the infiltration of these cells into the respiratory tract. Assessment of neutrophil infiltration was carried out using an assay to determine the activity of the neutrophil granule specific enzyme myeloperoxidase (MPO). MPO activity was greatly enhanced at 4 hpi in both the lung (Figure 3.1 F) and BALF (Figure 3.1 G) of RCAN1-deficient mice. These levels of MPO activity remained elevated in the lung tissue 24 hpi. However no differences in activity were observed in the BALF at this time point. These data stand in stark contrast to previous results in other gene knockout mice we and others have generated using this *P. aeruginosa* lung infection model where increased bacterial burden is usually associated with increased mortality[107, 134, 345]. Altogether, these data suggest that the increased mortality in RCAN1-deficient animals may not be directly caused by bacterial pneumonia.

RCAN1-deficient mice have greatly enhanced serum levels of inflammatory cytokines following P. aeruginosa lung infection. Due to the reported roles for RCAN1 as a regulator of inflammation, we next set out to determine if the discrepancies between the

enhanced bacterial clearance and increased mortality in RCAN1-deficient mice could be explained by susceptibility to systemic inflammation. In order to explore this possibility we examined the levels of four cytokines whose increased expression has been linked to mortality associated with systemic inflammation. These cytokines are TNF[346], IL-1β[347], IL-6[348] and a murine homologue of human IL-8, MIP-2[349]. Wild-type and RCAN1-deficient mice were infected intranasally with 1 LD_{50} of *P. aeruginosa* strain PAK. Mice were then left untreated, or were treated with the antibiotic ceftazidime every 8 hours, starting 2 hpi. Serum was then collected at various time points and analyzed for levels of inflammatory cytokines. We observed that the levels of IL-6 (Figure 3.2 A), MIP-2 (Figure 3.2 B) and IL-1β (Figure 3.2 C) started to increase in the serum of RCAN1-deficient mice as early as 3 hpi. Levels continued to increase at 6 and 12 hpi and were significantly increased in the serum of RCAN1-deficient mice when compared to wild type controls. Antibiotic treatment with ceftazidime modestly decreased IL-6 and IL-1β level in the serum in wild type mice. Similarly, MIP-2 and IL-1β level in the serum of RCAN1-deficient mice was modestly reduced after ceftazidime treatment. However the levels of these inflammatory mediators in RCAN1 deficient mice remained well above those seen in wild-type animals suggesting that the antibiotic therapy was unable to prevent aberrant systemic inflammation which occurs in the absence of RCAN1. Together these data demonstrate that in spite of enhanced neutrophil infiltration and bacterial clearance from the lungs following *P. aeruginosa* infection, RCAN1-deficient mice display enhanced mortality likely due to increased systemic inflammation.

We next further determined the inflammatory response in the lungs of RCAN1-deficient mice. Wild-type and RCAN1-deficient mice were infected intranasally with 10^7

or 10^9 CFU of *P. aeruginosa* strain 8821 for 4 and 24 hour infections respectively. A mock infection with saline was used as a control. At the indicated time points mice were sacrificed and lung tissue and BALF were collected and analyzed for the levels of pro-inflammatory, and immunoregulatory cytokines (Table 3.1). The levels of all the pro-inflammatory cytokines examined were greatly enhanced in both the lung and BALF of RCAN1-deficient mice infected with *P. aeruginosa* at the early 4 hpi time point when compared to wild-type mice. At the later 24 hpi time point few differences in inflammatory cytokine levels were observed, and the differences which persisted were smaller than those observed at 4 hpi. Interestingly the enhanced pro-inflammatory cytokine response observed at 4 hours was accompanied by decreased levels of the immunomodulatory cytokine TGF-β in both the lungs and the BALF of RCAN1-deficient mice. These results indicate that RCAN1 opposes the generation of an inflammatory environment through inhibiting pro-inflammatory cytokine production, and promoting immunomodulatory TGF-β, particularly during the early stages post-infection.

Cultured RCAN1-deficient macrophages display enhanced early production of inflammatory cytokines following infection with P. aeruginosa. Macrophages are important during host defense against *P. aeruginosa* infection. We set out to further characterize the inflammatory response in wild-type and RCAN1-deficient bone marrow derived macrophages *in vitro*. In order to examine RCAN1 expression in macrophages during *P. aeruginosa* infection bone marrow derived macrophages were cultured from wild-type and RCAN1-deficient mice. These macrophages were then infected with *P. aeruginosa* strain 8821 at a multiplicity of infection (MOI) of 1:10. Lysates were

prepared at various time points, and subjected to western blot analysis. The long form of RCAN1 (37 kDa) was found to be constitutively expressed in wild-type cells, however the short (25 kDa) form of the protein was rapidly induced in response to *P. aeruginosa* infection, supporting a role for the protein early in *P. aeruginosa* infection (Figures 3.3 A and 3.3 B). In order to assess the impact of this RCAN1 induction on inflammatory cytokine production, wild-type and RCAN1-deficient cultured macrophages were exposed to *P. aeruginosa* at various MOIs in order to determine the dose resulting in the optimal production of various inflammatory cytokines (Figure 3.4). The optimal MOI was determined to be 1:1 as higher doses resulted in substantial cell death. Thus, this dose was used for subsequent experiments.

Wild-type and RCAN1-deficient macrophages were then infected for various time points up to 48 hours. The levels of secreted inflammatory cytokines including IL-6, IL-1β, TNF and MIP2 were tested. The levels of all the inflammatory cytokines were significantly enhanced in the supernatants of RCAN1-deficient macrophages treated with *P. aeruginosa* at early time points (Figure 3.5 A-D). Interestingly, no significant differences were observed at the later 24 hpi time point. Similar results were obtained using *P. aeruginosa* strain 8821 at an MOI of 1:10 (Figure 3.6 A-D). These results suggest that RCAN1 plays an important negative regulatory role on inflammatory cytokine production early in infection.

Reports have implicated a role for RCAN1 in regulating two distinct pro-inflammatory transcription factors. Firstly, RCAN1 inhibits the activity of calcineurin, a phosphatase required for the activation of NFAT[187-189]. Secondly, calcineurin-independent and -dependent roles for RCAN1 have been characterized for the inhibition

of NF-κB through decreased phosphorylation of the inhibitory protein IκBα, leading to increase protein stability and inhibition of the NF-κB pathway[190, 191]. In order to assess the contribution of this second pathway to enhanced cytokine production in the absence of RCAN1, lysates were collected from wild-type and RCAN1-deficient bone marrow-derived macrophages treated with *P. aeruginosa* strain PAK at a 1:1 MOI for various times. These lysates were subjected to Western blot analysis for total IκBα as well as the phosphorylated form of the protein, and an actin loading control (Figure 3.5 E). Blots from three separate experiments were quantified using scanning densitometry, and the average fold increase relative to wild-type untreated samples was determined (Figure 3.5 F). IκBα was found to be significantly hyperphosphorylated at the early 3 and 6 hpi time points in the RCAN1-deficient cells, while no significant differences were observed with longer treatments. Similar results were obtained when cells were treated with *P. aeruginosa* strain 8821 at a 1:10 MOI (Figure 3.6 E and F), showing that RCAN1 impairs NF-κB activation through decreased phosphorylation of IκBα during *P. aeruginosa* infection.

Enhanced inflammatory cytokine production in RCAN1-deficient mice is accompanied by increased pro-inflammatory transcription factor activity following* P. aeruginosa *lung infection. NFAT and NF-κB are master regulators of cytokine and chemokine production. We next assessed the activity of these transcription factors by performing EMSAs on nuclear extracts prepared from the lungs of wild-type and RCAN1-deficient mice infected with *P. aeruginosa* strain 8821 for 4 or 24 hours. The activity of NF-κB was greatly enhanced in the lungs of RCAN1-deficient mice 4 hpi, while only a marginal

increase was observed at 24 hours that did not reach statistical significance (Figures 3.7 A and C). However, the activity of the pro-inflammatory transcription factor NFAT was greatly enhanced in RCAN1-deficient mice at both the early 4 hpi and late 24 hpi time points (Figure 3.7 B and D). Similar results were obtained for 4 hour infection with 1 LD_{50} of *P. aeruginosa* strain PAK for both NFAT and NF-κB (Figure 3.8). These results confirm that the RCAN1 greatly impacts the inflammatory response *in vivo* through temporally distinct negative regulation of two distinct pro-inflammatory transcription factors.

The ERK/STAT3 axis is regulated by RCAN1 following P. aeruginosa infection in vitro and in vivo. Recently, increased STAT3 activity has been reported in DS patients supporting a clinically relevant role for dysregulation of the pathway[350]. However a role for RCAN1 in the regulation of the STAT3 pathway has not been explored. In order to assess the activation of the STAT3 pathway *in vitro*, bone marrow-derived macrophages from wild-type and RCAN1-deficient mice were treated with *P. aeruginosa* strain 8821 at an MOI of 1:10. Lysates were collected at various times indicated, and Western blot analysis was performed. The MAPK ERK was found to be hyperphosphorylated in both untreated and RCAN1-deficient cells, as well as at early time points post-infection with *P. aeruginosa* (Figure 3.9 A and D). Two other MAPKs, JNK and p38, showed no differences activation state (data not shown).

As STAT3 is a target of ERK kinase activity at serine 727, a phosphorylation event required for transcriptional activity of the transcription factor[182], we next set out to determine the effects of increased ERK activation in RCAN1-deficient cells on the

STAT3 pathways. Western blot analysis indicated that STAT3 was hyperphosphorylated at serine 727 in untreated RCAN1-deficient macrophages, as well as at early time points post-infection with *P. aeruginosa* (Figure 3.9 A and B).

In addition to phosphorylation at serine 727, STAT3 activity also requires phosphorylation at tyrosine residue 705 to drive dimerization and nuclear translocation of the protein, which is induced via the IL-6 receptor (CD126) through a JAK/STAT dependent mechanism [181]. Western blot analysis showed greatly enhanced tyrosine 705 phosphorylation in both resting and *P. aeruginosa* infected RCAN1-deficient macrophages compared to wild-type controls (Figures 3.9 A and C). In order to assess the impact on aberrant STAT3 phosphorylation in the absence of RCAN1 on transcriptional activity, nuclear extracts collected from wild-type and RCAN1 deficient macrophages were prepared and subjected to EMSA. STAT3 transcriptional activity was found to be significantly impaired at early time points post infection in RCAN1-deficient macrophages (Figure 3.9 E and G). However, at later time points post infection STAT3 activity was significantly increased in RCAN1-deficient macrophages. Probe specificity was confirmed using competition and super shift assays (data not shown). Together these results indicate that the STAT3 pathway is dysregulated in RCAN1-deficient macrophages in both untreated and *P. aeruginosa* treated cells *in vitro*.

Having seen altered STAT3 activation *in vitro* we next set out to measure STAT3 transcriptional activity *in vivo*. EMSA was performed on lungs from wild-type and RCAN1-deficient mice treated with saline (NT) or infected with *P. aeruginosa* strain 8821 for 4 or 24 hours, using a DNA probe specific for STAT3. No STAT3 activity was observed in uninfected lungs of wild-type or RCAN1-deficient mice, and increased

activity was observed in the lungs of wild-type mice 4 hpi (Figure 3.9 F and H). However, at the later 24 hpi time point STAT3 activity was only observed in lungs of RCAN1-deficient mice. Together these data suggest that RCAN1 deficiency delays and prolongs STAT3 activation following *P. aeruginosa* infection *in vivo*.

3.5 Discussion:

DS is the most common chromosomal anomaly among live born infants, and is associated with a variety of immunological defects[301-304]. These defects combined with anatomical factors associated with DS lead to greatly increased risk of severe and persistent respiratory tract infections, including viral and bacterial pneumonias[311-314]. A handful of DS-associated genes have been implicated in these respiratory syndromes, and understanding their relative contributions to impaired immunological function is a critical step towards improving treatment and prevention of respiratory tract infections in these high risk patients. In the present study we demonstrate a critical role for the DS associated gene *RCAN1* as a negative regulator of inflammation in a *P. aeruginosa* model of bacterial pneumonia. RCAN1-deficiency was found to be associated with an inappropriate inflammatory response following *P. aeruginosa* infection both *in vitro* and *in vivo*. This unchecked inflammatory response was associated with dysregulation of three critical inflammatory pathways involving the transcription factors NFAT, NF-κB and STAT3. The resulting increase in inflammatory cytokine production led to increased neutrophil infiltration and bacterial clearance from the lungs of RCAN1-deficient mice. However, the end result of RCAN1-deficiency was increased mortality associated with systemic inflammation, which could not be corrected with antibiotic therapy. Together

these results show for the first time that RCAN1 is a central negative regulator of inflammation during *P. aeruginosa* lung infections.

It is worthy to note that the NFAT, NFκB and STAT3 pathways shown here to be regulated by RCAN1 are universally important during respiratory tract infections and have been extensively studied during *Pneumococcus* infection[351-353]. Thus, it is likely that the phenotype observed in RCAN1-deficient animals may not be specific to *P. aeruginosa* infection. RCAN1 may represent a general host defense mechanism in greater clinical settings such as *Pneumococcus* infection.

In addition to the novel insights into the regulation of the inflammatory response by RCAN1, this study also shed light on a previously unrecognized protective role for RCAN1 against systemic inflammation. Upon infection with one LD_{50} of *P. aeruginosa* RCAN1-deficient mice all succumbed within the first 18 hours post-infection. This mortality was associated with elevated levels of serum cytokines demonstrating wide spread systemic inflammation which could not be corrected with antibiotic therapy. Collectively our findings help to elucidate the protective biological functions of RCAN1 in the context of infection and inflammation and implicate dysregulation of NFAT, NF-κB and STAT3 signaling in the enhanced mortality observed in RCAN1-deficient mice.

RCAN1 is widely expressed in various tissues throughout the body[189, 318, 319], and is over-expressed in DS patients[317]. Intriguingly, previous studies have proposed RCAN1 as both an inhibitor[269, 354-356] and an activator[187, 321, 357-359] of inflammation via calcineurin, suggesting that the biological activity of the protein might be context dependent. Our findings here provide the first evidence of a negative regulatory role for RCAN1 in inflammation during respiratory tract infections using an *in vivo* model of bacterial

pneumonia. Interestingly, we found temporally distinct effects of RCAN1 on the kinetics of two important pro-inflammatory transcription factors. NFAT activity was greatly enhanced in the lungs of RCAN1-deficient mice throughout the course of bacterial infection. However NF-κB activity was only observed to be enhanced early post-infection in the lungs of RCAN1-deficient mice. The differential control exerted by RCAN1 over these pathways likely reflects distinct molecular regulatory mechanism. RCAN1 can exert sustained inhibition of NFAT via direct interactions with calcineurin[187-189]. By contrast RCAN1 controls NF-κB activation through poorly understood mechanisms leading to the stabilization of the IκB-NF-κB complex[190, 191]. It is perhaps the differential regulation of these transcription factors that accounts for the cytokine profiles observed during *P. aeruginosa* lung infection *in vivo*. While all inflammatory cytokines monitored were greatly elevated in RCAN1-deficient mice early in infection, fewer differences were observed later in infection, and those which remained were less pronounced.

Recently, increased STAT3 expression has been reported in DS patients implying a clinically relevant role for dysregulation of the pathway[350]. As STAT3 represents an important regulator of inflammation, we set out to see what effect, if any, RCAN1 had on the pathway. Surprisingly, we identified a previously unknown regulatory role for RCAN1 in STAT3 activation, both *in vitro* and *in vivo*. Hyperactivation of the ERK MAPK was observed *in vitro* coinciding with increased phosphorylation of the ERK target STAT3 at serine 727 in untreated cells, as well as at early time points post-infection. By contrast phosphorylation of STAT3 at tyrosine 705, which was also enhanced in RCAN1-deficient cells, was only observed following *P. aeruginosa*

infection. These patterns of aberrant STAT3 phosphorylation lead to delayed and prolonged STAT3 activation following *P. aeruginosa* infection *in vitro*. Similarly, the *in vivo* kinetics of STAT3 activation was substantially altered in RCAN1-deficient mice which displayed impaired early STAT3 activation which persisted later in infection, after the wild-type response had disappeared. These delayed STAT3 kinetics correlated with the levels of the immunomodulatory cytokine TGF-β suggesting that RCAN1 mediated STAT3 activation may be playing primarily an anti-inflammatory role during *P. aeruginosa* lung infection. These results are not entirely unanticipated as, in contrast to NFAT and NF-κB which are strictly pro-inflammatory transcription factors, STAT3 acts both to drive the inflammatory response, and participate in its resolution. Indeed various *in vivo* knockout models have found that inflammatory cytokine production is not impaired by STAT3-deficiency, but is instead enhanced leading to a variety of inflammatory pathologies[185] including increased susceptibility to endotoxemia[186], as is observed in RCAN1-deficient animals. Taken together our study demonstrates that along with inhibition of NFAT and NF-κB activity, RCAN1 also opposes the generation of a pro-inflammatory environment through the promotion of immunomodulatory STAT3 activity.

RCAN1-deficient mice provide a useful tool for studying the role of RCAN1 in respiratory tract infections *in vivo* and allow us to clearly define the role of the protein in various inflammatory pathways. Such studies would not be possible in traditional DS mouse models due to the extremely complex interplay of DS associated genes, and the intricate effects on protein expression and function resulting from trisomy of chromosome 21. Recently, a transgenic mouse model of human RCAN1 over-expression

has been published where the authors achieved expression of the protein at the elevated levels, and with a similar tissue distribution, to that seen in DS[360]. It will be interesting to use these mice to test the hypothesized immunosuppressive functions of RCAN1 in a clinically relevant model of over-expression.

Here we identify a novel role for RCAN1 as a central regulator of inflammation during respiratory tract infection *in vivo*. As a result, pharmacological intervention by partially modulating RCAN1 expression or function may represent a promising strategy for treating and preventing respiratory infections. Although no RCAN1 inhibitors are currently available, it may be possible to normalize expression levels by manipulating protein stability and turnover. RCAN1 turnover occurs via both proteasomal and lysosomal pathways[361, 362]. However a recent report has also shown that RCAN1 stability, and its inhibitory effects on calcineurin, are enhanced through a ubiquitination like process known as neddylation involving the ubiquitin like protein NEDD8[363]. Importantly, a well-studied first in class neddylation inhibitor called MLN4924 is currently in phase 1 clinical trials in cancer patients[364]. As inhibition of neddylation would both enhance RCAN1 turnover, and inhibit the proteins negative regulatory effects on calcineurin, MLN4924 represents a promising avenue of investigation for relieving RCAN1 mediated immune suppression.

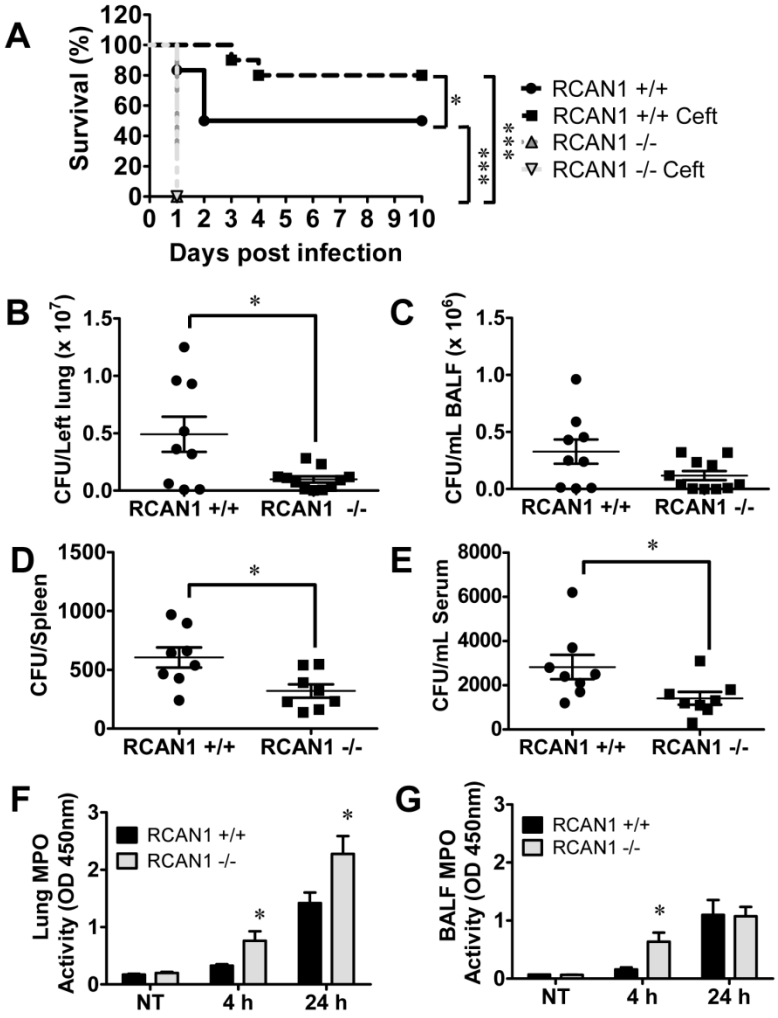

Figure 3.1: RCAN1-deficient mice display increased mortality but decreased bacterial burden following *P. aeruginosa* lung infection. RCAN1 wild type (+/+) and knockout (-/-) mice were infected intranasally with 1 LD_{50} (2.25 x 10^7 CFU) of *P. aeruginosa* strain PAK. Mice were then left untreated, or were treated with 200 mg/kg ceftazidime (Ceft) subcutaneously every 8 hours. Animal survival was monitored daily (A). (n = 10, *p<0.05, ***p<0.005). Wild-type and RCAN1-deficient mice were also infected intranasally with 10^7 (4 h) or 10^9 (24 h) CFU of *P. aeruginosa* strain 8821, or an equivalent volume of saline (NT). Lungs and bronchoalveolar lavage fluid (BALF) were collected 4, or 24 hours after infection. Serial dilution of homogenized lung tissue (B), BALF (C), spleens (D) and blood (E) were streaked on LB agar plates and incubated 24 hours at 37ºC. The resultant colonies were counted to determine bacterial load (n = 8-10 ± SEM, *p<0.05). Samples were then lysed and MPO activity was assayed in the lungs (F), and BALF (G). (n = 6-11 ± SEM, *p<0.05).

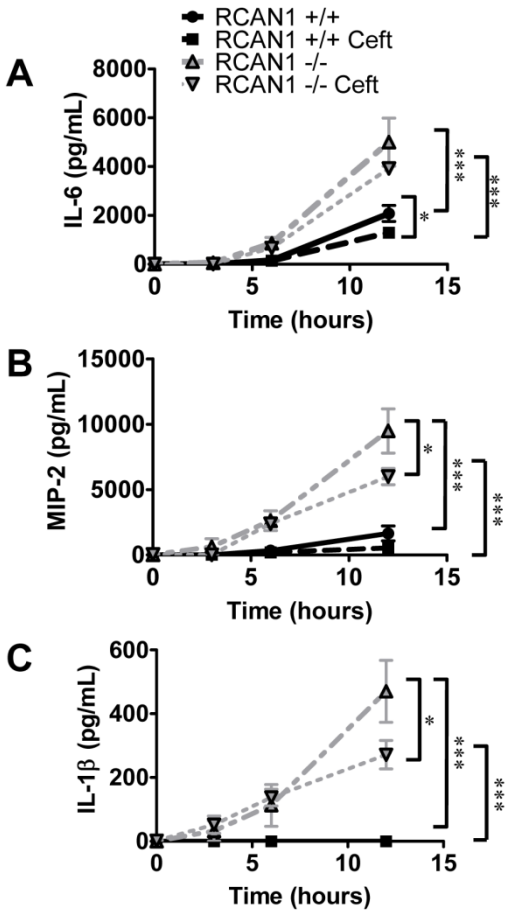

Figure 3.2: RCAN1-deficient mice have elevated serum cytokine levels following *P. aeruginosa* lung infection. RCAN1 wild type (+/+) and knockout (-/-) mice were infected intranasally with 1 LD_{50} (2.25×10^7 CFU) of *P. aeruginosa* strain PAK. Mice were then left untreated, or were treated with 200 mg/kg ceftazidime (Ceft) subcutaneously every 8 hours. Serum was monitored by ELISA at various points over the first 12 hours post infection for IL-16 (A), MIP-2 (B) and IL-1β (C). (n = 8-10 ± SEM, *$p<0.05$, ***$p<0.001$)

Table 3.1. RCAN1 deficient mice display enhanced early inflammatory cytokine production

		Uninfected[A]		4h[B]		24h[C]	
		RCAN1 +/+	RCAN1 -/-	RCAN1 +/+	RCAN1 -/-	RCAN1 +/+	RCAN1 -/-
Lung							
	IL-6	93.7 +/- 44.4	63 +/- 25.6	117.8 +/- 17.4***	523.6 +/- 60.0***	2753.4 +/-701.4	2492.6 +/-1018.1
	IL-1β	N.D.	N.D.	1793.9 +/- 201.6***	3611.9 +/- 289.0***	6348.7 +/-244.6*	7581.4 +/- 262.3*
	TNF	17.1 +/- 2.4	19.5 +/- 3.1	89.6 +/- 4.4**	208.8 +/- 30.2**	138.7 +/-25.0*	214.5 +/- 23.3*
	MIP-2	24.7 +/- 7.4	66.9 +/- 2.4	1912.1 +/- 207.9***	3143.2 +/- 231.8***	4313.9 +/- 489.4	4207.6 +/- 294.0
	LIX	197.8 +/- 86.2	498.1 +/- 196.6	1135.3 +/- 55.0**	1361.0 +/- 15.2**	5304.8 +/- 490.1	5390.8 +/- 428.7
	KC	318.2 +/- 116.3	819.5 +/- 207.3	6811.5 +/- 329.8**	8166.0 +/- 91.4**	7502.2 +/- 282.6	7687.3 +/- 97.5
	TGF-β	241.2 +/- 99.9	379.2 +/- 57.6	350.3 +/- 35.2*	248.4 +/- 13.2*	259.3 +/-26.6	234.1 +/- 18.6
BALF							
	IL-6	57.4 +/- 4.5	64.1 +/- 8.6	330.8 +/- 81.0**	961.0 +/- 115.0**	6523.5 +/- 2107.6	3658.3 +/- 1267.6
	IL-1β	N.D.	N.D.	134.3 +/- 3.1*	192.8 +/- 21.0*	147.1 +/- 50.4	377.7 +/- 98.0
	TNF	19.9 +/- 1.2*	32.4 +/- 2.9*	502.3 +/- 35.3***	1002.2 +/- 67.5***	819.5 +/- 97.8	854.2 +/- 73.9
	MIP-2	49.4 +/- 18.0	91.9 +/- 36.4	2475.5 +/- 467.3**	4961.8 +/- 119.4**	5493.1 +/- 337.0	5728.6 +/- 92.0
	LIX	191.3 +/- 102.4	77.8 +/- 24.4	402.7 +/- 136.3*	1198.8 +/- 285.5*	1018.6 +/- 252.6*	3517.0 +/- 265.9*
	KC	14.4 +/- 7.5*	87.8 +/-16.0*	6454.8 +/- 348.7*	7637.2 +/- 119.4*	4315.7 +/- 729.7*	6701.2 +/- 757.1*
	TGF-β	N.D.	30.0 +/- 18.1	139.2 +/- 28.2*	42.6 +/- 28.1*	50.7 +/- 17.2***	210.4 +/- 16.5***

N.D. Not detected

*p<0.05, **p<0.01, ***p<0.001

[A]Data are the mean +/- SE of 4 mice per group (pg/mL)

[B]Data are the mean +/- SE of 6 mice per group (pg/mL)

[C]Data are the mean +/- SE of 10 mice per group (pg/mL)

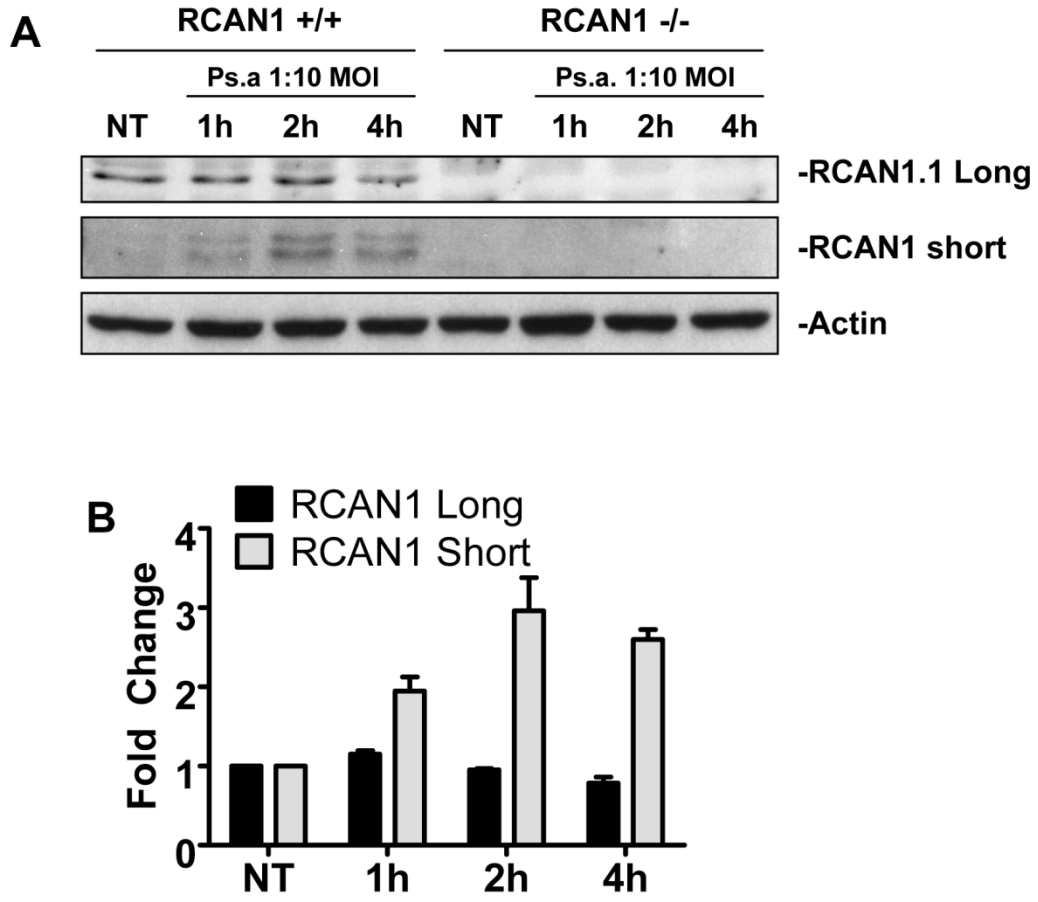

Figure 3.3: RCAN1 is induced in response to *P. aeruginosa* infection. Wild-type (+/+) and RCAN1-deficient (-/-) bone marrow derived macrophages were treated with *P. aeruginosa* strain 8821 at and MOI of 1:10. At the indicated time point lysates were prepared, and western blot analysis was performed (A). The blots were subjected to scanning densitometry for quantification (B). (n=2 +/- SEM).

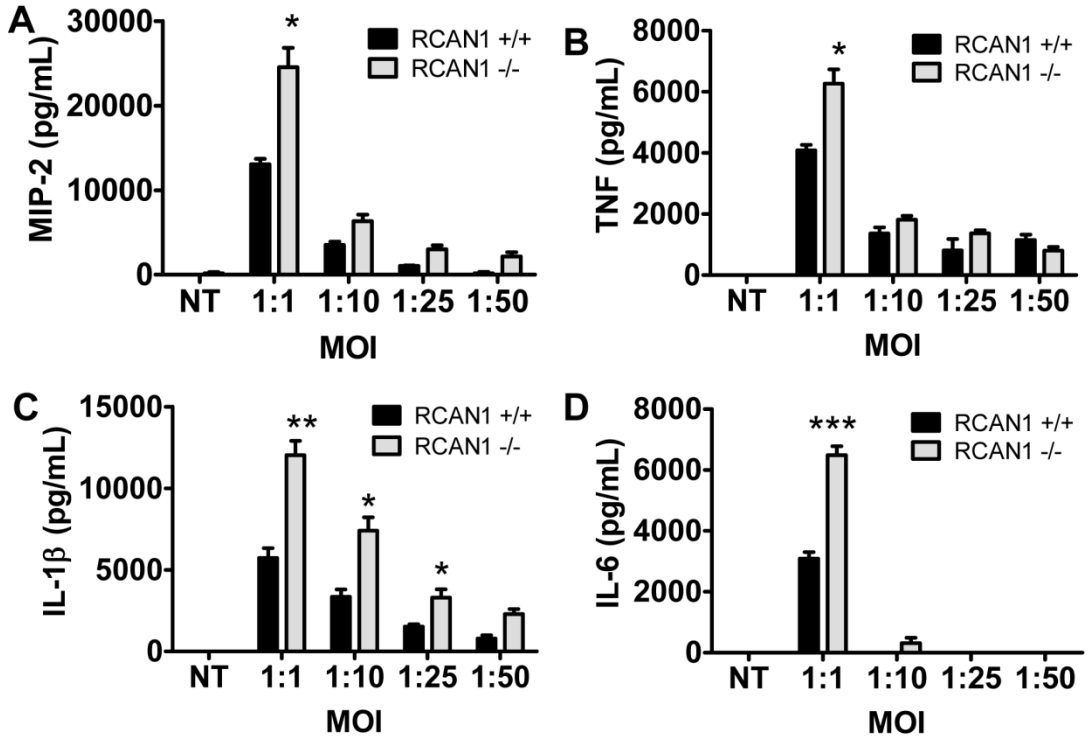

Figure 3.4: RCAN1-deficient macrophages display enhanced production of proinflammatory cytokines in response to *P. aeruginosa* infection: Wild-type (+/+) and RCAN1-deficient (-/-) bone marrow derived macrophages were left untreated (NT) or exposed to *P. aeruginosa* strain PAK at increasing MOIs for 6 hours. Supernatants were collected and analyzed for the pro-inflammatory cytokines MIP-2 (A), TNF (B), IL-1β (C) and IL-6 (D). (n=3 +/- SEM,*$p<0.05$, **$p<0.01$)

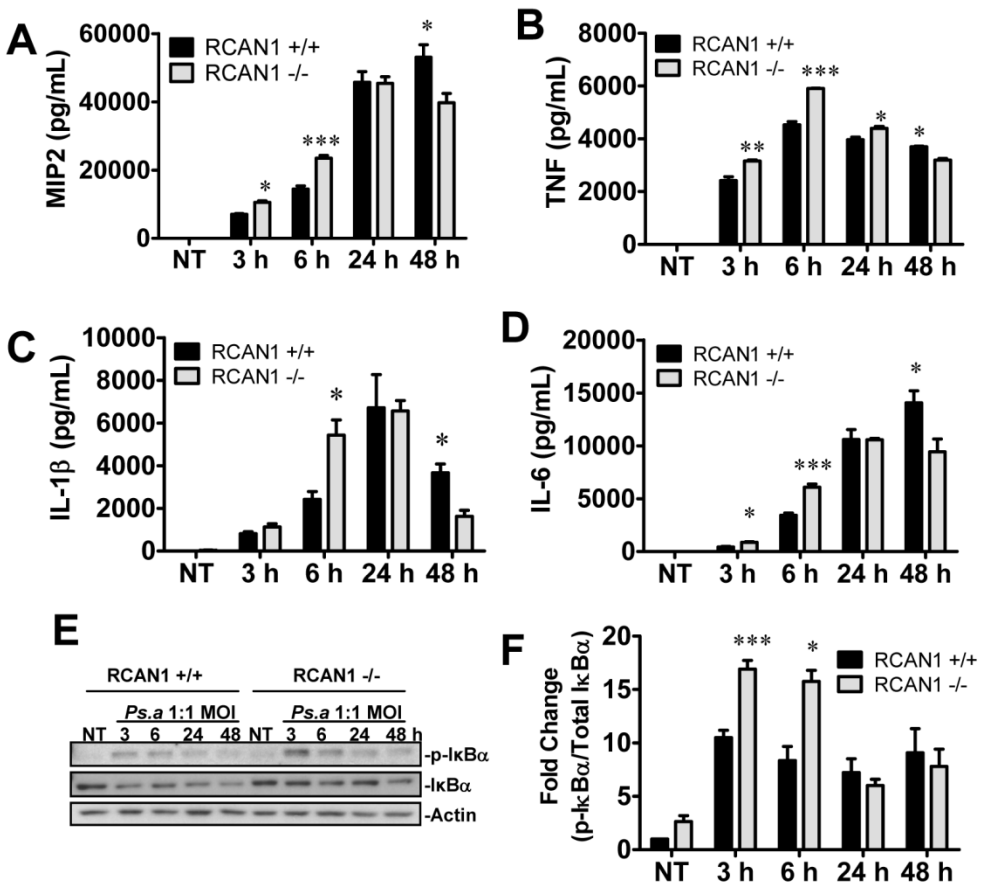

Figure 3.5: RCAN1-deficient macrophages display enhanced production of pro-inflammatory cytokines in response to *P. aeruginosa* infection: Wild-type (+/+) and RCAN1-deficient (-/-) bone marrow derived macrophages were left untreated (NT) or exposed to *P. aeruginosa* strain PAK at an MOI of 1:1. Supernatants were collected at various time points and analyzed for the pro-inflammatory cytokines MIP-2 (A), TNF (B), IL-1β (C) and IL-6 (D). (n = 3 ± SEM, *p<0.05, *p<0.01, ***p<0.001). Lysates were prepared and subjected to Western blot for phospho and total-IκBα as well as actin loading control (E). Blots are representative of 3 separate experiments, which were quantified by scanning densitometry (F). (n = 3 ± SEM, *p<0.05, ***p<0.001)

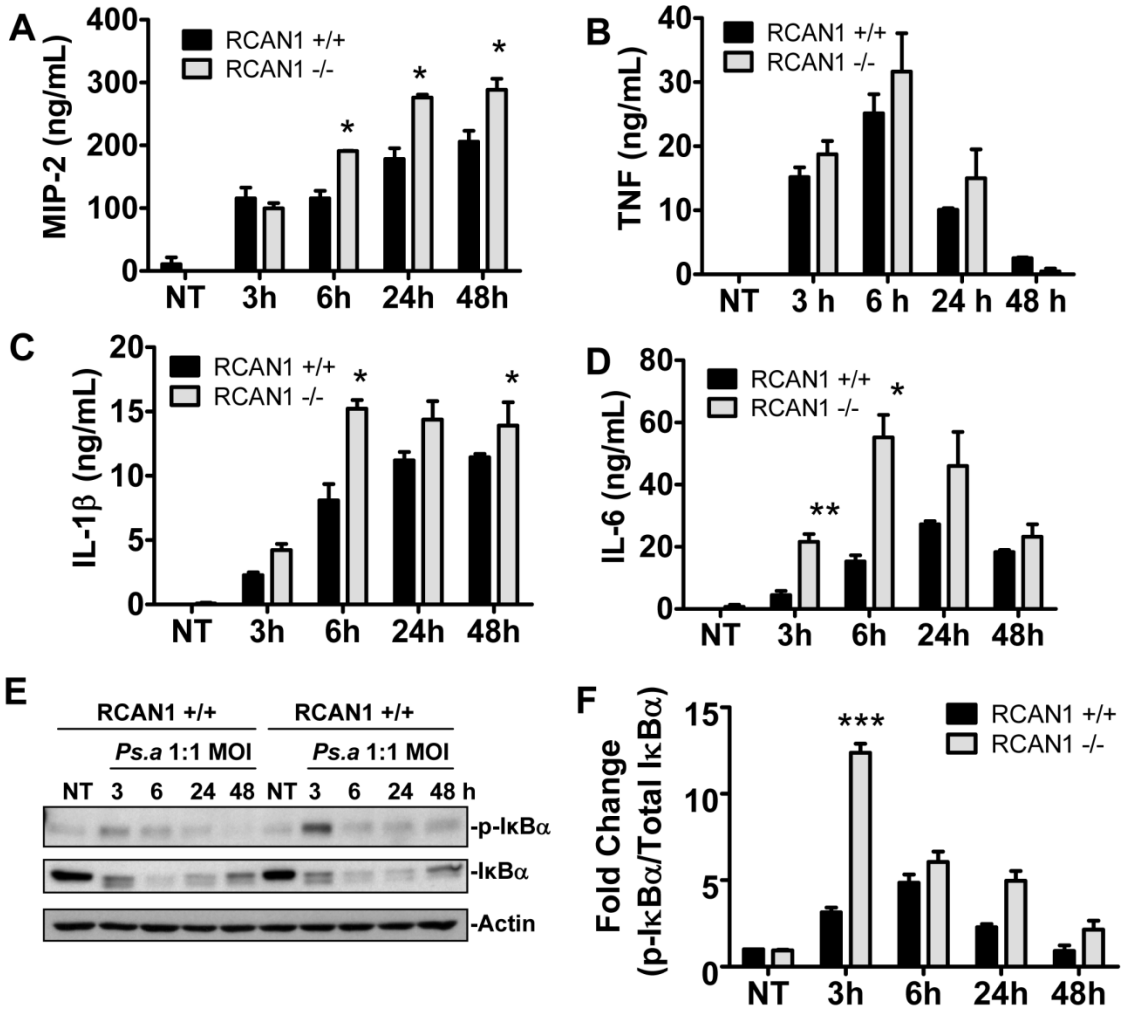

Figure 3.6: RCAN1-deficient macrophages display enhanced cytokine production and IκBα phosphorylation following *P. aeruginosa* infection. Wild-type (+/+) and RCAN1 deficient (-/-) bone marrow derived macrophages were treated with *P. aeruginosa* strain 8821 at and MOI of 1:10. Supernatants were collected at various time points and analyzed for inflammatory cytokines by ELISA (A-D). (n=3 +/-SEM, *p<0.05, **p<0.01). Lysates were also collected at each indicated time point and western blot analysis was performed for phospho and total IκBα as well as actin loading control (E). The blots were subjected to scanning densitometry for quantification (F). (n=3 +/-SEM, *p<0.05, ***p<0.001).

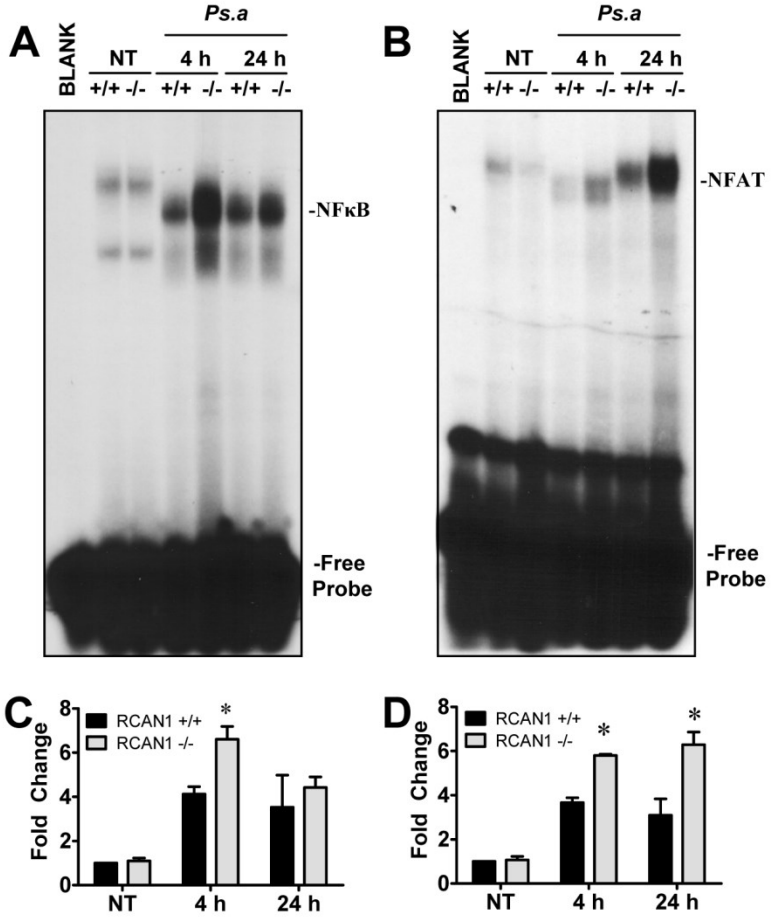

Figure 3.7: RCAN1-deficient mice display enhanced inflammatory transcription factor activation *in vivo* following *P. aeruginosa* infection. RCAN1 wild type (+/+) and knockout (-/-) mice were infected intranasally with 10^7 (4h) or 10^9 (24h) CFU of *P. aeruginosa* strain 8821, or an equivalent volume of saline. Nuclear proteins were extracted from lung tissues obtained from individual mice and subjected to EMSA by incubation with ^{32}P-labeled NF-κB (A), or NFAT (B) DNA probes. Data is representative of three individual experiments. Scanning densitometry was performed (C and D) and data is expressed as fold change vs. WT untreated lungs. ($n = 3 \pm SEM$, $^*p < 0.05$)

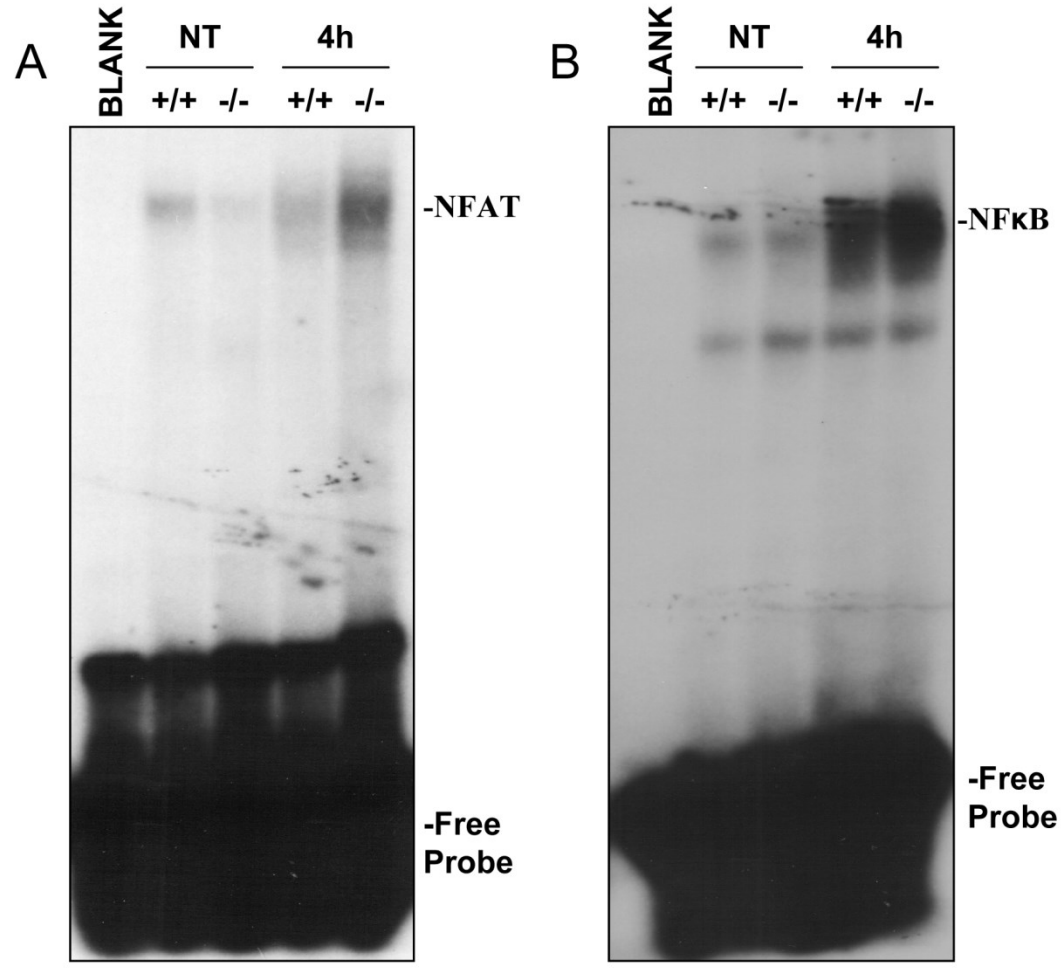

Figure 3.8: RCAN1-deficient mice display enhanced inflammatory transcription factor activation *in vivo* following *P. aeruginosa* infection. RCAN1 wild type (+/+) and knockout (-/-) mice were infected intranasally with 2.25 x 10_7 *P. aeruginosa* strain PAK, or an equivalent volume of saline (NT). Nuclear proteins were extracted from lung tissues obtained from individual mice and were subjected to EMSA by incubation with ^{32}P-labeled NFAT (A) or NF-κB (B), or DNA probes.

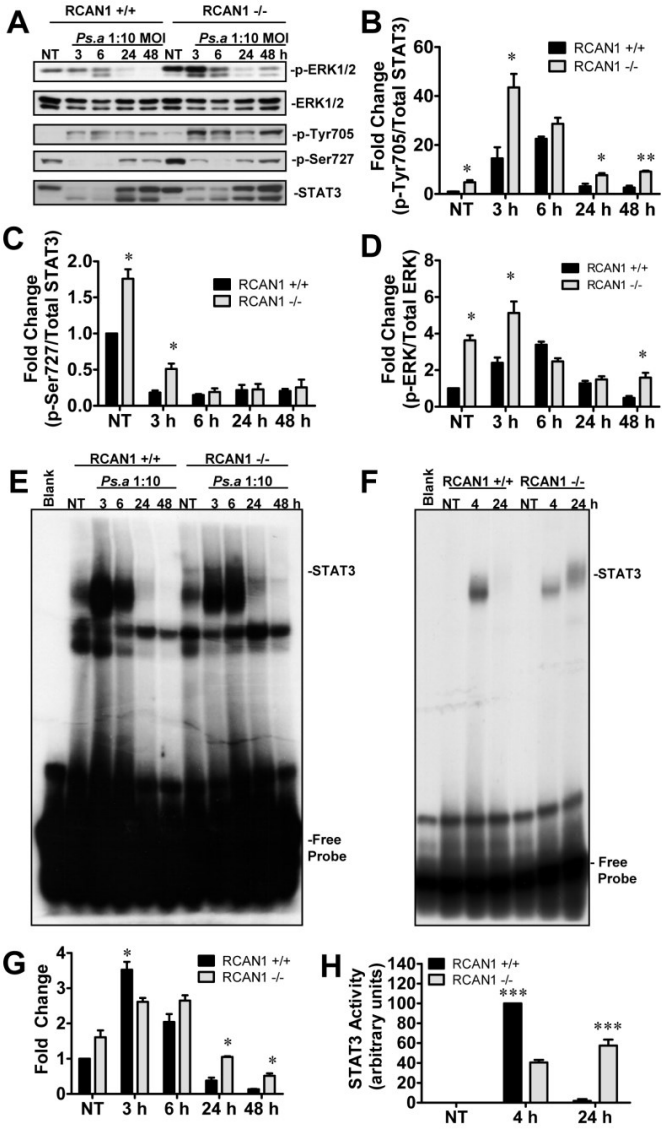

Figure 3.9: The ERK/STAT3 pathway is differentially regulated by RCAN1 following *P. aeruginosa* infection *in vitro* and *in vivo*. Bone marrow derived macrophages from WT (+/+) and RCAN1-deficient (-/-) mice were treated with *P. aeruginosa* strain 8821 at an MOI of 1:10 for various time points. Lysates or nuclear extracts were prepared and subjected to Western blot (A) or EMSA analysis respectively (E). RCAN1 wild type (+/+) and knockout (-/-) mice were infected intranasally with 10^7 (4 h) or 10^9 (24 h) CFU of *P. aeruginosa* strain 8821, or an equivalent volume of saline. Nuclear proteins were extracted from lung tissues obtained from individual mice and were subjected to EMSA by incubation with ^{32}P-labeled STAT3 DNA probe (F). Data is representative of three individual experiments. Scanning densitometry was performed (B-D, G-H) and data is expressed as fold change vs. WT untreated lungs or lysates (n = 3 ± SEM *p<0.05, **p<0.01, ***p<0.001).

CHAPTER 4: THE CALCINEURIN-NFAT AXIS CONTRIBUTES TO HOST DEFENSE DURING *P. AERUGINOSA* LUNG INFECTIONS

The calcineurin-NFAT axis contributes to host defense during *P. aeruginosa* lung infection

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Unsubmitted manuscript

Author contributions:

Robert D Junkins: Designed and executed experiments and analyzed data for all figures and tables. Wrote and edited manuscript.

Adam J. MacNeil: Provided technical expertise for EMSA and super shift assays. Contributed to experiment design and interpretation of data.

Tong-Jun Lin: Oversaw study design and data analysis, provided funding and participated in manuscript editing and revisions

4.1 Abstract:

Infection with the opportunistic pathogen *P. aeruginosa* is effectively controlled through tightly coordinated inflammation in healthy individuals. However dysregulation of the inflammatory response in disease states such as cystic fibrosis greatly increase susceptibility to *P. aeruginosa*, as well as resultant lung damage. Recently we identified regulator of calcineurin 1 as a central negative regulator of multiple inflammatory transcription factors following *P. aeruginosa* lung infection, implicating a role for the canonical calcineurin-nuclear factor of activated T cell (NFAT) pathway in *P. aeruginosa* infection. Calcineurin is a calcium/calmodulin sensitive phosphatase which dephosphorylates NFAT leading to nuclear translocation and transcriptional activity. However the contribution of this pathway to host defense against *P. aeruginosa* remains poorly characterized. In order to elucidate the role of the calcineurin-NFAT axis during *P. aeruginosa* infection an acute lung infection model was employed in wild-type and calcineurin Aβ (CnAβ) deficient mice. NFAT was found to be rapidly and transiently activated following *P. aeruginosa* infection both *in vitro* and *in vivo*. CnAβ-deficiency resulted in impaired activation of NFAT and decreased inflammatory cytokine production *in vivo*. Finally, non-canonical, calcineurin-independent activation of NFAT was identified in cultured macrophages. Together these results demonstrate for the first time that NFAT is activated through calcineurin dependent, and IKK dependent mechanisms following *P. aeruginosa* lung infection, and contributes to the host inflammatory response.

4.2 Introduction:

P. aeruginosa is an environmentally ubiquitous, opportunistic Gram negative bacterial pathogen which is commonly associated with ventilator associated pneumonia[8], and represents the leading cause morbidity and mortality among Cystic Fibrosis (CF) patients and immune compromised patients[12, 247]. In healthy individuals, *P. aeruginosa* elicits a tightly controlled innate immune response associated with significant neutrophil recruitment, which is sufficient to clear the infection[344]. However dysregulation of the inflammatory responses is associated with increased susceptibility to infection, and considerable damage to host tissues[107, 134, 345, 365]. This phenomena is highlighted in disease states such as CF where *P. aeruginosa* induced inflammation is both delayed and prolonged, resulting in chronic colonization associated with deteriorating lung function[69, 292]. Hence, understanding the components of an effective host defense against the bacteria is critical for the development of novel preventative and therapeutic strategies for the treatment of patients susceptible to *P. aeruginosa* infection[258, 259].

The nuclear factor of activated T cells (NFAT) family of transcription factors arose relatively recently in evolutionary history, and can be separated into calcineurin dependent (NFATc1, NFATc2, NFATc3 and NFATc4) and calcineurin independent (NFAT5) family members[150]. Canonical activation of NFATc family members requires an increase in intracellular levels of calcium, leading to activation of the Ca^{2+}/calmodulin dependent phosphatase calcineurin (Cn) which dephosphorylates multiple residues on NFATc proteins and exposes a nuclear localization sequence, leading to nuclear import[151]. Once in the nucleus, NFATc proteins interact with nuclear binding partners

(NFATn) to coordinate the expression of NFAT dependent genes, including a variety of inflammatory, and immunomodulatory cytokines[152, 153].

Calcineurin consists of both catalytic (CnA) and regulatory (CnB) subunits[154]. The catalytic subunit exists as the ubiquitously expressed alpha (CnAα) and beta (CnAβ) isoforms, as well as a gamma isoform (CnAγ) which is expressed primarily in the brain and testes. Similarly, the regulatory subunit consists of both the ubiquitously expressed CnBα and the testes specific CnBβ isoforms. In spite of their overlapping tissue expression and considerable sequence homology, CnAα and CnAβ have been found to exert biologically distinct roles *in vivo*, particularly with respect to NFAT activation where CnAβ plays a dominant role, and CnAα is largely dispensable[155-161].

Activation of the calcineurin-NFAT axis has long been known play critical roles in the acquired immune response, and it represents a frequent target of immunosuppressive therapy among transplant recipients[162, 163]. More recently roles for NFATc family members in bridging the innate and adaptive immune responses, as well as in the regulation of inflammatory responses have also been identified[164, 165]. However the role of the calcineurin-NFAT pathway during *P. aeruginosa* infection remains completely uncharacterized.

Recently, our group identified a critical role for regulator of calcineurin 1 (RCAN1) as a central negative regulator of *P. aeruginosa* induced inflammation *in vivo*, where RCAN1-deficient mice displayed uncontrolled systemic inflammation resulting in greatly increased mortality[365]. The enhanced inflammatory responses were associated with dysregulation of multiple inflammatory transcription factors including NFAT, raising the possibility that the calcineurin-NFAT axis contributes to host defense against

P. aeruginosa. In the current study we confirm that the calcineurin dependent NFAT isoforms NFATc1 and NFATc3 are rapidly and transiently activated following *P. aeruginosa* infection both *in vitro* and *in vivo*. Furthermore, we demonstrate that CnAβ-deficiency leads to impaired *P. aeruginosa* mediated inflammatory responses associated with decreased NFAT and NFκB activity. Finally, we uncover evidence of non-canonical activation of NFAT in *P. aeruginosa* infected macrophages. Together these results demonstrate that NFAT contributes to host defense against *P. aeruginosa* via both calcineurin dependent, and calcineurin independent mechanisms.

4.3 Materials and Methods:

Animals

Calcineurin (Cn) Aβ-deficient mice were purchased from Jackson Laboratories (Bar Harbor, ME) and have been described previously[366]. Heterozygous breeders were used to establish separate wild-type (CnAβ +/+) and CnAβ-deficient (CnAβ -/-) breeding colonies which were maintained in the same specific pathogen free facility. All animal protocols were approved by the University Committee on Laboratory Animals, Dalhousie University, in accordance with guidelines of the Canadian Council on Animal Care.

Animal survival studies

For survival experiments the laboratory strain of *P. aeruginosa* PAK (a gift from Dr. J Boyd (Institute of Marine Bioscience, National Research Council, Halifax, NS, Canada)) was used. Mice were intranasally infected with 1 LD_{50} of *P. aeruginosa* (1.0×10^7 CFU/mouse). Animals were monitored daily for disease score, weight loss and body

temperature. Moribund animals were sacrificed according to the guidelines of the Canadian Council on Animal Care.

Antibodies

Antibodies for NFATc1 (sc-13033X), NFATc2 (sc-7296X), NFATc3 (sc-8321X) and NFAT5 (sc-13035X) were purchased from Santa Cruz Biotechnology (Dallas, TX).

Bacterial preparation

P. aeruginosa were cultured as described previously[134]. Briefly, suspension cultures were grown until reaching the early stationery phase. Bacteria were washed in phosphate buffer and resuspended in saline for *in vivo* experiments or PBS for *in vitro* assays. Mice were infected as described below.

Cell culture

Bone marrow derived macrophages were cultured as described previously[367].

Cytokine production

Concentrations of cytokines in the lungs, BALF, serum and culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) as described previously using antibody pairs from R&D Systems (Minneapolis, MN)[77].

Lung infection with *P. aeruginosa* and collection of lung and bronchoalveolar lavage fluid (BALF)

For bacterial clearance experiments *P. aeruginosa* strain 8821 (a gift from Dr A. Chakrabarty, University of Illinois, Chicago, IL), a mucoid strain isolated from a cystic fibrosis patient was used[264]. Mice were infected with 10^9 CFU of *P. aeruginosa* intranasally. After 4 or 24 hours, BALF was obtained by lavaging the lung with 3 × 1 mL phosphate buffer saline containing soybean trypsin inhibitor (100 μg/mL). The lung tissue, spleens and blood were obtained for detection of cytokines, myeloperoxidase (MPO) and bacterial colony-forming unit (CFU) counting.

Lung tissue and spleens were homogenized in 50 mM HEPES buffer (4 μL/1 mg lung) containing soybean trypsin inhibitor (100 μg/mL). For counting bacterial CFU, 10 μL of the homogenate, BALF or blood was plated onto an agar dish and incubated for 24 h at 37°C. The lung homogenate was centrifuged at 4°C for 30 min at 18000g. The supernatant was stored at −80°C for later cytokine analysis. The pellet was resuspended and homogenized in 0.5% cetyltrimethylammonium chloride (CTAC) (4 μL/mg lung) and centrifuged as above. The cleared extract was used for MPO assay. For detection of cytokines and MPO activity, BALF was centrifuged at 480g for 5 minutes at 4°C. The supernatants were used for cytokine analysis. The pellets were resuspended in 1 mL NH_4Cl (0.15M) and centrifuged as before to lyse red blood cells. The supernatants were discarded and the pellets were resuspended in 0.5% CTAC (250 μL/mouse) then centrifuged. The cleared extracts were used for MPO assay.

Myeloperoxidase (MPO) assay

The MPO assay was used to determine the infiltration of neutrophils into the lungs of the mice as described previously[270]. Briefly, samples in duplicate (75 μL) were

mixed with equal volumes of the substrate (3,3',5,5'-tetramethyl-benzidine dihydrochloride, 3 mM; Resorcinol, 120 μM; and H_2O_2, 2.2 mM) for 2 minutes. The reaction was stopped by adding 150 μL of 2 M H_2SO_4. The optical density was measured at 450 nm.

Nuclear extract preparation and electrophoresis mobility shift assay (EMSA)

Nuclear protein extracts were obtained using a nuclear extract kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. EMSA was performed as previously described [343]. Briefly probe labeling was accomplished by treatment with T4 kinase (Life Technologies Inc, Burlington, ON) in the presence of [^{32}P] adenosine triphosphate (Perkin Elmer, Waltham, MA). Labeled oligonucleotides were purified on a Sephadex G-25M column (GE healthcare, Pittsburgh, PA). Six micrograms of nuclear protein was added to a 10 μL volume of binding buffer supplemented with 1μg of poly-(dI-dC) (GE healthcare, Pittsburgh, PA) for 15 min. Labeled double-stranded oligonucleotide was added to each reaction mixture, which was incubated at room temperature for 30 min and separated by electrophoresis on a 6% polyacrylamide gel in 0.5× Tris-boric acid-EDTA buffer. Gels were vacuum-dried and subjected to autoradiography. The following synthesized double-stranded oligonucleotides were used: NFAT binding consensus sequence on mouse IL-13 promoter 5'-AAGGTGTTTCCCCAAGCCTTTCCC-3 (Sigma-Aldrich, St. Louis, MO) and NF-κB consensus sequence on the IL-6 promoter 5'-AGTTGAGGGGACTTTCCCAGGC-3' (Promega, Madison, WI).

Super shift assay was performed as described previously[269]. Briefly, samples were prepared as described above and then incubated with 4 μg of the indicated antibody on ice for 45 minutes prior to incubation with ^{32}P-labeled double stranded DNA probes. Samples were resolved and developed as described above.

Statistics

Data are presented as mean ± S.E.M. of the indicated number of experiments. Statistical significance was determined by ANOVA and post hoc Tukey's honest significant test. Differences were considered significant at *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

Scanning Densitometry

Scanning densitometry was performed using Scion Image (Scion Corporation, Frederick, MD).

4.4 Results:

Calcineurin dependent NFAT family members are rapidly activated* in vitro *and* in vivo *following* P. aeruginosa *infection: Macrophages are abundant within the alveolar space of the lungs where they play important roles in recognizing and coordinating the immune response against invading pathogen, and have been implicated as critical components of host defense against *P. aeruginosa*[98, 99]. In order to examine whether NFAT is involved in macrophage mediated host responses to *P. aeruginosa,* bone-marrow-derived macrophages (BMMs) were cultured from wild-type mice. Cells were then left uninfected or treated for various times with *P. aeruginosa* strain 8821, a clinical

isolate from the lungs of a cystic fibrosis patient[264]. Nuclear extracts were prepared and subjected to EMSA for NFAT activity (Figure 4.1 A). Scanning densitometry of multiple batches of cells was performed, and a consistent rapid activation of NFAT transcriptional activity was observed, starting within 20 minutes post infection (Figure 4.1 B). These results suggest that NFAT is involved in macrophage mediated host defense against *P. aeruginosa*.

NFAT family members can be split into calcineurin dependent (NFATc1-4) and calcineurin independent family members (NFAT5). In order to determine which NFAT family members are activated upon *P. aeruginosa* stimulation a super shift assay for NFATc1, NFATc2, NFATc3 and NFAT5 was performed on nuclear extracts from BMMs collected 1 hour post-infection (Figure 4.1 B). Scanning densitometry of multiple batches was performed and showed that *P. aeruginosa* primarily induces the transcriptional activity of the calcineurin dependent NFAT family members NFATc1 and NFATc3 (Figure 4.1 D).

Having observed rapid activation of calcineurin dependent NFAT family members in macrophages, we next set out to determine whether this phenomena occurs *in vivo* following *P. aeruginosa* lung infection. Mice were left untreated or infected intranasally with 10^9 CFU of *P. aeruginosa* strain 8821 for 4 to 48 hours. Lung tissue was collected and nuclear extracts were prepared, and then subjected to EMSA for NFAT. A rapid induction of NFAT activity was observed following *P. aeruginosa* infection, which subsided within 24 hours (Figure 4.2 A). Activation was quantified across multiple animals by scanning densitometry (Figure 4.2 C). Super shift assays were used to identify which family members were activated in the lungs following *P.*

aeruginosa infection. As was observed *in vitro*, the calcineurin dependent NFAT family members NFATc1 and NFATc3 are primarily responsible for *P. aeruginosa* induced NFAT activity in the lung (Figure 4.2 B and D).

Calcineurin Aβ deficiency impairs NFAT and NFκB transcriptional activity following* P. aeruginosa *lung infection: Based on the observation that calcineurin-dependant NFAT family members are rapidly activated following *P. aeruginosa* infection *in vitro* and *in vivo*, we explored the biological contribution of the calcineurin-NFAT axis to host defense against the bacteria using mice deficient in the calcineurin Aβ (CnAβ -/-). CnAβ was chosen as previous reports have indicated that this is the subunit primarily involved in calcineurin mediated immune responses[159], and is responsible for upwards of 80% of all cellular calcineurin activity[366]. Wild type (CnAβ +/+) and CnAβ -/- mice were left uninfected, or infected intranasally with 10^9 *P. aeruginosa* strain 8821 for 4 or 24 hours, after which nuclear extracts were prepared from lung tissue, and subjected to EMSA for NFAT (Figure 4.3 A and B). CnAβ-deficiency decreased, but did not abolish *P. aeruginosa* induced NFAT activity.

In addition to modulating NFAT activity, calcineurin has also been implicated as a regulator of the pro-inflammatory transcription factor NFκB[190, 365]. In order to determine whether CnAβ-deficiency impacts NFκB activity following *P. aeruginosa* infection, NFκB EMSA was performed on nuclear extracts from the lungs of CnAβ +/+ and CnAβ -/- mice left uninfected, or infected with *P. aeruginosa* for 4 or 24 hours (Figures 4.4 A and B). Similar to what was observed for NFAT, CnAβ-deficiency decreased, but did not abolish NFκB activity *in vivo*. These findings demonstrate that

CnAβ plays an important role in the regulation of the pro-inflammatory transcription factors NFAT and NFκB following *P. aeruginosa* infection *in vivo*.

P. aeruginosa *induced NFAT activity occurs via an IKK dependent mechanism in macrophages :* Macrophages are an important source of inflammatory cytokines during *P. aeruginosa* infection. In order to assess the role of CnAβ in the activation of NFAT *in vitro* BMMs were cultured from CnAβ +/+ and CnAβ -/- mice, then infected with *P. aeruginosa* for various time points. Nuclear extract were prepared and subjected to EMSA for NFAT (Figure 4.5 A and C). In opposition to observations made *in vivo*, no impact on NFAT activation, or inflammatory cytokines production (Figure 4.6) was observed in CnAβ-deficient macrophages.

Calcineurin-independent activation of "calcineurin dependant" NFAT family members has been reported previously to occur via a IKK-Tpl-2 dependant mechanism which leads phosphorylation of NFAT family members at unique residues leading to nuclear translocation and transcriptional activity[368, 369]. In order to examine whether a similar mechanism may be involved in NFAT activation in macrophages, cells from CnAβ +/+ mice were infected with *P. aeruginosa* in the presence or absence of 3 different IKK inhibitors: BMS345541, sc-514 and suldinac (Figure 4.5 B and D). All three inhibitors significantly decreased *P. aeruginosa* induced NFAT activity, demonstrating that activation proceeds via an IKK dependant mechanism. However unlike previously proposed mechanisms for IKK mediated NFAT activation, *P. aeruginosa* induced NFAT activation in macrophages is not dependent upon the kinase activity of tumour progression locus 2 (Tpl-2), suggesting the existence of a calcineurin-

independent, IKK-dependent, Tpl-2 independent pathway for the activation of NFAT in murine macrophages (Figure 4.7).

Calcineurin Aβ deficiency impairs inflammatory cytokine production following* P. aeruginosa *lung infection. NFAT and NFκB are critical regulators of inflammatory cytokine production *in vivo*. In order to determine whether dysregulation of these transcription factors observed in CnAβ-deficient mice impacts inflammatory responses during *P. aeruginosa* lung infection CnAβ +/+ and CnAβ -/- were left uninfected, or infected with *P. aeruginosa* for 4 or 24 hours after which lung tissue (Figures 4.8 A-D), bronchoalveolar lavage fluid (BALF) (Figure 4.8 E-H) and serum (Figures 4.8 I-L) were collected and subjected to ELISA analysis for the levels of the inflammatory cytokines TNF, IL-6, IL-1β and the neutrophil chemoattractant MIP-2. Overall inflammatory cytokine production was significantly reduced in the lungs and BALF in CnAβ-deficient animals. A trend towards decreased systemic production of inflammatory mediators was also observed in the serum, although these results failed to reach significance. Critically, the production of immunoregulatory cytokines previously shown to be induced via NFAT-dependant mechanisms in various cell types, including IL-2[152, 322], IL-3[370], IL-4[371], IL-10[152, 372, 373], IL-12p70[152] and IL-13[374], were not induced following *P. aeruginosa* induced NFAT activation, suggesting a pro-inflammatory role for the transcription factor during early host responses to the bacteria (Figure 4.9).

CnAβ-deficiency does not impact bacterial clearance or survival following* P. aeruginosa *lung infection The recruitment of neutrophils to the site of infection is

critical for effective host defence against *P. aeruginosa* infection[344]. Given the impaired inflammatory responses in CnAβ -/- animals, we next set out to examine the impact of CnAβ-deficiency on neutrophil infiltration *in vivo*. CnAβ +/+ and CnAβ -/- mice were left uninfected, or infected with *P. aeruginosa* for 4 or 24 hours after which neutrophil infiltration into the lungs (Figure 4.10 A) and BALF (Figure 4.10 B) was assessed using an assay for the neutrophil granule specific enzyme myeloperoxidase (MPO). CnAβ-deficiency markedly impaired early recruitment of neutrophils in both the lungs and BALF following *P. aeruginosa* infection. This effect persisted in the lungs 24 hours post infection. These results demonstrate that CnAβ plays an important role in regulating the inflammatory response, and neutrophil recruitment following *P. aeruginosa* infection. In spite of these differences, no impact was observed on bacterial burden 24 hours post infection (Figure 4.10 C and D). Similarly, no impact on survival or disease severity was observed in CnAβ-deficient animals when challenged with one LD_{50} of the laboratory strain of *P. aeruginosa* PAK (Figure 4.11). These results suggest that while the CnAβ contributes to *P. aeruginosa* induced inflammation it is ultimately dispensable for bacterial clearance and animal survival.

4.5 Discussion:

The calcineurin-NFAT axis plays critical roles in cellular immunity. However the contribution of the pathway to innate immunity remains poorly defined. Recent work conducted in our lab elucidated a critical role for RCAN1 as a negative regulator of inflammation during early host defense against the opportunistic bacterial pathogen *P. aeruginosa*. Given the well characterize role of RCAN1 as a negative regulator of

calcineurin-NFAT signaling, we set out to characterize the role of this axis in host defense against *P. aeruginosa*. In the current study we demonstrate that calcineurin dependent family members (NFATc1 and NFATc3) are rapidly and transiently activated following *P. aeruginosa* infection *in vitro* and *in vivo*. Utilizing mice deficient in calcineurin Aβ we identified that the calcineurin-NFAT pathway contributes to *P. aeruginosa* induced inflammation. However, ultimately CnAβ was dispensable for bacterial clearance and animal survival.

Although CnAβ clearly contributes to *P. aeruginosa* induced inflammation, considerable NFAT activity remained in the lungs of CnAβ deficient mice. This observation can likely be attributed to two factors. First, there exists multiple isoforms of calcineurin, including two other catalytic subunits CnAα and CnAγ. Hence it is possible that these isoforms either compensate for CnAβ deficiency, or are responsible for *P. aeruginosa* induced NFAT activity under normal biological conditions. Previous reports have demonstrated that CnAβ is responsible for greater than 80% of calcineurin activity in cardiac tissue[366] and is the primary isoform required for effective cellular immunity[157, 159]. However the relative contribution of calcineurin isoforms during early innate immune responses to bacterial pathogens is poorly understood raising the possibility that other catalytic subunits may play prominent roles in calcineurin mediated inflammation.

The second contributing factor to the residual NFAT activity in the lungs of CnAβ deficient mice is the novel observation that NFAT is activated in cultured macrophages through a CnAβ independent mechanism which is instead activated downstream of IKK activity. IKK dependant activation of NFAT has been reported previously and was proposed to be directly mediated by the kinase activity of the MAP3K Tpl-2[375, 376].

However pharmacological inhibition of Tpl-2 was unable to block *P. aeruginosa* induced NFAT activity in bone marrow derived macrophages, suggesting the existence of an alternative IKK dependant, Tpl2 and calcineurin independent pathway for activation of "calcineurin dependent" NFAT family members. Accordingly, CnAβ deficiency did not impact inflammatory cytokine production from *P. aeruginosa* stimulated BMMs suggesting that calcineurin may play cell type specific roles in NFAT activation, and that residual NFAT activity observed *in vivo* may be attributed in part to calcineurin independent pathways mediating NFAT activation.

The observation that the calcineurin-NFAT axis is ultimately dispensable for bacterial clearance and animal survival during *P. aeruginosa* infection is not entirely surprising when viewed from an evolutionary perspective. Relative to other components of the innate immune response, NFAT family member arose very recently in evolutionary history[150]. As a result, it stands to reason that NFAT would play its most prominent roles in more recent branches of the immune system, such as cellular immunity. Although the calcineurin-NFAT axis undoubtedly contributes to *P. aeruginosa* induced inflammation, it appears its role is primarily to enhance existing inflammatory pathways, and that it is ultimately dispensable for the evolutionarily more ancient innate immune response.

Recent work has shown that activation of NFAT family members (NFATc1 and NFATc3) play a critical role in neutrophil mediated innate immunity during fungal infections with *C. albicans*[377]. However the results present here, combined with previous studies which used the calcineurin inhibitor cyclosporine A (CsA)[378] indicate that while the calcineurin-NFAT axis contributes to *P. aeruginosa* induced inflammation, it does not impact disease severity or progression. These observations reflect clinical data which

suggests that patients taking calcineurin inhibitors are at increased risk of fungal, but not bacterial pneumonias[379]. There are at least two possible explanations for these kingdom specific roles for the calcineurin NFAT-axis. First, the NFAT activity may play an as yet uncharacterized role in innate antifungal immunity which is not required from host defense against bacterial pathogens. Second, as is highlighted in this study, there appears to exist multiple pathways for NFAT activation in response to bacterial stimuli, some of which are independent of calcineurin signaling. As a result of this redundancy, even in the presence of calcineurin inhibitors there may be sufficient residual NFAT activity for an effective inflammatory response to bacterial pathogens.

This work has highlighted an evolving theme of crosstalk between the NFκB and NFAT pathways. Previous work has demonstrated that calcineurin can enhance NFκB activity through promoting dissociation and degradation of the inhibitory molecule IκB[190]. Consistent with this view, in the current study CnAβ was found to be required for maximal NFκB activity. Conversely, activation of the IKK complex, a critical component of the canonical NFκB pathway, has been demonstrated both previously[375, 376], and in the current study to be capable of mediating transcriptional activation of NFAT. In our model of acute *P. aeruginosa* lung infection, this cross talk appear to serve important roles both in the enhancement of inflammatory responses against the bacteria, and in creating redundancies to ensure activation of NFAT even when the canonical calcineurin-NFAT axis is impaired.

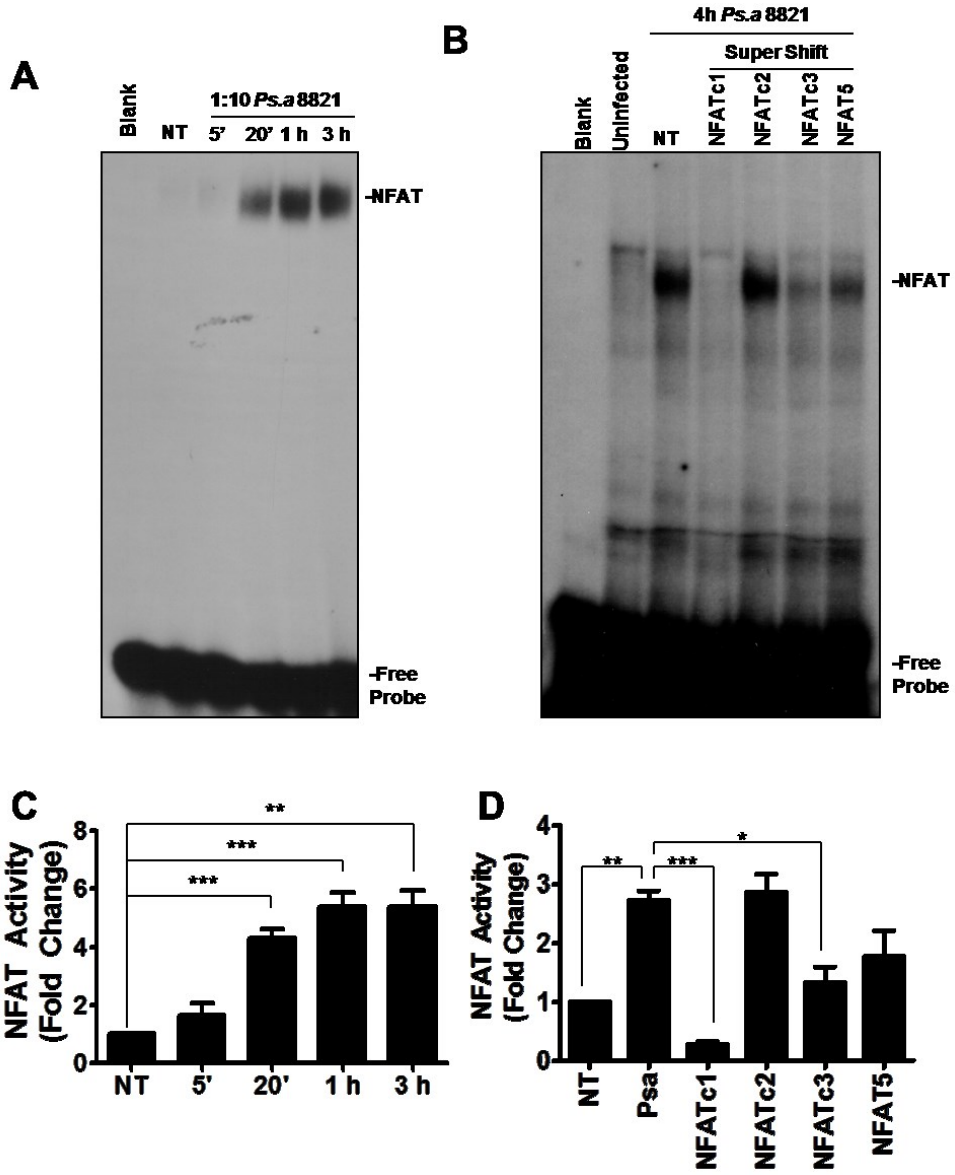

Figure 4.1: Calcineurin dependant NFAT family members are rapidly activated in bone marrow derived macrophages *in vitro* following *P. aeruginosa* infection. Bone marrow derived macrophages were isolated from CnAβ +/+ mice and used on culture day 7. Cells were left untreated (NT) or infected with *P. aruginosa* strain 8821 at an MOI of 1:10 for time points indicated then nuclear extracts were prepared and subjected to EMSA for NFAT (A), and supershift assay to identify which NFAT family members were activated (B). Scanning densitometry of multiple batches was performed (C and D). (n=3-4 ± SEM, *p < 0.05, **p < 0.01, ***p < 0.005).

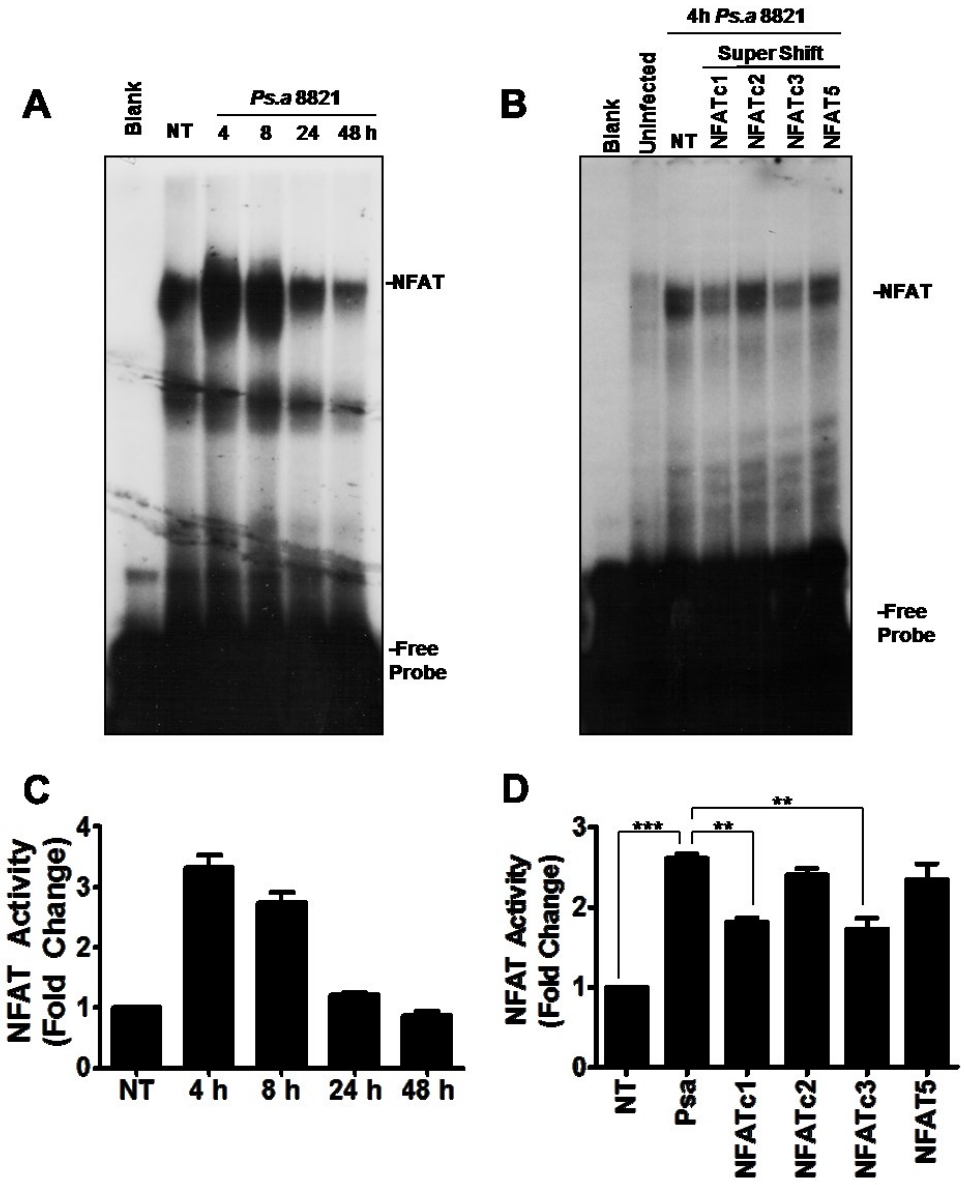

Figure 4.2: Calcineurin dependent NFAT family members are rapidly and transiently activated in the lungs following *P. aeruginosa* infection *in vivo*. CnAβ +/+ mice were infected intranasally with 10^9 *P. aeruginosa* strain 8821. Mice were sacrificed at the indicated time points and nuclear extracts were prepared from their lungs which were subjected to EMSA for NFAT (A). Nuclear extracts from uninfected lungs and the lungs of mice infected for 4 hours were subjected to EMSA and supershift to identify which isoforms of NFAT were activated following *P. aeruginosa* infection (B). Scanning densitometry was performed on samples from multiple animals and expressed as a fold change vs. uninfected controls (C and D). (n=2-4 ± SEM, **p < 0.01, ***p < 0.005)

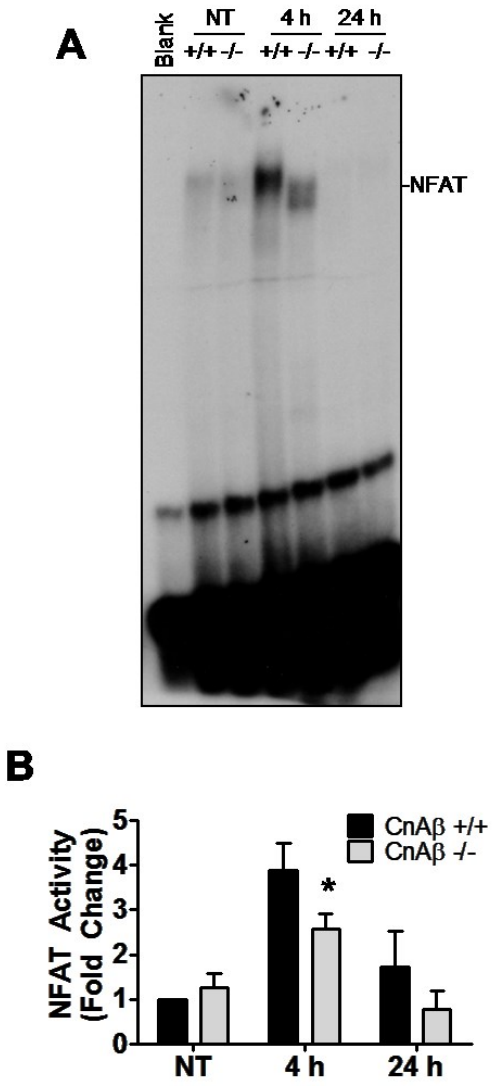

Figure 4.3: Calcineurin deficiency impairs NFAT activation *in vivo*. Age and sex matched CnAβ +/+ and CnAβ -/- mice were left uninfected or infected intranasally with 10^9 *P. aeruginosa* 8821 for 4 or 24 hours. Mice were sacrificed and lung tissue was collected. Nuclear extracts were prepared from lung tissue and subjected to EMSA assay for NFAT (A). Transcription factor activity was quantified using scanning densitometry (B). (n=5 ± SEM, *p < 0.05)

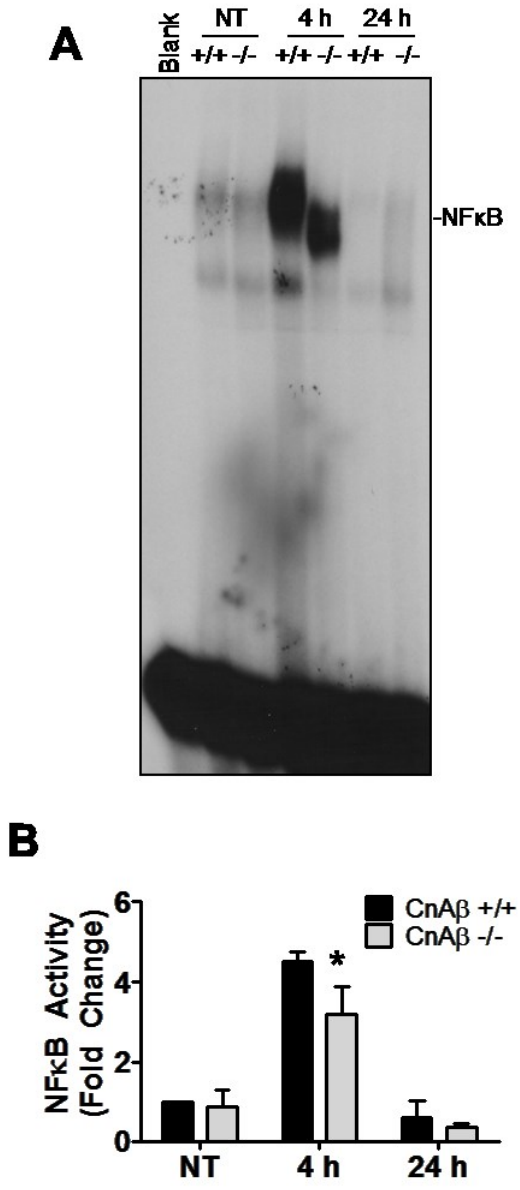

Figure 4.4: Calcineurin deficiency impairs NFκB activation *in vivo*. Age and sex matched CnAβ +/+ and -/- mice were left uninfected or infected intranasally with 10^9 *P. aeruginosa* 8821 for 4 or 24 hours. Mice were sacrificed and lung tissue was collected. Nuclear extracts were prepared from lung tissue and subjected to EMSA assay for NFκB (A). Transcription factor activity was quantified using scanning densitometry (B). (n=5 ± SEM, *$p < 0.05$)

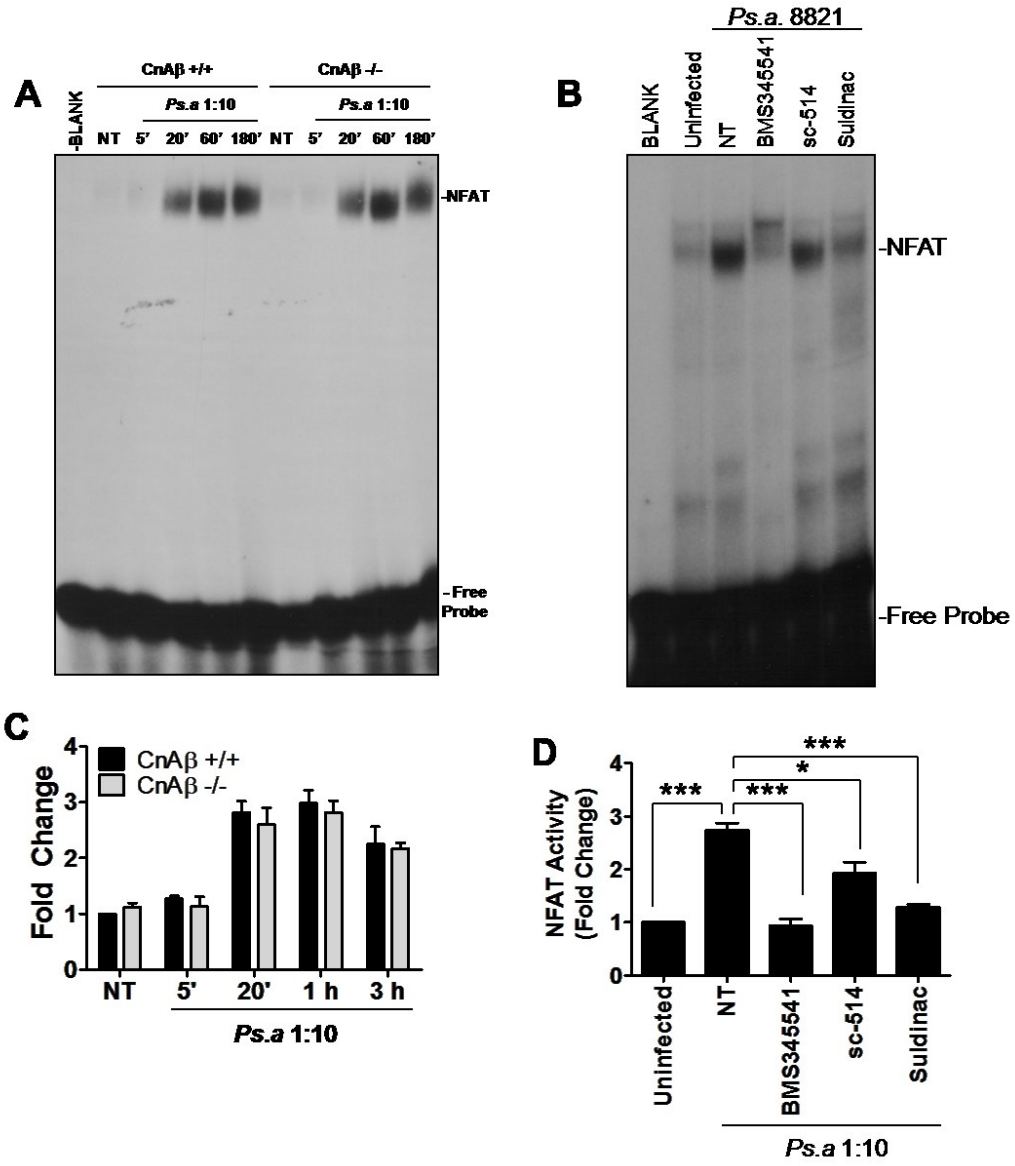

Figure 4.5. *P. aeruginosa* induced NFAT activity in macrophages is calcineurin Aβ independent and IKK dependent: Bone marrow derived macrophages from CnAβ +/+ or -/- mice were left untreated (NT) or infected with *P. aeruginosa* strain 8821 at an MOI of 1:10. Nuclear extracts were collect at the time points indicated and subjected to EMSA for NFAT (A). Alternatively macrophages from CnAβ +/+ mice were pretreated for 1 hour with 100 μM BMS345541, 100 μM sc-514 or 5 mM Suldinac then infected with *P. aeruginosa* strain 8821 at an MOI of 1:10 for 30 minutes after which nuclear extracts were collected and subjected to EMSA for NFAT (B). NFAT activity was quantified using scanning densitometry (C and D). (n=3 ± SEM, *$p < 0.05$, ***$p < 0.005$)

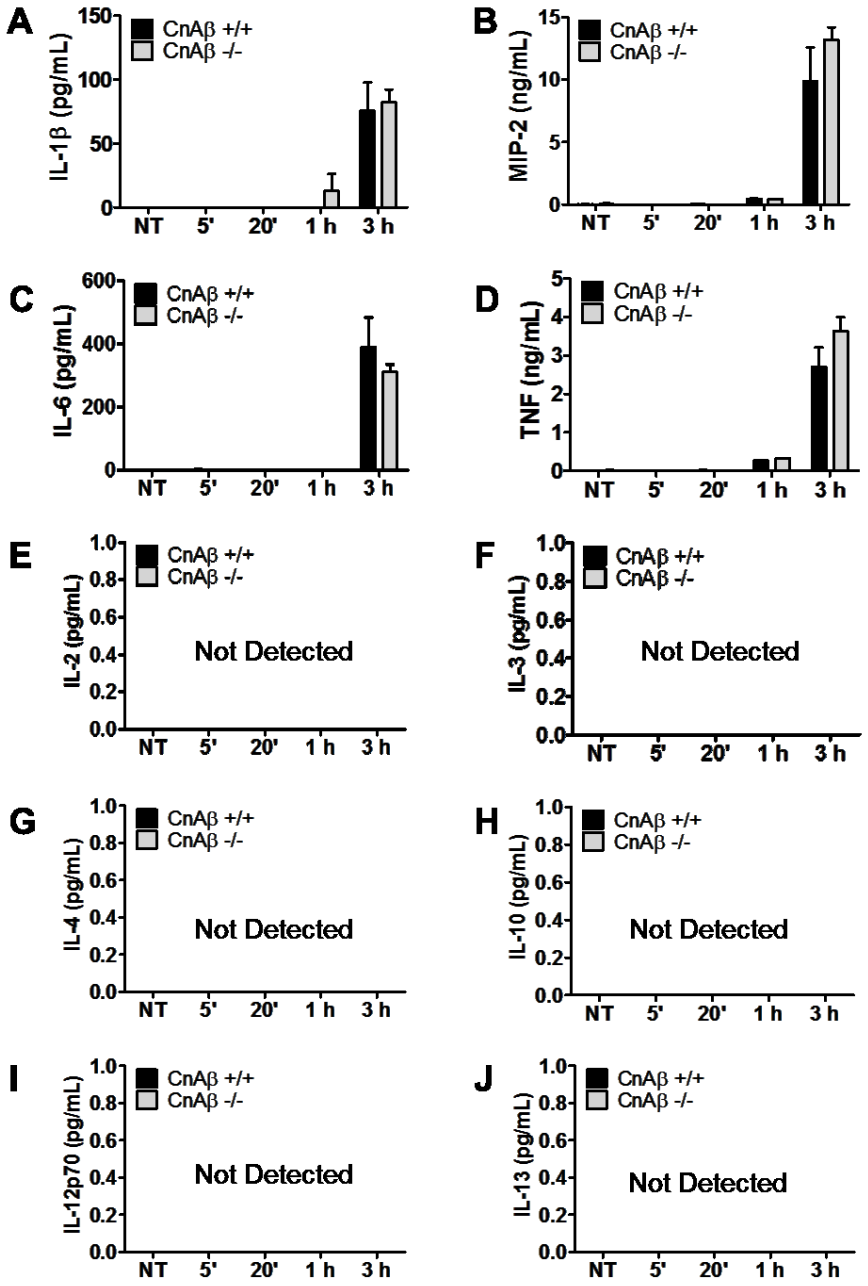

Figure 4.6: *P. aeruginosa* induced NFAT activity correlates with inflammatory, but not immunoregulatory cytokines production from cultured macrophages: Bone marrow derived macrophages were cultured from CnAβ +/+ and CnAβ -/- mice. Macrophages were left uninfected (NT) or infected with *P. aeruginosa* strain 8821 at an MOI of 1:10. Supernatants were collected at the indicated time points, and were subjected to ELISA for the indicated pro-inflammatory and immunoregulatory cytokines. (n= 3 ± SEM)

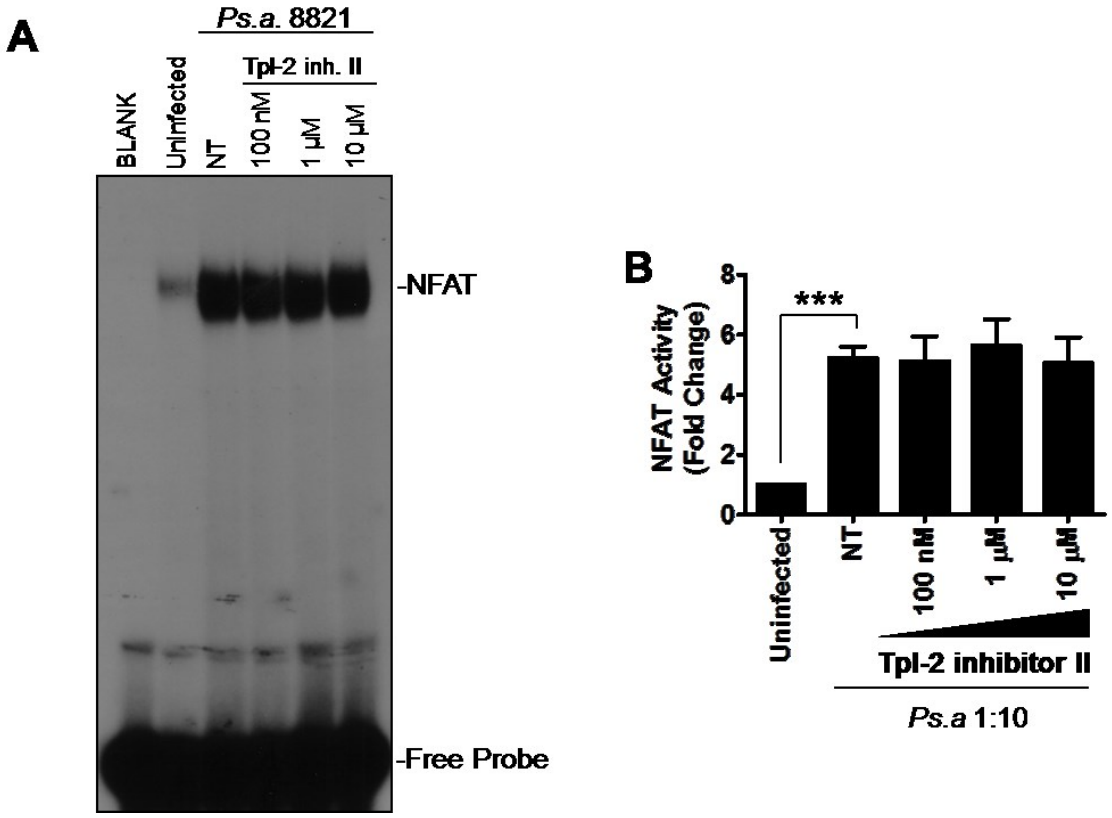

Figure 4.7: NFAT activation in macrophages following *P. aeruginosa* infection is Tpl-2 independent: Bone marrow derived macrophages were cultured from CnAβ +/+ mice. On culture day 7 cells were left untreated or pretreated for 1 hour with the indicated doses of Tpl-2 inhibitor II then infected with *P. aeruginosa* strain 8821 at an MOI of 1:10 for 30 minutes. Nuclear extracts were prepared and subjected to EMSA for NFAT (A). Scanning densitometry was performed and changes were expressed as fold change vs. uninfected samples (B). (n=3 ± SEM,***p<0.001)

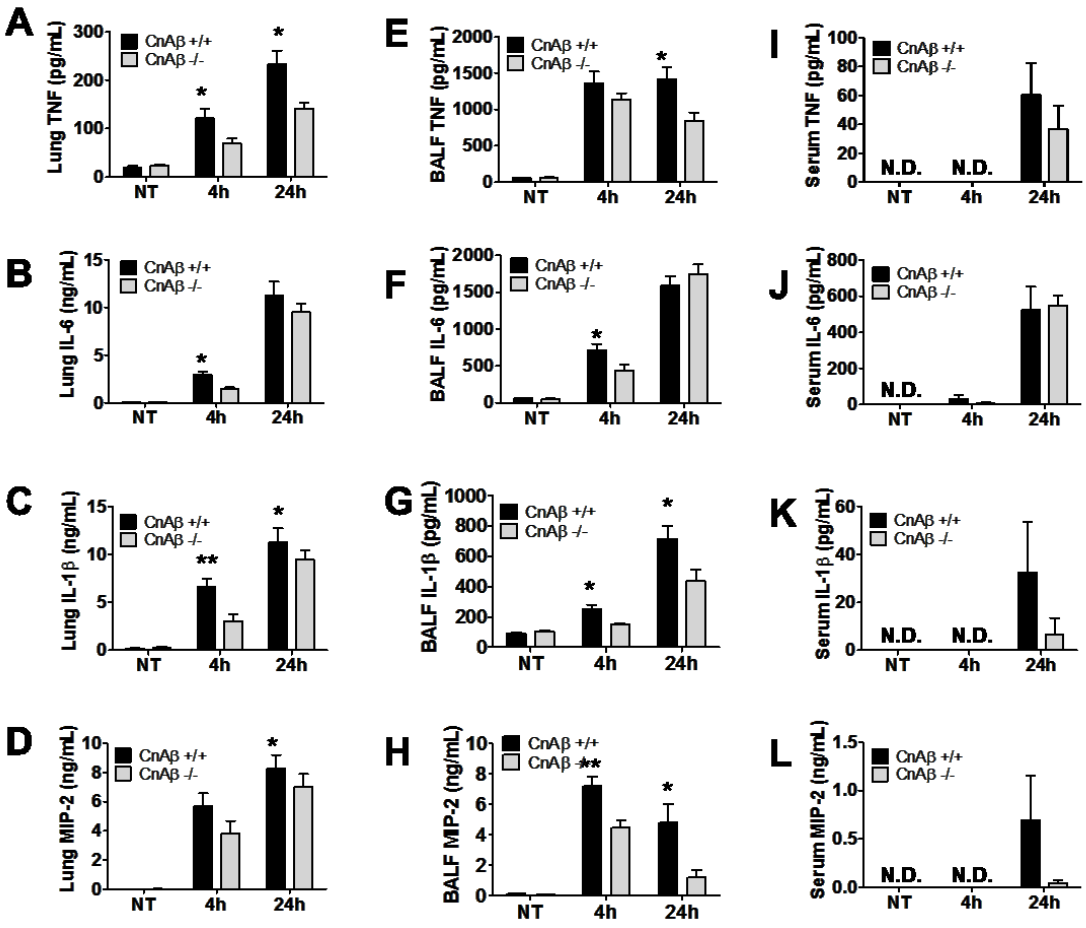

Figure 4.8: CnAβ-deficiency impairs inflammatory cytokine responses during *P. aeruginosa* lung infection. CnAβ +/+ and CnAβ -/- mice were infected intranasally with saline (NT), 10^7 (4h) or 10^9 (24 h) CFU of *P. aeruginosa* strain 8821. Mice were sacrificed 4 or 24 hours post infection and lung tissue (A-D), BALF (E-H) and serum (I-L) was collected and analyzed for inflammatory cytokines by ELISA. n=5-10 ± SEM, *p<0.05, **p<0.01. N.D. not detected.

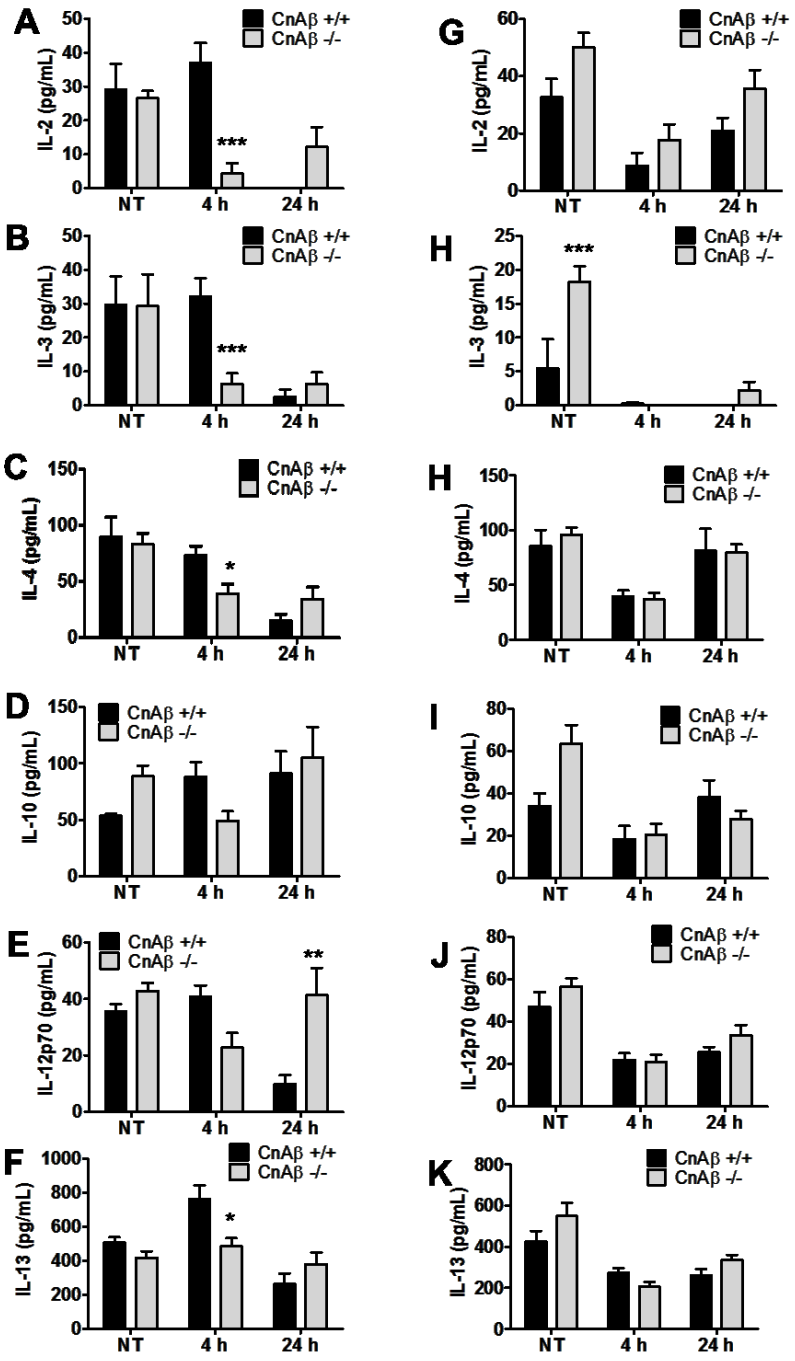

Figure 4.9: Immunoregulatory NFAT dependent cytokines are not strongly induced following *P. aeruginosa* lung infection: CnAβ +/+ and CnAβ -/- mice were infected intranasally with saline (NT), 10^7 (4h) or 10^9 (24 h) CFU of *P. aeruginosa* strain 8821. Mice were sacrificed 4 or 24 hours post infection and lung tissue (A-F), and BALF (G-K) was collected and analyzed for immunoregulatory cytokines by ELISA. n=5-10 ± SEM, *p<0.05, **p<0.01, ***p <0.005).

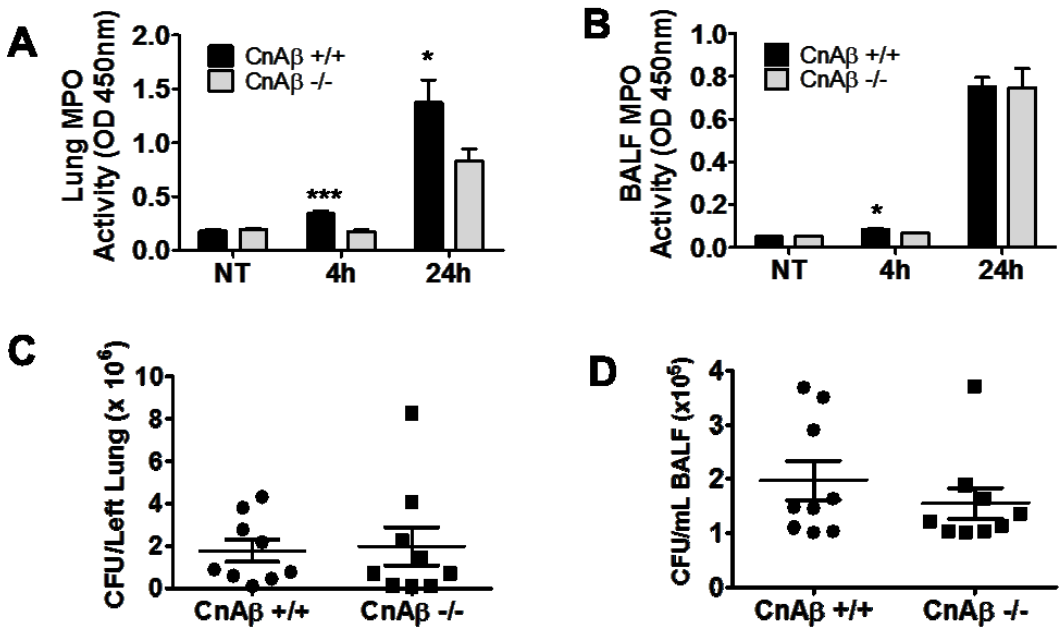

Figure 4.10: CnAβ-deficiency impairs neutrophil recruitment, but does not impact bacterial clearance following *P. aeruginosa* lung infection. Age and sex matched CnAβ +/+ and CnAβ -/- mice were left uninfected (NT) or infected intranasally with 10^9 *P. aeruginosa* 8821 for 4 or 24 hours. Mice were sacrificed and lung tissue and BALF were collected in order to assess activity of the neutrophil granule specific enzyme MPO (A and B) and bacterial load (C and D). (n=5-10 ± SEM, *$p < 0.05$, ***$p < 0.005$)

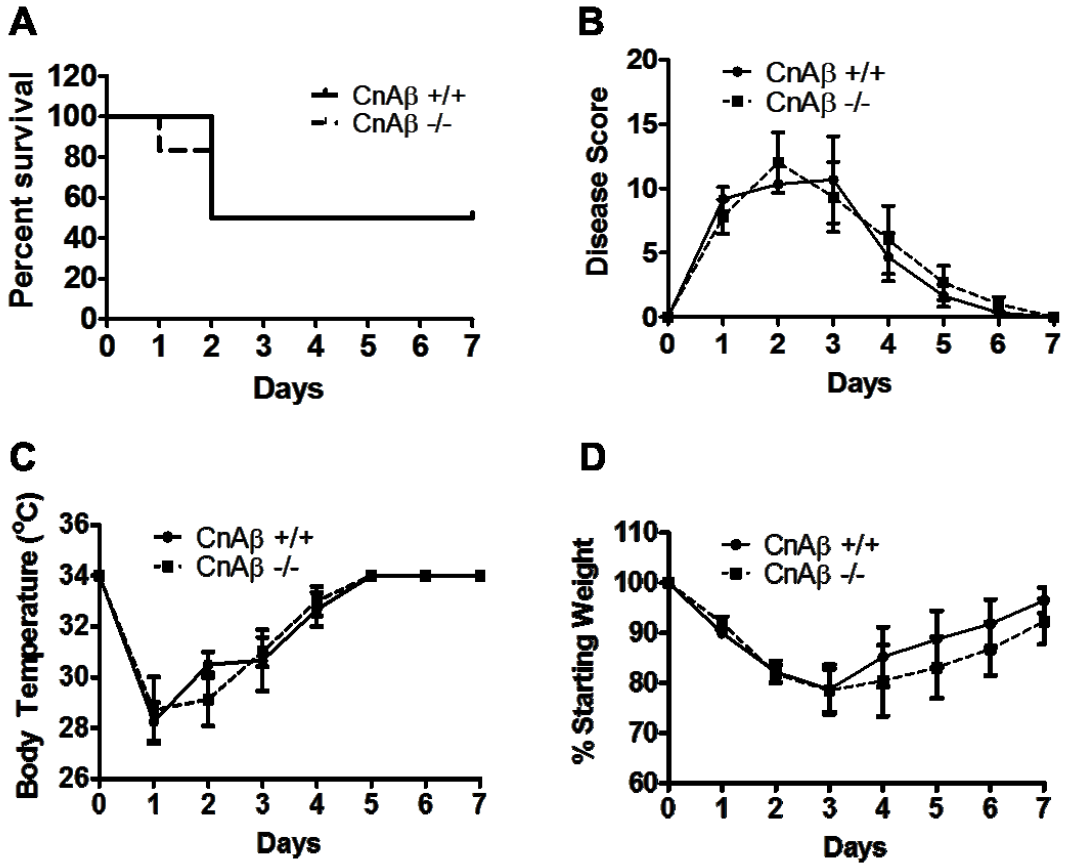

Figure 4.11: CnAβ-deficiency does not impact animal survival following *P. aeruginosa* infection. CnAβ +/+ and CnAβ -/- mice were infected intranasally with 10^7 CFU of *P. aeruginosa* strain PAK. Animals survival (A), disease score (B), body temperature (C) and body weight (D) were monitored for 7 days post infection. (n=10 ± SEM)

CHAPTER 5: MAST CELLS PROTECT AGAINST P. AERUGINOSA INDUCED LUNG INJURY

Mast cells protect against *P. aeruginosa* induced lung injury

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*These authors contributed equally to this manuscript

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Author contribution:

Robert D. Junkins: Designed, executed and analyzed data from figures 5.2, 5.3, 5.4, 5.5 panel B, 5.6, 5.7, 5.8, 5.9 and 5.10 panel B. Contributed data to figure 5.1. Co-authored the first draft of the manuscript, and was the primary author of subsequent revisions.

Svetlana O. Carrigan: Designed, executed and analyzed data from figure 5.1, 5.5 panel A and C, 5.10 panel B. Contributed data figure 5.3. Provided initial observation that mast cell-deficient mice display enhanced acute lung injury.

Zhengli Wu: Provided technical assistance with immunofluorescence for figure 5.8.

Andrew Stadnyk: Participated in study design, interpretation of data, and manuscript preparation.

Elizabeth Cowley: Participated in study design, interpretation of data, and manuscript preparation.

Thomas Issekutz: Participated in study design, interpretation of data, and manuscript preparation.

Jason Berman: Participated in study design, interpretation of data, and manuscript preparation.

Tong-Jun Lin: Oversaw study design and data analysis, provided funding and participated in manuscript editing and revisions

5.1 Abstract

Pseudomonas aeruginosa is a gram-negative opportunistic pathogen which represents the leading cause of morbidity and mortality in immune compromised individuals. Understanding mechanisms of the host response to *P. aeruginosa* is of particular importance for the development of successful preventative and treatment strategies. Maintaining the integrity of the respiratory epithelium is critical for an effective host response to *P. aeruginosa*. Mast cells are abundant throughout the respiratory tract where they are closely associated with respiratory epithelium. Mast cells have been implicated in a number of aspects of both the innate and adaptive immune responses, but their function *in vivo* during *P. aeruginosa* lung infection is poorly understood, and their impact on epithelial integrity in the lungs remains undefined. We found that mast cells prevented epithelial permeability changes following *P. aeruginosa* infection *in vitro*. Mast cell-deficient $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice displayed greatly increased epithelial permeability, bacterial dissemination and neutrophil accumulation compared to wild type animals following *P. aeruginosa* infection. In an *in vitro* transwell co-culture model mast cells decrease epithelial cell apoptosis and TNF production following *P. aeruginosa* infection. Together, our data demonstrates a previously unrecognized role for mast cells in the maintenance of epithelial integrity during *P. aeruginosa* infection, through a mechanism which likely involves prevention of epithelial apoptosis and TNF production.

5.2 Introduction

The respiratory epithelium acts as the first line of defense against invading pathogens within the lungs through not only acting as a physical barrier to infection, but also promoting clearance of pathogens from the lungs through mucociliary clearance[380, 381]. Maintenance of epithelial integrity is achieved primarily through the interaction between two adjacent epithelial cells. Disruption of epithelial integrity is a hallmark of acute lung injury, such as that observed in acute respiratory distress syndrome which occurs at a rate of 62-78 cases per 100,000 people each year and is associated with mortality rates between 29-42%[382, 383]. During bacterial infection, lung injury is further characterized by accumulation of neutrophils within the lungs, increased dissemination of bacteria from the sight of infection and lung epithelial apoptosis[384, 385]. Dysregulation of epithelial integrity is associated with various disease states such as cystic fibrosis where patients have persistent chronic inflammation in their airways which leads to increased lung epithelial permeability and further dysregulation of inflammatory response in these patients[381, 386, 387].

Bacterial and viral pathogens have evolved mechanisms to disrupt epithelial integrity, resulting in enhanced pathogenicity[388-390]. *Pseudomonas aeruginosa* is a Gram negative opportunistic bacterial pathogen resistant to many commonly used antibiotics. Chronic lung infection with *P. aeruginosa* is a major concern among immunocompromised patients and represents the leading cause of morbidity and mortality in cystic fibrosis[247, 381, 388, 389]. Furthermore acute *P. aeruginosa* infection is the second most common cause of ventilator associated pneumonia where it is associated

with poor clinical outcomes[391]. Given the damaging effects of *P. aeruginosa* infection and the emergence of multi-antibiotic resistant strains, understanding mechanisms involved in host defense against the bacteria will assist in developing preventative and therapeutic strategies for treating patients susceptible to *P. aeruginosa* infection[258, 259]. During *P. aeruginosa* infection, epithelial barrier function has been shown to play a particularly important role as intact epithelial layers increase resistance to bacterial internalization and infiltration[59, 392, 393]. Apoptosis occurs in the early phase of *P. aeruginosa* pneumonia and has been recognized as a feature of *P. aeruginosa*-induced lung injury[385]. Blockade of *P. aeruginosa*-induced apoptosis improves permeability and lung fluid balance[385].

P. aeruginosa expresses a plethora of virulence factors which disrupt host defense mechanisms and promote bacterial invasion and survival[15]. For example, exotoxins secreted through the type III secretion system[394], quorum sensing molecules[395], rhamnolipids[396], exotoxin A[24] and elastase[24, 397] have all been implicated in increasing epithelial permeability during *P. aeruginosa* infection. However, host factors involved in maintaining airway epithelial integrity during *P. aeruginosa* infection remain incompletely understood.

Mast cells are long-lived bone-marrow-derived cells resident in tissues throughout the body. Within the lung, they are abundant, representing up to 2 % of the alveolar wall where they protrude into the airspace of the lung, ideally placing them as sentinel cells of the immune system and first responders to respiratory tract infections[75]. Upon encountering microbes, mast cells play critical roles both in direct killing of pathogens, as well as in initiation and coordination of the innate and acquired immune responses[76, 77].

Depending upon the stimulation encountered, mast cells are capable of producing numerous inflammatory and immunomodulatory factors which include preformed granules containing histamine and proteases as well as *de novo* synthesized cytokines and arachidonic acid metabolites. Furthermore, mast cells can influence surrounding cells through both the production of soluble mediators and cell to cell contact, mediating both innate and adaptive immune responses[79-81]. Although mast cells are classically considered as a proinflammatory cell type, mounting evidence also suggests that these cells have immunomodulatory functions[398, 399]. The role of mast cells during bacterial infection *in vivo* has been studied in a variety of models using mast cell deficient mice or mast cell stabilizers[400-406]. In some models mast cell mediators have been implicated in neutrophil recruitment and function as well as bacterial clearance[15, 393]. However due to the diversity of immunomodulatory mediators produced by mast cells and the arsenal of Fc and pattern recognition receptors expressed on their surface, the contribution of mast cells to host immune responses is highly variable and often pathogen specific[407-409]. *In vivo*, whether mast cells modulate the host response to *P. aeruginosa* lung infection has not been reported previously.

Given the close spatial relationship between mast cells and the respiratory epithelium, and the importance of tightly regulated epithelial permeability during lung infections, we set out to examine whether mast cells influence airway epithelial integrity during *P. aeruginosa* lung infection. *P. aeruginosa* lung infection was induced in wild-type and mast cell deficient $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice. Increased epithelial permeability, bacterial dissemination, and an accumulation of neutrophil within the lungs of mast cell-deficient animals demonstrate a biologically critical role for mast cells in opposing

bacteria induced acute lung damage. Using an *in vitro* Transwell model we further confirmed that mast cells and mast cell-derived factors promote epithelial integrity. We further showed that mast cells reduced *P. aeruginosa*-mediated epithelial cell apoptosis and epithelial derived TNF production. Thus, mast cells contribute to maintaining epithelial integrity in the airways during *P. aeruginosa* lung infection.

5.3 Materials and Methods

Animals

Mast cell-deficient $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice and C57BL/6 mice were purchased from Charles River Laboratories (Saint Constant, QC). Mice received food and water *ad libitum* and were housed in pathogen free facilities at the IWK Health Centre animal facility. $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice were matched with C57BL/6 mice for age and sex. The protocols were approved by the University Committee on Laboratory Animals, Dalhousie University, in accordance with the guidelines of the Canadian Council on Animal Care.

Antibodies:

Rabbit anti-caspase 3 (#9661) was purchased from New England Biolabs (Whitby, ON). Rabbit anti-ZO-1 (402200), rabbit anti-occludin (404700) and Alexa 594 conjugated goat anti-rabbit IgG (A11072) were purchased from Life Technologies (Burlington, ON). Goat anti-actin (sc-1616), Horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (sc-2004) and HRP conjugated rabbit anti-goat IgG (sc-2768) were purchased from Santa-Cruz Biotechnology (Dallas, TX).

Bacterial preparation and infection of mice

P. aeruginosa mucoid strain 8821 was a gift from Dr. A. Chakrabarty (University of Illinois, Chicago, IL). *P. aeruginosa* were cultured as described previously[345]. Briefly, suspension cultures were grown until reaching the early stationery phase. Bacteria were washed in phosphate buffer and resuspended in saline. Mice were anesthetized and intranasally infected with *P. aeruginosa* strain 8821 (1×10^9 CFU/mouse) in 20 μL of total inoculums volumes followed by 10 μL saline.

Mice were sacrificed at various time points post infection and bronchoalveolar lavage fluid (BALF) was obtained by lavaging the lung with 3 × 1 mL phosphate buffer solution containing 100 μg/mL soybean trypsin inhibitor (Sigma Aldrich, St. Louis MO). Lung tissue was also collected and homogenized for detection of cytokines, as well as MPO activity and histology as described previously[107, 134].

Cell Culture

The human epithelial cell line 16HBE14o$^-$ cells were maintained on flasks coated with 10 μg/mL human fibronectin (BD Biosciences, Mississauga ON Canada), 100 μg/mL bovine serum albumin (Sigma Aldrich) and 30 μg/mL type I bovine collagen (BD Biosciences). For transwell experiments cells were grown on 0.4 μm transwell filters (Fisher Scientific, Ottawa ON), coated as described above, for 6-8 days at a density of 150000 cell/0.33 cm^2 filter in MEM containing 10% fetal bovine serum (FBS) and 50 U/mL of both penicillin and streptomycin. Confluence of the monolayers was confirmed by measurement of transepithelial electrical resistance (TEER). The human mast cell line HMC-1 was cultured in IMDM containing 10% FBS and 50 U/mL of both penicillin and streptomycin.

Highly purified cord blood-derived mast cells (CBMC) (> 95% purity) were obtained by long term culture of cord blood progenitor cells as previously described[248]. The percentage of mast cells in the cultures was determined by toluidine blue staining of cytocentrifuged samples. Mature mast cells after more than 8 weeks in culture were identified by their morphological features and the presence of metachromatic granules, at which time they were used for this study.

Cytokine and chemokine production

The concentration TNF in culture supernatants or cell free lung supernatants were determined by ELISA as previously described using antibody pairs from R&D Systems[107, 134].

Image analysis:

Area analysis was performed from TIFF images of monolayers stained for caspase-3 as described below. Unedited images captured using identical microscope settings were analyzed using Photoshop (Adobe, San Jose, CA).

Immunofluorescence Microscopy:

Immunofluorescence of 16HBE14o- cells grown on transwell filters was carried out as described previously with a few modifications[410]. Briefly cells were grown on transwell filters for 6-8 days at a density of 150000 cell/0.33cm^2 filter in DMEM containing 10% FBS. Confluence of the monolayers was confirmed by measurement of TEER. HMC-1 cells were added at a density of 5×10^5 cells/mL to the basolateral (bottom) chamber of half of the transwells in a total volume of 800 µL. The other half of

the wells received 800 µL of IMDM containing 10% FBS as a mast cell free control. The indicated number of *P. aeruginosa* bacteria were added to the apical (top) chambers of the transwells in 200 µL of IMDM containing 10% FBS. Twenty four hours later cells were fixed and permeabilized using BD Cytofix/Cytoperm according to the manufacturer's directions (BD Biosciences). Cells were blocked in 5% goat serum (Santa Cruz), and then incubated with the indicated primary antibodies, followed by Alexa 594 conjugated goat anti-rabbit IgG (Life Technologies). Images were captured using fluorescence microscopy (Nikon Eclipse E600; Nikon, Tokyo, Japan) or by confocal laser scanning microscopy (Zeiss LSM510, Zeiss, Toronto, ON).

***In vivo* epithelial permeability measurement**

Vascular and epithelial permeability in lungs of mice infected with *P. aeruginosa* was determined using Evan blue dye. Mice were intranasally infected with *P. aeruginosa* strain 8821 as described above. Eighteen hours before sacrifice mice were injected i.p. with 400 µL of 0.5 % Evan blue dye (Sigma Aldrich) in phosphate buffer solution. Twenty-four hours post-infection mice were sacrificed and serum, lung tissue and the first 1 mL wash of BALF were collected as described above. Serum was diluted 1/20 in phosphate buffer solution in all samples and used to calculate relative permeability indexes in individual mice. BALF and lung homogenates were centrifuged for 10 minutes at 18000g to remove cells and bacteria. Permeability changes were calculated as a ratio of optical density at 620 nm of BALF or lung supernatants to diluted serum.

***In vitro* epithelial permeability measurement**

16HBE14o$^-$ cells were grown on 0.4 μm transwell filters (Fisher Scientific) for 6-8 days at a density of 150000 cell/0.33mm filter in DMEM containing 10% FBS. Monolayers were considered confluent when TEER exceeded 600 Ohm*cm^2 at which point transwells were used for experiments. HMC-1 cells were added at a density of 5 × 10^5 cells/mL to the lower, basolateral chamber of half of the transwells in 24 well plate in a total volume of 800 μL. The other half of the wells received 800 μL of IMDM containing 10% FBS as a mast cell free control. The indicated number of *P. aeruginosa* bacteria were added to the apical (top) chambers of the transwells in 200 μL of IMDM containing 10% FBS. TEER was measured 24 hours later using an EVOM Epithelial Voltohmeter according to the manufacturer's instructions (World Precision Instruments, Sarasota, FL).

For the determination of monolayer permeability, cells were grown on transwell filters and treated as described above. Twenty four hours after bacterial inoculation, 5 μL of four kDa dextran conjugated to fluorescein isothiocyanate (FITC-dextran 4000, Sigma Aldrich) was added to the apical (top) chamber to achieve a final concentration of 5 mg/mL, and the chamber was incubated for 3 hours at 37^{o}C and 5% CO_2. After incubation, media from the bottom chambers were collected and fluorescence measured at 518nm using a fluorometer (Fluoroscan Ascent F, Thermo Scientific, Waltham MA). Transepithelial FITC-dextran 4000 diffusion was calculated using bare filter diffusion as 100%.

Mast cell reconstitution:

Mast cell-deficient $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice were reconstituted with bone marrow derived mast cells cultured from C57BL/6 mice as described previously[411]. Briefly, mice were reconstituted with 5×10^6 BMMCs in 200 μL RPMI delivered i.v. Animals were used for experiments 8 weeks after reconstitution. Reconstitution of mast cell-deficient $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice with BMMCs was confirmed by Alcian blue staining of paraffin embedded lung sections as described previously[411].

Statistics

Data are presented as mean ± SEM of the indicated number of experiments. Statistical significance between multiple treatments was determined by ANOVA and post-hoc Tukey's honest significance test. Alternatively significance between only two groups was determined using an unpaired t test.

Western Blot and Scanning Densitometry:

Western blot analysis and scanning densitometry was performed as described previously[412]. Briefly cell lysates (10–20 μg) were subjected to electrophoresis in 10% or 12% SDS-polyacrylamide gels. Gels were transferred to polyvinylidene difluoride membrane, blotted with primary and secondary antibodies as indicated, and detected by an enhanced chemiluminescence detection system (Western Lightning Plus-ECL; PerkinElmer, Waltham, MA). Scanning densitometry analysis was performed using Scion Image (Scion Corporation, Frederick, MD).

5.4 Results:

Mast cells oppose bronchial epithelium permeability changes induced by* P. aeruginosa *infection: In order to the examine the impact of mast cells on epithelial barrier function we established an *in vitro* model in which 16HBE14o- human bronchial epithelial cells were grown on 0.4 μm pore size transwell filters. The basolateral chambers of each well received either HMC-1 human mast cells, or media control. Monolayers were then infected apically with increasing doses of *P. aeruginosa* bacteria for 24 hours, after which epithelial permeability and barrier function were measured by transepithelial electrical resistance (TEER) (Figure 5.1A) and FITC-dextran diffusion (Figure 5.1 B) respectively. Monolayers displayed a dose dependent decrease in TEER, and a dose dependent increase in FITC-dextran diffusion indicating a loss of epithelial integrity and increased permeability. Importantly, changes in TEER and FITC-dextran diffusion were significantly decreased by the presence of basolateral mast cells, suggesting that mast cells play an important role in maintaining epithelial integrity following *P. aeruginosa* infection. Similar results were obtained using primary human cord blood derived mast cells from two separate donors demonstrating that this phenomenon is not restricted to the HMC-1 mast cell line (Figure 5.2).

Mast cell-deficient mice display increased epithelial permeability following* P. aeruginosa *lung infection: Increased epithelial permeability is a hallmark of acute lung injury. Having observed an essential role for mast cells in maintaining epithelial integrity *in vitro*, we next set out to explore the biological contribution of mast cells to epithelial

integrity *in vivo* following *P. aeruginosa* lung infection. Wild-type C57BL/6, $Kit^{W\text{-}sh}$/ $Kit^{W\text{-}sh}$ mast cell-deficient mice reconstituted with C57BL/6 bone marrow derived mast cells and $Kit^{W\text{-}sh}$/ $Kit^{W\text{-}sh}$ mast cell-deficient mice were infected intranasally with *P. aeruginosa* 8821, a mucoid strain isolated from the lungs of a cystic fibrosis patient [264]. Eight hours later mice were injected intraperitoneally with Evan blue dye. Twenty four hours post infection mice were sacrificed and leakage of the dye into the BALF was measured and expressed as a ratio to serum concentration in order to examine endothelial and epithelial permeability respectively (Figure 5.3 A). Mast cell-deficient mice displayed increased Evan's blue dye leakage in the BALF, supporting an important role for mast cells in maintaining epithelial integrity *in vivo* following *P. aeruginosa* infection. Reconstitution of mast cell-deficient mice with wild-type C57BL/6 BMMCs repopulated the lungs with mast cells (Figure 5.4), and was able to significantly decrease *P. aeruginosa* induced changes in epithelial permeability.

Intact respiratory epithelium plays an essential barrier function in the lung and prevents bacterial dissemination into the blood. In order to examine the biological impact of mast cells on epithelial barrier function wild-type and mast cell-deficient mice were infected intranasally with *P. aeruginosa* strain 8821. Dissemination of the bacteria into the blood stream was monitored 4, 24, 48 and 72 hours post infection (Figure 5.4 B). Few bacteria were isolated from the blood of wild-type animals following *P. aeruginosa* lung infection, suggesting the infection was effectively contained in the lung. In contrast, significant dissemination of the bacteria into the blood of mast cell-deficient mice was observed starting 24 hours post-infection. In spite of enhanced bacterial dissemination in mast cell-deficient animals, the numbers of bacteria in the blood eventually began to

decrease 72 hours post-infection, and no mortality was observed in wild-type or mast cell-deficient mice.

In order to ensure increased bacterial dissemination was due to the absence of mast cells in $Kit^{W\text{-}sh}$/ $Kit^{W\text{-}sh}$ mast cell-deficient mice, bacterial counts in the blood 24 hours post infection was monitored in wild-type C57BL/6, $Kit^{W\text{-}sh}$/$Kit^{W\text{-}sh}$ mast cell-deficient mice, and $Kit^{W\text{-}sh}$/$Kit^{W\text{-}sh}$ mast cell-deficient mice reconstituted with wild-type BMMCs (Figure 5.4 C). Bacterial dissemination was significantly reduced following reconstitution of $Kit^{W\text{-}sh}$/ $Kit^{W\text{-}sh}$ mast cell-deficient mice with BMMCs, demonstrating that impaired epithelial barrier function is due to the absence of mast cells.

Increased epithelial permeability also contributes to the increased accumulation of neutrophils in the lung[384]. In order to examine the impact of mast cell deficiency on neutrophil infiltration into the lungs wild-type and mast cell deficient mice were infected intranasally with *P. aeruginosa* strain 8821. Mice were sacrificed 4, 24, 48 or 72 hours later and lung tissue was collected and assayed for activity of the neutrophil specific MPO (Figure 5.5 A), or fixed for hematoxylin-eosin (H&E) staining (Figure 5.5 C). MPO activity was significantly increased in the lungs of mast cell-deficient mice at all time points post-infection compared to wild-type animals, and histological samples displayed significantly increased numbers of infiltrating leukocytes. Furthermore, as with epithelial permeability and bacterial dissemination, increased neutrophil infiltration into the lungs was restored to near wild-type levels in mast cell-deficient mice following reconstitution with wild-type mast cells (Figure 5.5 B). Together with observed effects on epithelial permeability and bacterial dissemination, these data suggest that mast cells

play a critical role in maintaining epithelial integrity following *P. aeruginosa* infection *in vivo*.

Enhanced epithelial integrity following* P. aeruginosa *infection is mediated by mast cell-derived factors. To further confirm a role of mast cells in promoting epithelial integrity, cell free supernatants were prepared from the basolateral chamber of 16HBE14o- transwells with or without HMC-1 cells, and apical *P. aeruginosa* stimulation for 24 hours by high speed centrifugation to remove residual mast cells. Additional cell free supernatants were prepared as described above from HMC-1 cells cultured in the presence or absence of *P. aeruginosa* for 24 hours. These supernatants contained mast cell secreted factors and were added to the basolateral chamber of 16HBE14o- transwells which were then infected apically with *P. aeruginosa* bacteria. The impact of each treatment on TEER (Figure 5.6 A) and FITC-dextran diffusion (Figure 5.6 B) were measured 24 hours later. All supernatants from samples containing HMC-1 cells were able to improve epithelial integrity to levels observed when mast cells were present. These results suggest that mast cell-derived factors are involved in maintaining epithelial integrity, and that stimulation with *P. aeruginosa* and epithelial co-culture are not required for the production of the mast cell product. Similar results were obtained with cell free supernatants from CBMC cells, and CBMC-16HBE14o- transwell co-culture supernatants prepared as described above, again demonstrating that this phenomenon is not restricted to the HMC-1 mast cell line (Figure 5.7).

Mast cells do not significantly impact tight junction integrity following* P. aeruginosa *infection: Mast cells have previously been implicated as key regulators of epithelial integrity in the intestine[94, 413, 414], where tight junction proteins have a prominent role in maintaining epithelial integrity. In order to determine if mast cells impact tight junction organization *in vitro* during *P. aeruginosa* infection, 16HBE14o- monolayers were grown on transwells filters until TEER was established. Half of the monolayers received HMC-1 mast cells in the basolateral chamber while the other half received media control. Cells were left untreated, or infected apically with increasing doses of *P. aeruginosa* bacteria for 24 hours, after which monolayers were fixed and stained for ZO-1 (Figure 5.8 A) or occludin (Figure 5.8 B) by immunofluorescence. A loss of tight junction integrity was observed following *P. aeruginosa* infection as indicated by the decreased localization of occludin and ZO-1 staining at cell interfaces. The presence of mast cells in the basolateral chamber had no significant impact on the localization of either tight junction protein. Lysates were also collected from transwell monolayers treated with increasing doses of *P. aeruginosa* in the presence or absence of mast cells, and were subjected to Western blot analysis for ZO-1 and occludin (Figure 5.8 C). Consistent with previous reports *P. aeruginosa* resulted in a dose dependent decrease in the levels of ZO-1 and occludin[24, 397]. However the presence of basolateral mast cells had little impact on the expression of either tight junction protein. Together these data demonstrate that mast cells do not affect tight junction protein localization or expression during *P. aeruginosa* infection.

Mast cells decrease* P. aeruginosa *induced epithelial cell apoptosis: *P. aeruginosa* has previously been shown to induce significant epithelial cell apoptosis via caspase-3

dependent mechanisms[385, 415]. Caspase-3 activation has been used as an apoptosis marker. We examined the impact of mast cell co-culture on *P. aeruginosa* induced epithelial cell apoptosis. 16HBE14o- human bronchial epithelial cells were grown on transwell filters until significant TEER was established. Basolateral chambers received either HMC-1 human mast cells or media control. Monolayers were left untreated, or infected apically with increasing doses of *P. aeruginosa* bacteria. Twenty four hours later monolayers were fixed and stained for active caspase-3 (Figure 5.9 A). Area analysis was used to quantify the percentage of apoptotic cells (Figure 5.9 B). Caspase-3 activation was further monitored by Western blot in cell lysates collected from transwell monolayers treated with increasing doses of *P. aeruginosa* in the presence or absence of basolateral mast cells (Figure 5.9 C and 5.9 D). A dose dependent activation of caspase-3 was observed following *P. aeruginosa* infection and was significantly reduced by co-culture with mast cells. These results suggest that mast cells reduce caspase-3 activation following *P. aeruginosa* infection.

Mast cells decrease epithelial derived TNF production following* P. aeruginosa *infection: TNF is a key mediator of both acute lung injury[416, 417] and apoptosis of bronchial epithelial cells in the lungs[418]. In order to determine the impact of mast cell co-culture on TNF production following *P. aeruginosa* infection apical supernatants were collected from 16HBE14o- cells grown on transwells and infected with increasing doses of *P. aeruginosa* bacteria in the presence or absence of basolateral mast cells. ELISA was used to assay apical secretion of TNF (Figure 5.10 A). Apical TNF levels increased in a dose dependent manner following *P. aeruginosa* infection. However co-culture with

basolateral mast cells was able to almost completely abrogate *P. aeruginosa* induced TNF production. Similar results were obtained *in vitro* using primary human CBMCs (Figure 5.2 C). *In vivo* mast cell-deficient $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice displayed significantly elevated levels of TNF in their lungs 48 and 72 hours post infection (Figure 5.10 B). Together these results suggest that mast cells reduce TNF production following *P. aeruginosa* lung infection.

5.5 Discussion

The epithelium is the first cellular barrier encountered by invading bacteria. In the lung the healthy epithelium with complex junctional structures allow for the selective passage of molecules and maintenance of well-defined polarized functions[419]. Dysfunctions in epithelial permeability affect bacterial dissemination and contribute to lung injury[420, 421]. *P. aeruginosa* is the leading cause of morbidity and mortality among cystic fibrosis patients[247, 381, 388, 389] and employs a range of strategies to disrupt host lung epithelial permeability[15, 24, 394-397]. How host factors involved in counteracting the pathogen-induced lung epithelial permeability changes is not entirely understood. In this study using an *in vitro* transwell co-culture model we found that mast cells and mast cell-derived factors reduced *P. aeruginosa*-induced increases in epithelial permeability. Using mast cell deficient $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice we confirmed that mast cell-deficiency led to increased lung epithelial permeability, increased *P. aeruginosa* dissemination into the blood stream and increased neutrophil infiltration into the lung. Further studies using the *in vitro* transwell co-culture model showed that mast cells decreased *P. aeruginosa*-induced epithelial cell apoptosis and epithelial derived TNF production. Together these findings demonstrate a previously unrecognized role for mast cells in maintaining the

integrity of the lung epithelium and opposing lung injury during *P. aeruginosa* lung infection.

The role of mast cells in acute lung injury has previously been addressed in a number of different models, which yielded conflicting results. In models of ischemia-reperfusion injury mast cell derived tryptase and mast cell protease 7 have been shown as key mediators of acute lung injury[116, 422], and pharmacological inhibition of mast cell function has been shown to reduce lung injury following liver transplantation[423]. A similar role for mast cells has also been proposed during H5N1 influenza infection where mast cell stabilizers reduced lung injury leading to reduced mortality *in vivo*[424]. These studies suggest that mast cells contribute to lung injury likely involves mast cell-derived proteinases. In contrast, mast cells have recently been shown to reduce acute lung injury in a model of pulmonary LPS challenge[425]. In this model local production of TGF-β1 stimulated production of IL-6 from mast cells which induced neutrophil apoptosis, and decreased acute lung injury[425]. Similarly, we found that mast cells reduce *P. aeruginosa*-induced lung injury *in vitro* and *in vivo*. However, our findings point towards and alternative mechanism of action as we did not see any differences in the levels of TGF-β1 or IL-6 in the lungs of the mast cell-deficient animals when compared to wild-type animals following *P. aeruginosa* lung infection (data not shown). Furthermore, we were able to replicate mast cell-mediated improvements in epithelial integrity using an *in vitro* transwell model in which no neutrophils were present. Hence, our findings demonstrate for the first time that mast cells directly oppose acute lung injury during *P. aeruginosa* lung infection through maintaining epithelial integrity.

In addition, our study highlights the divergent biological roles of mast cells at different mucosal surfaces throughout the body. Mast cells arrive at mucosal sites as immature precursor cells, and only complete their differentiation once recruited to the tissue in which they become resident [70]. As a result mast cell phenotypes are extremely plastic, and are largely determined by the biochemical nature of the tissue in which they reside[71-74]. In the current study we demonstrated a previously unrecognized role for mast cells in opposing changes in epithelial permeability in the lung. These findings stand in contrast to extensive research demonstrating that mast cell proteases are responsible for the increasing epithelial permeability in the intestines in response to diverse stimuli[77, 93, 94, 258, 344, 395, 396]. It is noteworthy that mast cells do not secrete proteases in response to stimulation with bacterial products[426, 427]. In addition, we and others[95] found that unlike in the intestines mast cells do not impact tight junction integrity in the lungs. Thus, our results highlight a unique and protective role of mast cells in regulating epithelial permeability in the lung.

The localization of mast cells to mucosal interfaces ideally positions them to be sentinel cells of the immune system, recognizing and coordinating immune responses against invading pathogens. In order to effectively respond to diverse infectious agents, mast cell possess a wide array of pattern recognition and Fc receptors, and are capable of rapidly producing a plethora of bactericidal, pro-inflammatory and immunomodulatory mediators[78]. As a result, the role of mast cells during infections is highly pathogen specific. For example, during *Klebsiella pneumoniae* lung infection mast cells are required for neutrophil recruitment and bacterial clearance[401]. In contrast, during *Mycoplasma pulmonis* lung infection mast cell-deficient mice showed a significant and

persistent accumulation of neutrophils, suggesting that mast cells dampen neutrophil recruitment in the lung[398]. Recently, Chan *et al* described a broad immunosuppressive role for mast cells in the bladder during *E. coli* infection[425]. Taken together with the observed role for mast cells in protecting against *P. aeruginosa* induced acute lung damage, these findings demonstrate the ability of mast cells to respond to lung infections with a pathogen specific and biologically essential immune responses.

Our results appear stand in contrast with other groups who used the mast cell stabilizer cromolyn to inhibit mast cell function in rats treated with LPS from *P. aeruginosa*[82]. Administration of cromolyn reduced LPS-induced neutrophil accumulation in the rat lung, suggesting that mast cells promote LPS-induced lung inflammation[82]. These differences can be explained by the fact that cromolyn has selective effects on some aspects of mast cell functions such as degranulation but has limited effects on others such as cytokine and chemokine production[428]. Furthermore, in addition to its partial effects on mast cells, cromolyn also has non-specific effects on other cell responses and has been reported to directly affect neutrophil activity[429]. In addition, due to the complex interplay of the arsenal of virulence factors expressed by *P. aeruginosa*, LPS alone may display very different effects in the lungs than those seen during infection with whole bacteria. These differences in experimental models, and the fact that here we used live infection, may explain the discrepancies observed. These studies provide interesting insights into the divergent roles of mast cells in response to various pathogenic stimuli, and highlight the complexity of the role of mast cells during *P. aeruginosa* lung infection.

The current study demonstrates a previously unappreciated and biologically important role for mast cells in opposing bacteria induced acute lung injury during *P. aeruginosa* lung infection. The absence of mast cells resulted in increased epithelial permeability, an increased accumulation of neutrophils within the parenchyma of the lung, and significant dissemination of bacteria from the site of infection. Although the nature of secreted mast cell factors responsible for counteracting bacteria induced changes in epithelial permeability remains elusive, we have provided a mechanistic insight demonstrating the ability of mast cells to suppress caspase-3 activation and TNF production by the respiratory epithelium in response to stimulation with *P. aeruginosa*.

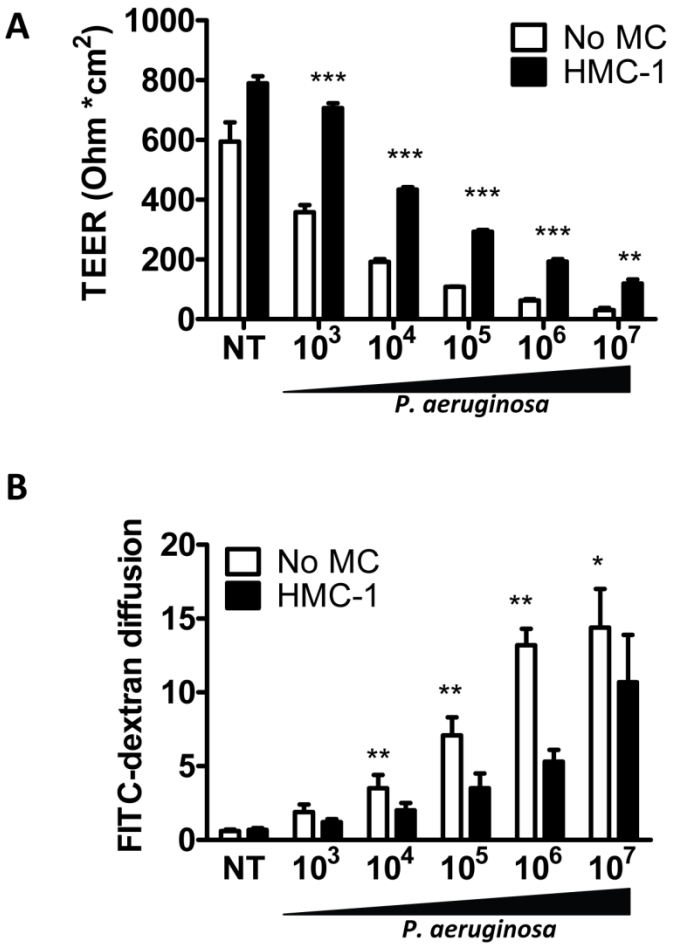

Figure 5.1: Mast cells prevent bronchial epithelium permeability changes induced by *P. aeruginosa in vitro*. 16HBE14o- human bronchial epithelial cells were grown to confluence on 0.4 μm pore size transwell filter. Half of the wells received basolateral HMC-1 cells at a density of 5×10^5 cells/mL in a total volume of 800 μL, while the remaining wells received only media as a no mast cell control. Monolayers were infected apically with increasing doses of *P. aeruginosa* as indicated. Transepithelial electrical resistance was measured 24 hours later. (A). Alternatively, cells were grown and treated as described above then twenty four hours after bacterial inoculation, 5μl of FITC-dextran 4000 was added to each apical chamber and incubated for 3 hours. A sample of fixed volume from the basolateral chambers was collected and fluorescence was measured (B). Data are represented as a percentage of FITC-dextran 4000 diffusion across bare filter (n=5 ± SEM, *$p < 0.05$, **$p<0.01$, ***$p < 0.005$).

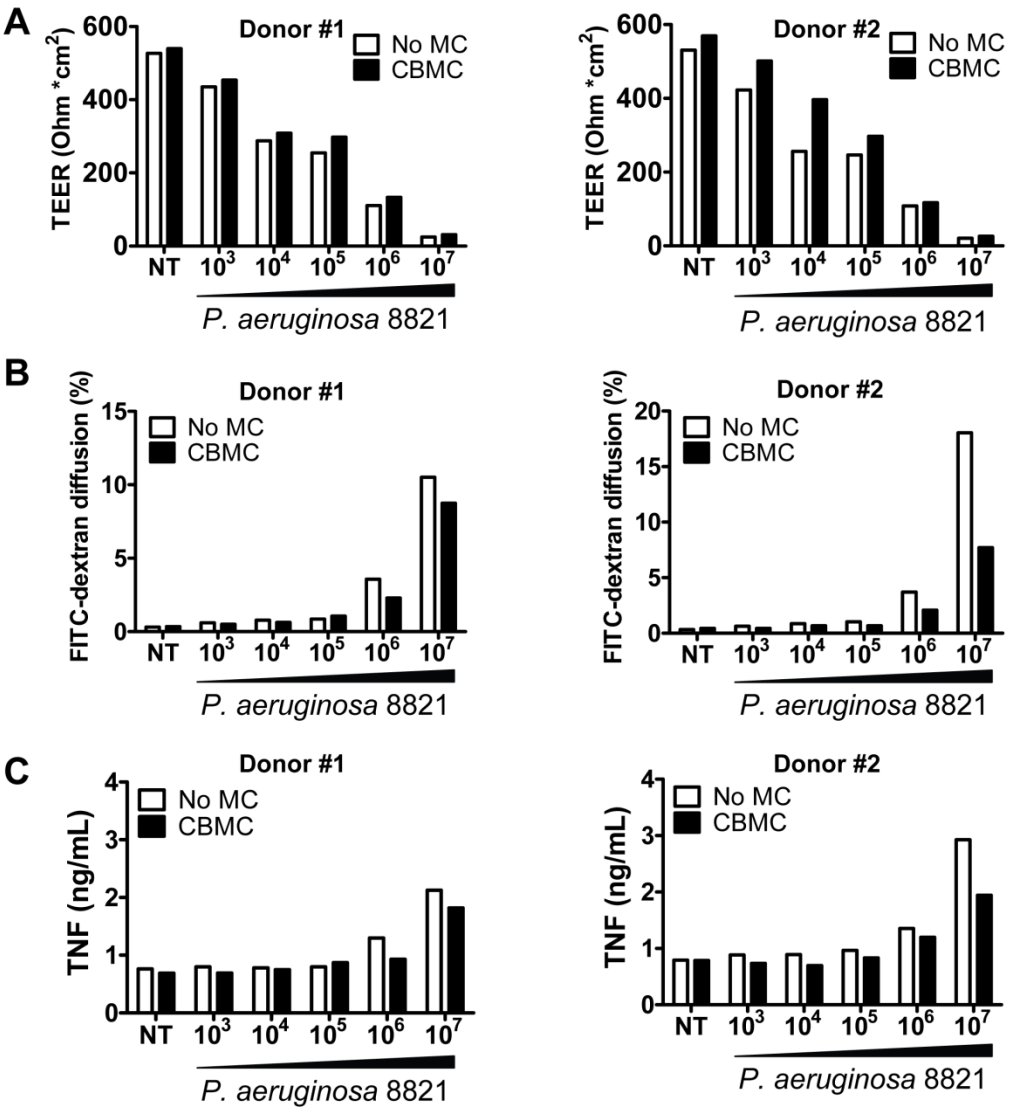

Figure 5.2: CBMCs promote epithelial integrity during *P. aeruginosa* infection- HBE cells were grown to confluence on transwells for 6 days. Epithelial integrity was confirmed using TEER. Basolateral wells were filled with 800 µL CBMC cells from two separate donors at a density of 0.5 x 10^6 cells/mL in RPMI, or received 800 µL of RPMI as an no CBMC control. Monolayers were then left uninfected (NT) or infected with increasing doses of *P. aeruginosa* strain 8821 in the apical chamber. Twenty one hours later 10 µL of FITC-Dextan was added apically. Twenty four hours post infection TEER was measured (A), then basolateral and apical supernatants were collected to measure FITC-Dextran diffusion (B) and TNF production respectively (C).

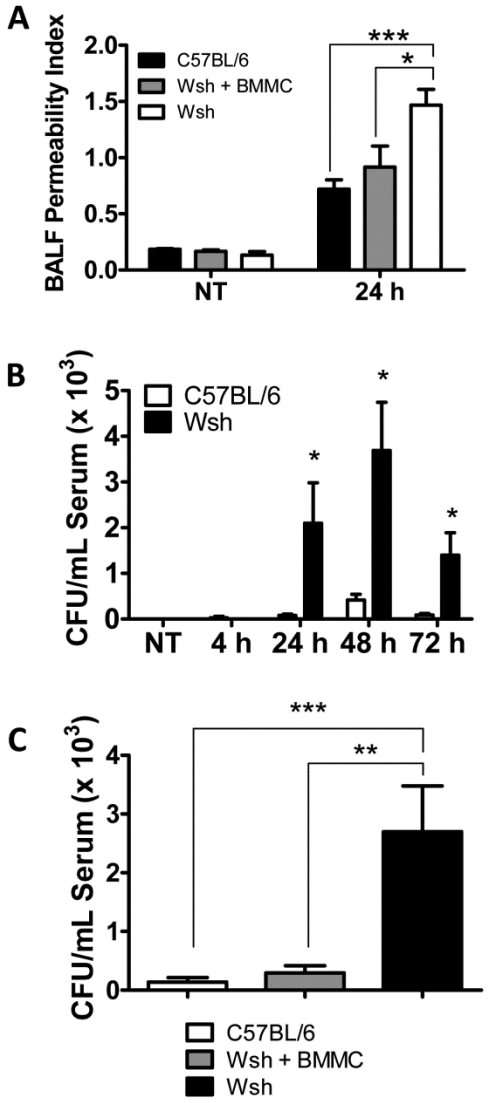

Figure 5.3: Mast cell-deficient mice displayed increased lung epithelial permeability and bacterial dissemination following *P. aeruginosa* lung infection. C57BL/6, $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ reconstituted with C57BL/6 BMMCs (Wsh + BMMC) and $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ (Wsh) mice were left uninfected (NT, n=3) or infected intranasally with 10^9 *P. aeruginosa* strain 8821 for 24 hours (24 h, n=8-18). Eighteen hours prior to sacrifice mice received intraperitoneal injections of Evan blue dye. Dye leakage into the BALF was assessed and is presented as the $OD_{620\ nm}$ ratio of BALF to a 1:20 dilution of serum (permeability index) (A). Serum was also cultured overnight to determine bacterial dissemination into the blood (C). Alternatively C57BL/6 and $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ (Wsh) mice were infected intranasally with 10^9 *P. aeruginosa* strain 8821 then serum was collected and cultured 4, 24, 48 and 72 hours post infection to monitor bacterial dissemination (B) (n=10). All data are present as mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$).

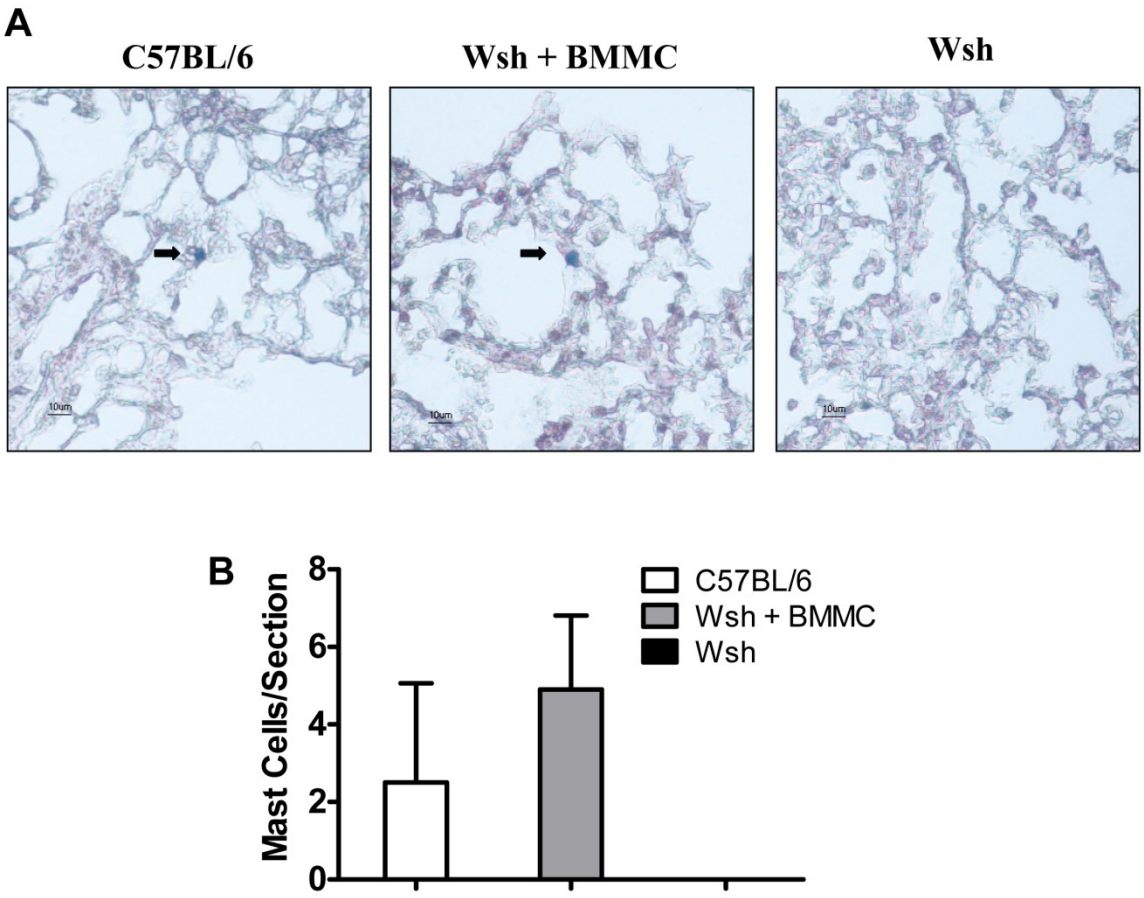

Figure 5.4: Reconstitution of Wsh mice with BMMCs restores mast cell numbers in the lungs: Lungs from C57BL/6, mast cell-deficient Kit^{Wsh}/Kit^{Wsh} mice (Wsh), or mast cell-deficient Kit^{Wsh}/Kit^{Wsh} mice reconstituted with BMMCs (Wsh + BMMCs) were collected 10 weeks post-reconstitutions. Lung samples were fixed in Carnoy's fixative and mast cells were stained with Alcian blue (200 x magnification, scale bars indicate 10 µm) (A). Mast cells are identified with arrows. The number of mast cells per lung section was counted (B). (n=6-8 ± SD).

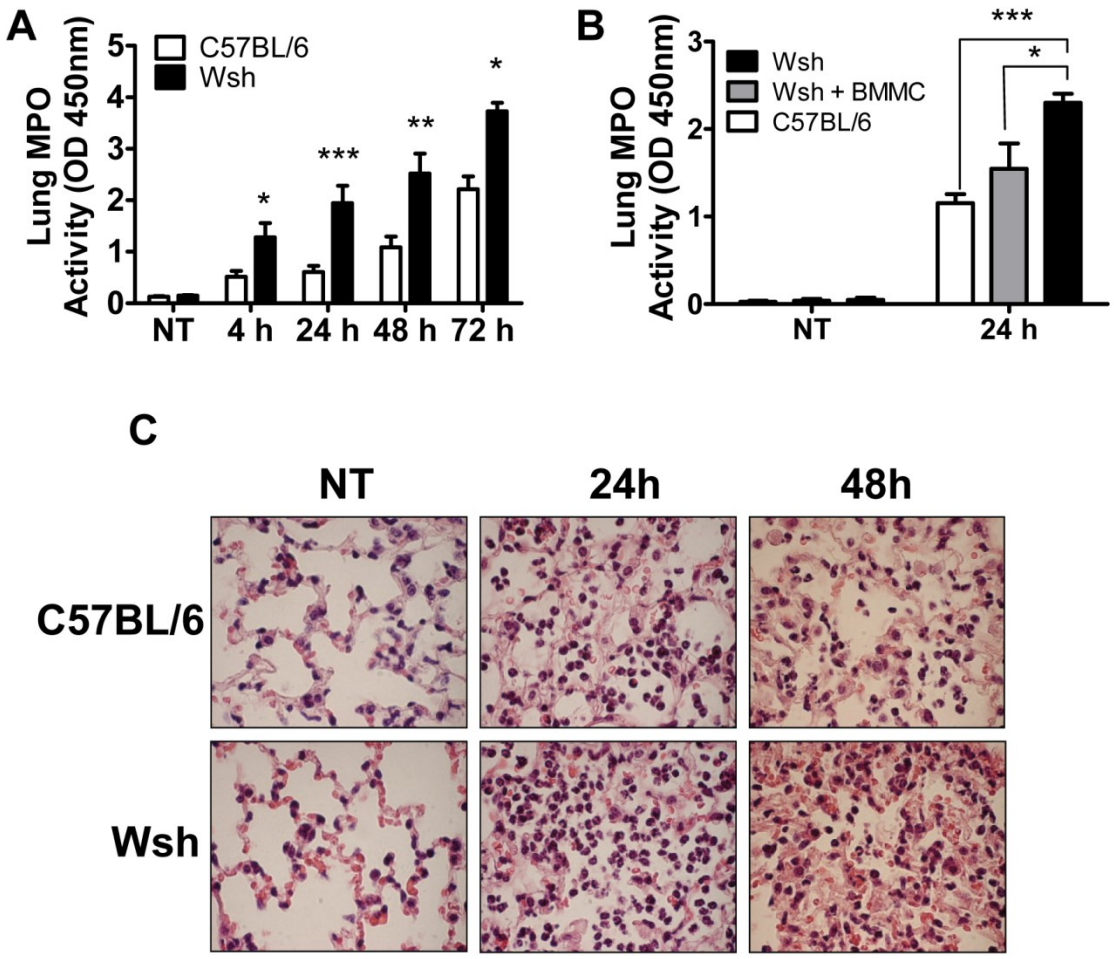

Figure 5.5: Mast cell deficient mice display enhanced neutrophil recruitment in their lungs following *P. aeruginosa* lung infection. C57BL/6 and $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ (Wsh) mice were intranasally infected with *P. aeruginosa* (strain 8821, 10^9 CFU/mouse) or left untreated (NT). After 4, 24, 48 or 72 hours mice were sacrificed and neutrophil accumulation in the right lung (A) was measured using an MPO assay (n = 8 ± SEM. *p<0.05, **p<0.01,*p<0.001). The upper lobe of the left lung was also collected at each time point for hematoxylin-eosin staining (×100), and greater leukocyte infiltration in lungs of $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice is apparent (C). Alternatively C57BL/6, $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ reconstituted with C57BL/6 BMMCs (Wsh + BMMC) and $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ (Wsh) were left uninfected (NT) or infected as described above for 24 hours after which neutrophil infiltration into the lungs (B) was assessed using an MPO assay (n=8 ± SEM, *p < 0.05, ***p < 0.005).

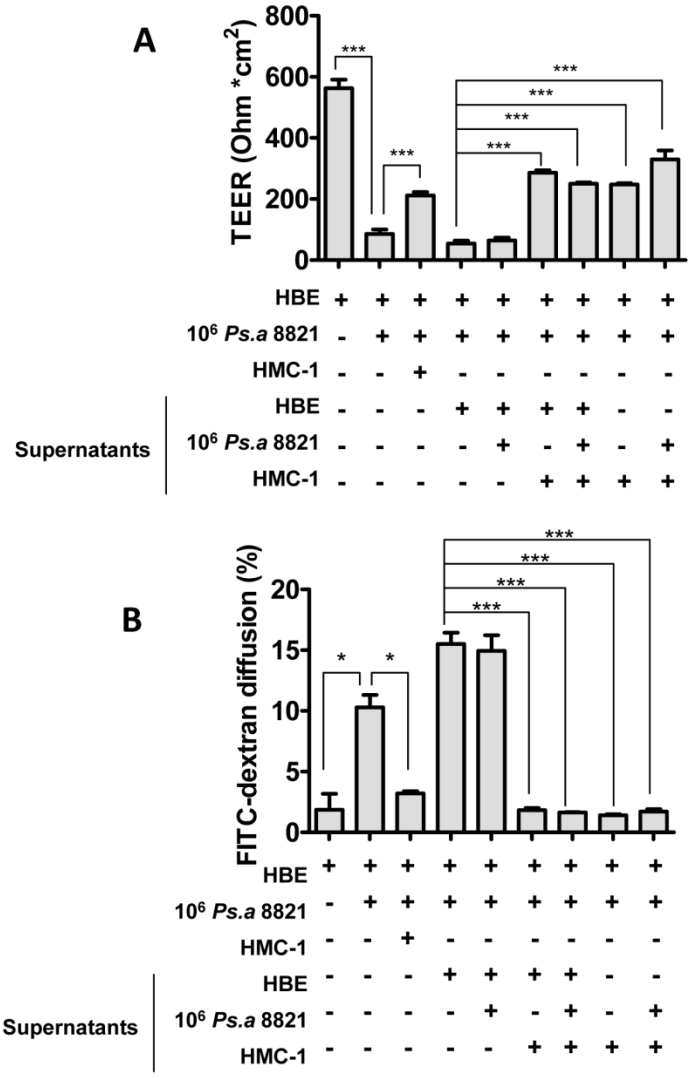

Figure 5.6: Enhanced epithelial integrity following *P. aeruginosa* infection is mediated by secreted mast cell factors. 16HBE14o- cells were grown to confluence on transwells for 6 days. Epithelial integrity was confirmed using TEER. Basolateral wells received 800 µL of IMDM, HMC-1 cells at a density of 0.5 x 10^6 cells/mL, or the indicated cell free supernatants. Cell free supernatants were prepared from the basolateral chambers of 16HBE14o- transwells in the presence or absence of HMC-1 cells and apical infection with 1 × 10^6 *P. aeruginosa* strain 8821 for 24 hours. Alternatively cell free supernatants were collected from HMC-1 cells incubated directly with *P. aeruginosa* strain 8821 at a 1:10 MOI for 24 hours. Supernatants were cleared of cells by high speed centrifugation. Monolayers were left uninfected, or infected with 1×10^6 CFU *P. aeruginosa* strain 8821 in the apical chamber. Twenty four hours later 5 µL of FITC-Dextran was added apically. Twenty four hours post infection TEER was measured (A), then basolateral supernatants were collected and assayed for FITC-Dextran diffusion (B). (n=4 ± SEM, *$p < 0.05$, ***$p < 0.005$)

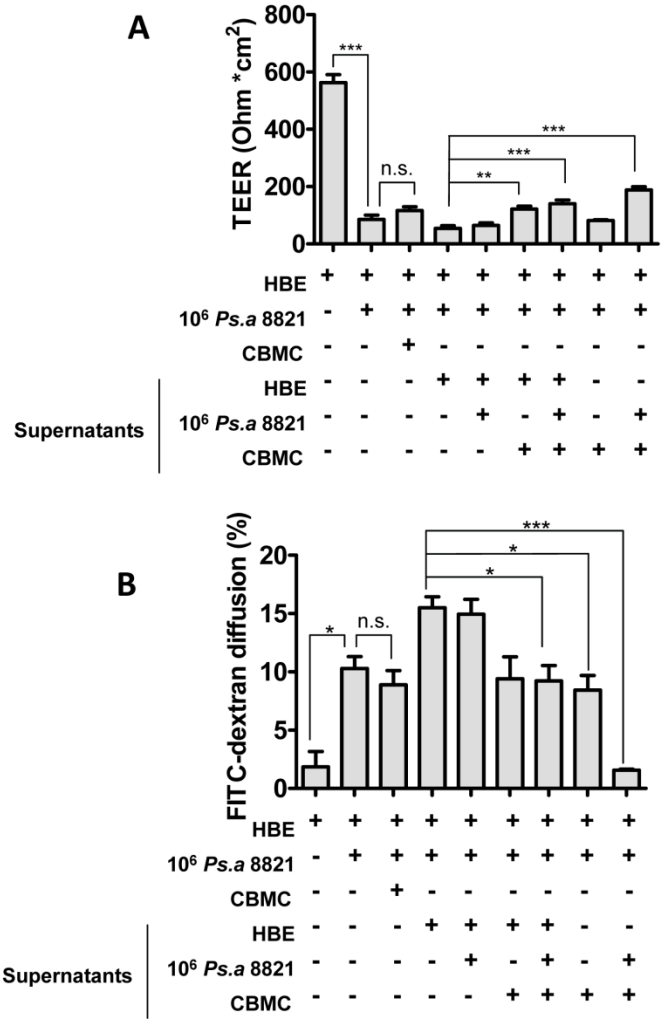

Figure 5.7: Enhanced epithelial integrity following *P. aeruginosa* infection is mediated secreted primary mast cell factors. 16HBE14o- cells were grown to confluence on transwells for 6 days. Epithelial integrity was confirmed using TEER. Basolateral wells received 800 µL of RPMI, CBMC cells at a density of 0.5 x 10^6 cells/mL, or the indicated cleared basolateral supernatant. Monolayers were left uninfected, or infected with 10^6 *P. aeruginosa* strain 8821 in the apical chamber. Twenty one hours later 10 µL of FITC-Dextran was added apically. Twenty four hours post infection TEER was measured (A), then basolateral supernatants were collected and assayed for FITC-Dextran diffusion (B). (n=3 ± SEM, *p < 0.05, **p < 0.01, ***p < 0.005)

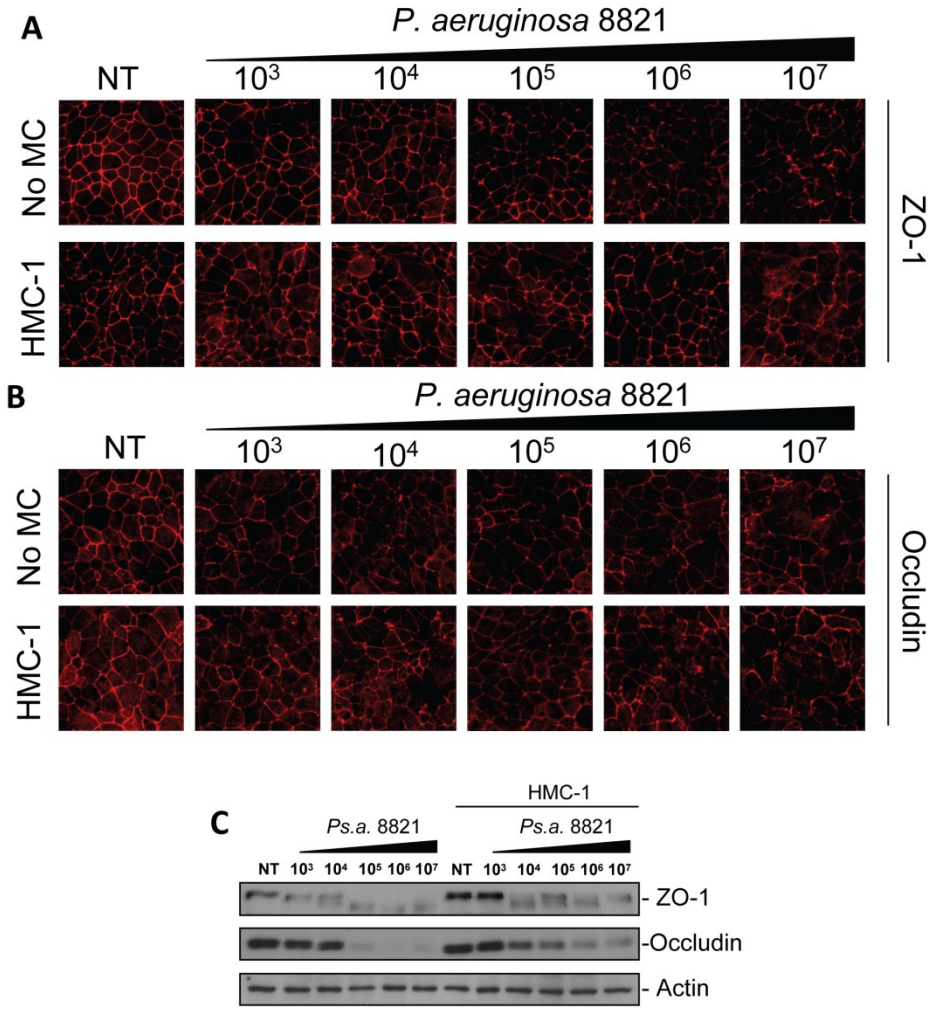

Figure 5.8: Mast cells do not significantly impact tight junction integrity following *P. aeruginosa* infection. 16HBE14o- cells were grown to confluence on transwells for 6 days. Epithelial integrity was confirmed using TEER. Basolateral wells received 800 µL of IMDM or HMC-1 cells at a density of 0.5 x 10^6 cells/mL. Monolayers were left uninfected (NT), or infected with increasing infectious doses of *P. aeruginosa* strain 8821 in the apical chamber. Twenty four hours later cells were fixed and strained for ZO-1 (A) or occludin (B) and imaged by confocal microscopy. Alternatively cells were grown and treated as described above, then lysates were prepared and subjected to Western blot analysis for ZO-1, occludin and actin (C). Representative images of 2 or 3 independent experiments shown.

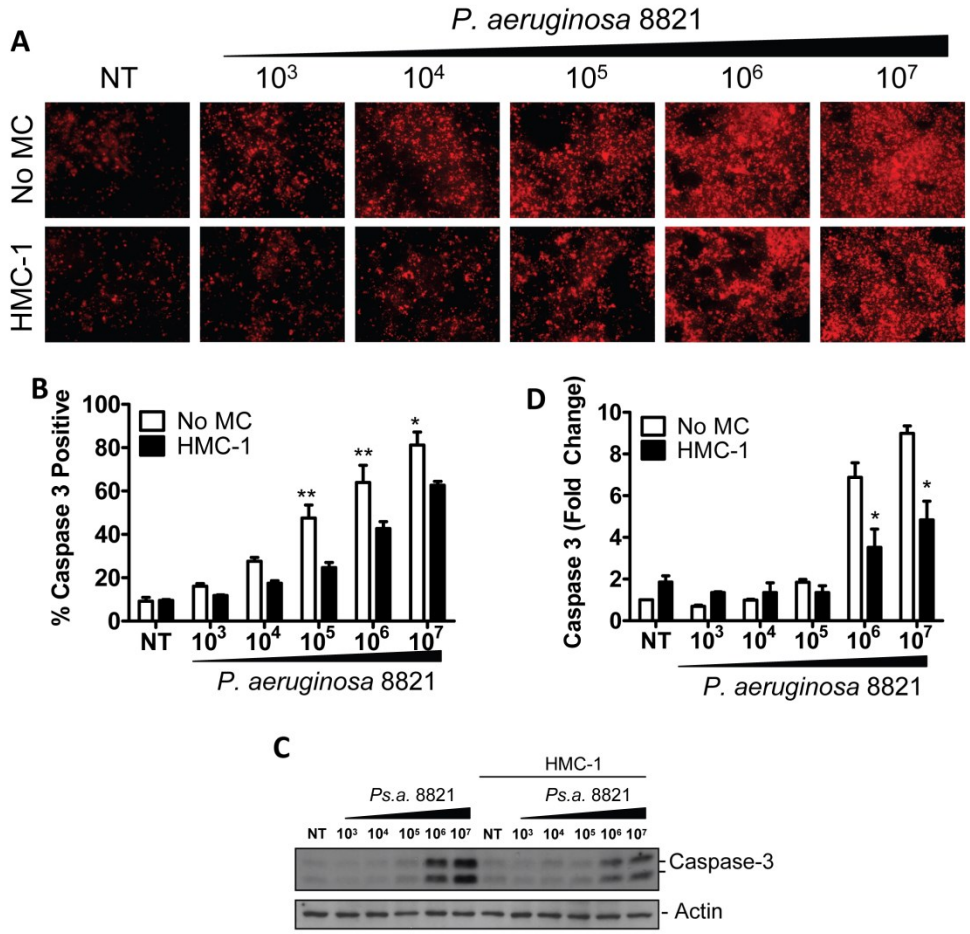

Figure 5.9: Mast cell co-culture decreases *P. aeruginosa* induced caspase-3 activity in bronchial epithelial cells: 16HBE14o- cells were grown to confluence on transwells for 6 days. Epithelial integrity was confirmed using TEER. Basolateral wells received 800 µL of IMDM or HMC-1 cells at a density of 0.5 x 10^6 cells/mL. Monolayers were left uninfected (NT), or infected with increasing doses of *P. aeruginosa* strain 8821 in the apical chamber. Twenty four hours later cells were fixed and stained for active caspase 3 (A). Area analysis was used to determine the percentage of caspase-3 positive cells (B). Alternatively cells were grown and treated as above, then cell lysates were prepared and subjected to Western blot analysis for active caspase-3 (C). Results were quantified using scanning densitometry (D). (n=3 ± SEM, *$p < 0.05$, **$p < 0.01$)

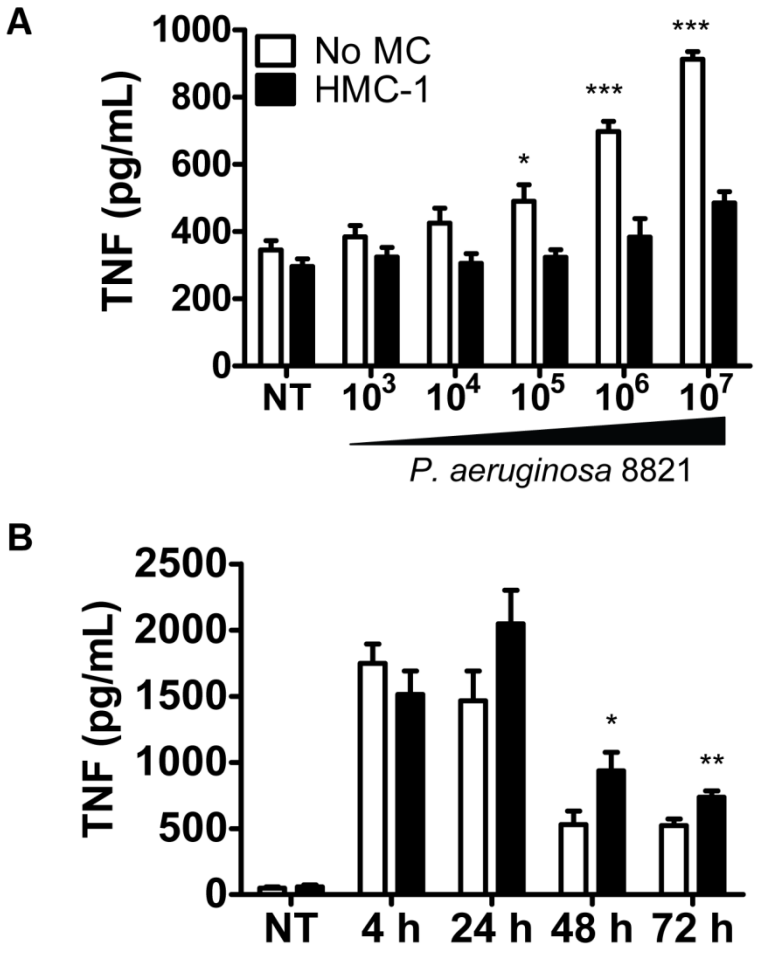

Figure 5.10: Mast cells decrease TNF production following *P. aeruginosa* infection *in vitro* and *in vivo:* 16HBE14o- cells were grown to confluence on transwells for 6 days. Epithelial integrity was confirmed using TEER. Basolateral wells received 800 µL of IMDM or HMC-1 cells at a density of 0.5 x 10^6 cells/mL. Monolayers were left uninfected (NT), or infected with increasing doses of *P. aeruginosa* strain 8821 in the apical chamber. Twenty four hours later apical supernatants were collected and TNF production was assessed by ELISA (A) (n=5±SEM, *p < 0.05, ***p < 0.005). Alternatively mice were intranasally infected with *P. aeruginosa* (strain 8821, 10^9 CFU/mouse) or left untreated (NT). After 4, 24, 48 or 72 hours animals were sacrificed, and cell free lung supernatants were collected and used to determine TNF concentrations by ELISA (B) (n=8 ± SEM, *p < 0.05, **p < 0.01).

CHAPTER 6: DISCUSSION

The emerging potential of autophagy based therapies in the treatment of cystic fibrosis lung infections

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Author contributions:

Robert D. Junkins: Conceived, wrote and edited manuscript. Designed and prepared figures.

Craig McCormick: Contributed to writing and editing manuscript

Tong-Jun Lin: Contributed to writing and editing manuscript

6.1 The emerging protential of autophagy based therapies in the treatment of cystic fibrosis lung infections

6.1.1 Abstract:

Cystic fibrosis (CF) is caused by mutations in the CF transmembrane conductance regulator (CFTR), a channel that normally transports anions across epithelial cell membranes. The most common manifestation of CF is buildup of mucus in the airways and bacterial colonization of the lower respiratory tract, accompanied by chronic inflammation. Antibiotics are used to control CF-associated opportunistic infections, but a risk of lengthy antibiotic treatment is the emergence of multiple drug resistant (MDR) strains. New antimicrobial strategies are needed to prevent and treat infections in these high-risk individuals. Autophagy contributes to the control of a variety of microbial infections. For this reason, the recent discovery of functional impairment of autophagy in CF provides a new basis for understanding susceptibility to severe infections. Herein, we review the role of autophagy in host defense against CF-associated bacterial and fungal pathogens, and survey pharmacologic approaches to restore normal autophagy function in these individuals. Autophagy restoration therapy may improve pathogen clearance and mitigate lung inflammation in CF airways.

6.1.2 Introduction:

Lung damage secondary to chronic infections is the leading cause of morbidity and mortality among patients with cystic fibrosis (CF). Antibiotics are commonly used to prevent and treat CF-associated infections, and have a generally positive track record with respect to improving quality of life and life expectancy. Unfortunately, prolonged antibiotic use can cause undesirable clinical outcomes including the creation of a niche for fungal pathogens which prove extremely difficult to treat, and the emergence of multiple drug resistant (MDR) strains of common CF-associated pathogens.[430, 431] The emergence of MDR strains threatens to undermine the advances made in CF treatment over the past thirty years. For this reason, new therapeutic approaches are needed to prevent and treat CF-associated lung infections.

Etiologic mutations in the CFTR/CF transmembrane conductance regulator contribute to defective immunity and increased lung pathology through a number of mechanisms. Chief among these is osmotic dysregulation in the airway, which results in the accumulation of thick mucus at the surface of respiratory epithelial cells, leading to impairment of pathogen clearance, disruption of TLR/toll like receptor signaling pathways, and dysregulated inflammatory responses. Protein aggregate accumulation has also been observed in CF airways and a recent landmark study by Luciani, *et. al.* provided striking molecular evidence that etiologic CFTR mutations trigger a cascade of events that culminate in BECN1/beclin-1 depletion and impairment of autophagy.[246] Because autophagy plays an important role in the control of a variety of microbial infections, the functional impairment of autophagy is undoubtedly a significant risk factor for opportunistic infections in CF airways.

Autophagy is an evolutionarily conserved catabolic process through which portions of the cytosol are sequestered and degraded within specialized double membrane bound vesicles termed autophagosomes. Over the past decade autophagy has emerged as a central component of the innate and adaptive immune responses where it plays roles in antigen presentation including cross-presentation, direct and indirect killing of intracellular and extracellular pathogens and the generation of bactericidal peptides.[432] Autophagy has also emerged as a central regulator of inflammatory responses where it plays roles in modulating inflammasome activation, NFκB activity and interferon production. A growing number of intracellular pathogens have been shown to be specifically targeted to the autophagosome for lysosomal degradation through a process known as xenophagy[433]. Furthermore, a distinct phenomenon called LC3-associated phagocytosis (LAP) has been implicated in the control of both extracellular and intracellular pathogens. LAP involves many components of the canonical macroautophagy pathway including ATG5, ATG7, LC3 and the BECN1 PtdIns3K complex,[228] but it does not involve the formation of a double membrane bound autophagosome. Instead LAP facilitates processing of phagosomes containing TLR4-, 6-, 9-, or T-cell immunoglobulin mucin 4-associated ligands through the recruitment of LC3 to the phagosomal membrane and delivery of cargo to lysosomes.

There is evidence for autophagy dysregulation in a variety of disease states, including cancer, neurodegenerative diseases, infectious diseases and autoimmune disorders. For this reason, therapeutic modulation of autophagy is of great interest. Perturbations of autophagy in CF airways suggest that therapeutic strategies aimed at restoring normal autophagy may help prevent and treat CF-associated opportunistic

infections. We recently reported a previously unrecognized role for autophagy in host defense against the CF-associated pathogen *P. aeruginosa*, and demonstrated that pharmacological induction of autophagy could enhance clearance of the pathogen *in vitro* and *in vivo*.[412] These findings highlight a growing body of evidence that supports the therapeutic potential of autophagy-inducing compounds, or 'autophagy restoration therapy' in combating CF-associated opportunistic infections.

6.1.3 Cystic fibrosis as a disorder associated with impaired autophagy:

CF is the most common life-threatening genetic disease in North America and Europe, afflicting approximately 1 in every 3600 live births.[434] CF is caused by mutations in the CFTR gene that disrupt anion channel activity. Oxidative stress is a hallmark of CF airways, and recent work has shown that etiologic mutations in CFTR increase the levels of intracellular reactive oxygen species (ROS). One consequence of increased ROS in airway epithelial cells is enhanced activity of TG2/tissue transglutaminase 2, a calcium-dependent enzyme that creates intra- or intermolecular covalent bonds between proteins by conjugating a reaction between the ε-amino group of a lysine residue and a γ-carboxamide group of a glutamine residue.[244] In cells bearing etiologic mutations in CFTR, normal ubiquitination and proteasomal degradation of TG2 is inhibited by ROS-dependent small ubiquitin like-modifier (SUMO)ylation mediated by PIAS4/protein inhibitor of activated STAT, 4.[244, 245] Inhibited turnover of TG2, combined with high intracellular concentrations of calcium, drive aberrant TG2 activity that crosslinks target proteins and results in aggresome formation. Many TG2 substrates have been identified, but one substrate that directly impacts autophagy is BECN1. TG2-mediated cross-linking

causes sequestration of BECN1 and its accumulation in HDAC6/histone deacetylase 6-, SQSTM1- and ubiquitin-containing cytoplasmic aggresomes. BECN1 sequestration in aggresomes results in the dislodgement of class III PtdIns3K complexes from the endoplasmic reticulum, thereby inhibiting autophagy.[246]

It is not yet known precisely how dysfunctional autophagy leads to the acquisition and persistence of opportunistic lung infections in CF patients, but the functional impairment of autophagy would be expected to undermine innate host defenses by compromising xenophagy and LAP. Therapies targeted at restoring autophagy in cells and animals harboring CFTR mutations restore trafficking of mutant CFTR to the plasma membrane,[246] attenuate hyperinflammatory responses,[291] and promote clearance of CF associated pathogens.[412, 11,272] Although the precise molecular details have not yet been fully elucidated, it appears that pharmacologic induction of autophagy may be a useful therapeutic option for CF, and one that may not so easily be thwarted by the emergence of resistance.

6.1.4 The role of autophagy in host defense against common CF associated pathogens:

It has long been recognized that individuals with CF have defects in immunity that render them susceptible to a variety of opportunistic airway infections. Accumulating evidence indicates that autophagy defects could underlie increased susceptibility to infection with certain microbes in CF, particularly those that establish intracellular infections which would normally be cleared by autophagy.[254] Our current knowledge

regarding the role of autophagy in host defense against a variety of CF-associated pathogens is summarized below (Figure 6.1.1).

6.1.4.1 *Pseudomonas aeruginosa:*

P. aeruginosa is the second most common pathogen isolated from CF airways, and MDR strains now infect approximately 10% of all CF patients[430, 431], underscoring the need for novel therapeutics. Although largely considered an extracellular pathogen, *P. aeruginosa* can invade host airway epithelial cells where the bacteria can reside for extended periods of time.[67] It has been proposed that this intracellular phase of infection may be involved in the development of antibiotic resistance and the acquisition of biofilm-like properties which aid the establishment of chronic infection.[67]

In light of these findings, we recently explored the therapeutic potential of pharmacological induction of autophagy *in vitro* and *in vivo* in the treatment of acute *P. aeruginosa* lung infection.[412] We demonstrated *in vitro* that clearance of intracellular bacteria from human airway epithelial cells was significantly enhanced through induction of autophagy with the mTOR inhibitor rapamycin. Similar observations were made in myeloid-lineage cells that play prominent roles in airway immune responses, alveolar macrophages,[255] and mast cells,[412] suggesting that autophagy represents a critical component of the innate immune response against *P. aeruginosa*. Furthermore, we observed that cells harboring the most common mutation in CF patients, ΔF508 CFTR, displayed impaired autophagic responses and failed to clear infection unless pre-treated with rapamycin, consistent with the notion that defects in autophagy in CF airways can be pharmacologically reversed. We further demonstrated that pretreatment with rapamycin was able to enhance bacterial clearance in a model of acute *P. aeruginosa*

lung infection *in vivo*. Further work will be required to determine whether pharmacological induction of autophagy will be equally effective in combating established *P. aeruginosa* infections.

The precise role of autophagy in host defense against *P. aeruginosa* remains to be elucidated. *P. aeruginosa* has a type III secretion system that delivers effector proteins into the host cell, including ExoS, an enzyme that inactivates a variety of target host proteins by ADP-ribosylation. ExoS targets include Rab5,[66] a small GTPase essential for phagolysosome maturation and autophagosome formation.[435] Thus, ExoS permits invasive *P. aeruginosa* to avoid acidified compartments in epithelial cells, promoting survival.[436] Our studies demonstrated that *P. aeruginosa* countermeasures could be overcome by rapamycin treatment, but the underlying mechanism of clearance remains obscure. By electron microscopy, we observed bacteria that had clearly been taken up into double membrane bound vesicles characteristic of autophagosomes, but these observations were infrequent, suggesting that xenophagy may not significantly contribute to *P. aeruginosa* clearance. It is possible that the enhanced killing of intracellular *P. aeruginosa* following induction of autophagy is actually mediated primarily through LAP, and xenophagy represents a relatively less common event. Our work suggests that *P. aeruginosa* ExoS activity can be at least partially overcome by rapamycin treatment *in vivo* and in cultured of airway epithelial cells and mast cells. Although the mechanistic details regarding the role of autophagy in host defense against *P. aeruginosa* remain to be defined, correcting defects in the autophagy pathway associated with defective CFTR has the potential to restore both xenophagy and LAP, since both processes depend on BECN1-class III PtdIns3K complexes.

6.1.4.2 *Burkholderia cepacia*

B. cepacia is an opportunistic bacterial pathogen which is capable of causing both extracellular and intracellular infections of host epithelial cells and macrophages. Although *B. cepacia* infections are not particularly common in CF patients, afflicting 3-5% of the population,[430, 431] they are extremely difficult to treat due to multi-drug resistance, and because hyperinflammatory responses triggered by the infection accelerate deterioration of pulmonary function, and in some cases lead to fatal necrotizing pneumonia.

The role of autophagy in host defense against *B. cepacia* was recently addressed by Abdulrahman *et. al.*[272] It was found that *B. cepacia* becomes targeted to autophagosomes in wild-type macrophages, but not macrophages harboring ΔF508 CFTR mutations, and that the recruitment of the bacteria to these structures targets them for lysosomal degradation. Killing of *B. cepacia* via autophagy could be enhanced through pharmacological induction of the pathway with rapamycin both *in vitro* and *in vivo*. Critically, rapamycin was also able to reduce *B. cepacia* induced lung inflammation in a CF mouse model, suggesting that autophagy therapy can both promote clearance of the bacteria from the lungs, and suppress the damaging inflammation responsible for deteriorating lung function and necrotizing pneumonia in CF patients. Considering the unavailability of conventional antibiotic therapies for *B. cepacia* infections, and encouraging pre-clinical results, autophagy-inducing drugs represent a promising therapeutic option.

6.1.4.3 Non-tuberculosis mycobacterium (NTM)

NTM infections are a growing concern among CF populations due to their increasing prevalence, MDR nature, and because infection is often associated with poor clinical outcomes. Current estimates suggest that NTM strains infect between 5-22% of CF patients.[437] Among these infections the predominant pathogens were found to be the slow-growing *Mycobacterium avium* complex (MAC) and the fast-growing *Mycobacterium abscessus*. As with *Mycobacterium tuberculosis* (Mtb), the ability to replicate and survive within host cells after infection is a critical determinant of NTM virulence.[438]

Compared to the well-studied role of autophagy in host defense against Mtb, relatively little is known about the role of autophagy during NTM infections. Our only insight comes from a study that noted that prolonged treatment with the antibiotic azithromycin inhibited autophagy and predisposed patients to infection with *M. abscessus.*[439] In this study impaired autophagy led to decreased clearance of *M. abscessus* from infected macrophages, and the establishment of chronic NTM infection. These findings suggest that as with Mtb, NTM can be targeted for degradation by autophagy. However it is important to note that this study did not address the role of autophagy in host defense against *M. avium* or other NTM strains and further research will be required to fully understand the therapeutic implications of autophagy-inducing drugs in the treatment of these infections.

6.1.4.4 *Haemophilus influenzae*

H. influenzae is a small Gram negative bacterial pathogen which includes both encapsulated and unencapsulated strains, the latter of which are referred to as nontypeable *Haemophilus influenzae* (NTHi).[440] NTHi often establishes biofilms within

the lower respiratory tract to chronically colonize the airways of CF patients at a very young age. Although the pathological effects of NTHi infection in CF patients remain incompletely understood, it is thought that the inflammation caused by these infections may predispose the host to later infection with *P. aeruginosa.*[436]

Currently the role of autophagy during host defense against NTHi infection remains undefined. However, a recent report has demonstrated that NTHi can establish long-term intracellular infection of human airway epithelial cells where it remains metabolically active, but non-proliferative.[441] It has been reported that NTHi bacteria persist in single membrane bound vesicles that co-localize with markers of the late endosome, but no co-localization with autophagosomal markers was observed, leading to speculation that autophagy may be actively subverted by as-yet-uncharacterized mechanisms.

6.1.4.5 *Aspergillus fumigatus:*

Although the majority of CF-associated infections are caused by bacterial pathogens, the rate of fungal infections with *A. fumigatus* have been steadily increasing and currently affect approximately 20% of all CF patients.[430] These infections are difficult to treat with conventional antifungal medications, and are associated with profound inflammation in the lungs. In healthy individuals *A. fumigatus* spores are phagocytosed and degraded by alveolar macrophages, neutrophils and monocytes. *A. fumigatus* has not been shown to invade host cells, but the autophagosomal marker LC3 is recruited to *A. fumigatus*-containing phagosomes, and the recruitment of this protein is essential for effective killing of internalized spores both *in vitro* and *in vivo.*[442] These findings suggest that novel CF therapeutics designed to augment or restore autophagy

may effectively target both bacterial and fungal opportunistic infections, achieving 'cross-kingdom killing'.

It is important to note that the spores did not appear to become incorporated into classical autophagosomes in this study. Instead, spores were recruited in an ATG5-dependent fashion to LC3-II-decorated single membrane bound phagosomes, strongly implicating LAP in spore clearance. The impact of etiologic CFTR mutations on LAP remains incompletely defined, but would be predicted to impair anti-fungal defense. Further study will be required to better understand the role of autophagy defects in the susceptibility to *A. fumigatus* infection in CF airways, and to explore the potential of autophagy promoting therapies in the treatment of fungal infections.

6.1.5 *Staphylococcus aureus*: A complication for CF autophagy restoration therapy?

Staphylococcus aureus is the single most common CF-associated opportunistic infection, colonizing between 50-68% of the population.[430, 431] The rate of colonization with methicillin resistant *Staphylococcus aureus* (MRSA) has skyrocketed over the past decade from just 2% of the CF population in 2001 to more than 25% of the same population in 2010.[431] MRSA infections in CF patients are associated with decreased lung function, frequent hospitalization, and increased mortality.

S. aureus is capable of establishing intracellular infections,[443] but there is no evidence that it is targeted for autophagic degradation.[444] Instead, the bacterium escapes the phagocytic pathway and transits to the autophagosome where it prevents lysosomal fusion to establish a replicative niche. Defects in autophagy have previously been reported to prevent *S. aureus* intracellular replication and host cell death.[444] For these reasons, it is curious that *S. aureus* can so efficiently colonize the CF airway, which

clearly has severe impairment of autophagy. A possible explanation for this apparently contradictory data comes from recent reports that demonstrate induction of LC3 conversion and envelopment of bacteria in LC3-positive vesicles is independent of BECN1 and PIK3C3, and instead proceeds through a non-canonical autophagy pathway induced by the *S. aureus* pore forming toxin α-hemolysin and regulated by intracellular levels of cAMP.[435] These results raise the possibility that defects associated with BECN1 activity in CF may not impact *S. aureus* induced autophagy. Indeed, other studies have demonstrated that *S. aureus* can reside and replicate within the cytosol of airway epithelial cells, and that this process is enhanced by mutations in the CFTR.[445] Thus, the role of autophagy in CF-associated *S. aureus* infections remains incompletely defined.

Without a more complete understanding of how defects in autophagy induced by mutations in CFTR impact *S. aureus* infection it is difficult to predict how pharmacological manipulation of the pathway might affect the course of infection. Given the ability of intracellular *S. aureus* to commandeer the autophagy pathway to create a replicative niche, it is uncertain whether autophagy-inducing therapy will improve clearance of the bacteria. Furthermore, it will be important to monitor whether the pharmacological induction of autophagy will accelerate *S. aureus*-mediated lung damage. *In vitro* evidence suggests that induction of autophagy with rapamycin increases numbers of intracellular bacteria, and enhances *S. aureus*-mediated host cell death.[444] However these effects were only seen at extremely high doses of rapamycin (80 μg/mL) that far exceeded realistic dosage for *in vivo* studies, and lower doses had no impact on the course of infection.

6.1.6 Autophagy based therapies

Many of the microbes associated with opportunistic CF airway infections have evolved mechanisms to undermine phagolysosomal maturation and/or autophagy, suggesting that these are potent barriers to be overcome. Reinforcing autophagy via rapamycin treatment has shown some efficacy in promoting clearance of certain microbes *in vitro* and *in vivo*, and the exploration of alternative autophagy-inducing drugs is in the early stages. With the recent elucidation of a mechanism underlying defective autophagy in CF airways, and the identification of a drug, cystamine, that can reverse these effects, there is a unique opportunity to regain control over opportunistic infections in CF. What follows is a brief summary of candidate therapeutic approaches intended to restore autophagy in CF airways. (Figure 6.1.2).

6.1.6.1 Non-specific inducers of autophagy

One possible approach to treating CF-associated autophagy defects with the aim of improving pathogen clearance and decreasing inflammation in the airways is to employ potent autophagy inducers in an attempt to force newly translated BECN1 into an active PtdIns3K complex before it can be functionally sequestered into HDAC6-positive aggresomes by aberrant TG2 activity. Therapeutic manipulation of autophagy has become a topic of considerable interest as research has revealed tantalizing links between autophagy and the etiology of many high-priority human diseases. Unfortunately, a limited selection of autophagy-inducing drugs is currently available, many of which suffer from undesirable off-target effects. Current autophagy inducers can be grouped into three classes: i) mechanistic target of rapamycin (mTOR) inhibitors, ii) modulators of calcium dependent signaling or iii) IP3 inhibitors.[446] Beyond their respective roles in

controlling autophagy, each of these targets play integral roles in multiple aspects of cell growth and homeostasis, making them poor targets for the specific induction of autophagy. This issue is highlighted in work exploring the ability of rapamycin, a well characterized mTOR inhibitor and potent inducer of autophagy, to enhance host defense against CF associated pathogens. Rapamycin has been shown to promote clearance of the CF associated pathogens *P. aeruginosa* and *B. cepacia* both *in vitro* and *in vivo*.[67, 272, 412, 447] However in addition to its ability to promote autophagy, rapamycin also has well characterized immunosuppressive effects. These effects could potentially be beneficial in the treatment of CF-associated lung infection as pathogen-induced lung inflammation is an important cause of decreased lung function in CF patients, and was significantly reduced following rapamycin therapy *in vivo*.[272, 412] However the use of immunosuppressive drugs to treat infections could also negatively impact the ability of the patient to fight the infection, and increase their susceptibility to other opportunistic infections. In addition rapamycin has been associated with significant lung toxicity in transplant recipients.[66] As a result rapamycin represents a poor candidate for autophagy inducing therapy in the treatment of CF associated lung infections, and other, more specific autophagy inducers are urgently needed.

To this end, a small molecule screen of 3717 FDA-approved drugs was recently performed in order to identify novel inducers of autophagy.[448] Among the most effective of these compounds were the anti-psychotic drugs bromperidol, metergoline, thioridazine and chlorpromazine. The authors demonstrated that these newly identified autophagy inducers were able to significantly reduce IFNγ/interferon-γ and LPS induced IL-1β/interleukin-1β production from macrophages, enhanced localization of intracellular

Salmonella to LC3 positive structures resulting in enhanced bacterial killing, promoted T_{reg} expansion and decreased T_H17 expansion *in vitro*. Previous screens have also identified other anti-psychotic drugs which were able to induce autophagy and effectively control mycobacterial infections.[449] The psychoactive nature of these compounds, and their potentially life threatening side effects, limit their utility, but this work nevertheless provides a strong theoretical basis for future drug development.

A similar drug screen for therapeutic compounds which improve prion disease outcomes unexpectedly identified the second generation selective histamine H_1-receptor antagonist astmeizole as a potent inducer of autophagy at biologically achievable concentrations.[450] Astemizole significantly improved survival outcomes in a mouse model of prion disease demonstrating efficacy *in vivo*. Although the mechanism through which astemizole modulates autophagy, and the ability of astemizole to restore autophagy in CF remain entirely uncharacterized, it represents an attractive option for autophagy induction therapy in CF associated lung infections due to its previously reported anti-fungal[451] and anti-malaria[452] effects. Although not widely used in North America and Europe today due to the availability of superior next-generation histamine receptor antagonists, astemizole has an extremely well characterized safety and drug interaction profile. In fact, other than the extremely rare occurrence of cardiac arrhythmias associated with astemizole overdose, the drug appears to be relatively safe, and suitable for long-term administration, with appropriate medical supervision.

Fundamental studies of autophagy have also recently led to the development of a novel cell-permeable peptide activator of autophagy.[294] This peptide was derived from BECN1 and fused to the HIV Tat protein transduction domain; it functions by binding to

a newly identified negative regulator of autophagy known as GLIPR2/Gli Pathogenesis-Related 2, and relieving autophagy suppression. Tat-BECN1 peptide has been demonstrated to selectively induce autophagy, improve clearance of protein aggregates, and improve survival outcomes following viral infection *in vivo*. Although the impact of the peptide has not been tested in a CF model, rescuing BECN1 expression or activity can effectively restore normal autophagy in cells harboring mutation in CFTR, suggesting that this peptide may have some utility in the treatment CF-associated lung infections.[246]

6.1.6.2 Antioxidant therapy

CF is now well established as a disease characterized by extensive systemic and pulmonary oxidative stress, caused in part by dysregulation of glutathione (GSH) homeostasis and poor adsorption of alimentary antioxidants, which has led to considerable interest in the use of antioxidant therapy in the treatment of the disease (reviewed in[243]). Interestingly, in addition to contributing to lung tissue damage, oxidative stress also appears to play a role in defective autophagy in the airways of CF patients. Excessive generation of ROS in the lungs of CF patients has been proposed as a mechanism underlying the crosslinking and functional sequestration of BECN1 by TG2. Increased TG2 activity in CF also leads to additional ROS production, creating a feed-forward loop that drives inflammation, tissue damage, and continued dysregulation of the autophagy pathway. For these reasons, antioxidant therapy could significantly reduce inflammation and promote clearance of CF associated lung infections.

The most well studied form of CF antioxidant therapy explored to date is direct supplementation with inhaled GSH, or N-acetylcysteine (NAC) which can either be inhaled or delivered orally. The aim is to restore levels of GSH in the lungs to those seen

in healthy individuals, where it represents a critical component of antioxidant defenses. Intraperitoneal administration of NAC has been shown to decrease TG2 SUMOylation, and restore normal levels of autophagy in two CF mouse models, supporting the therapeutic potential of antioxidant therapy in the treatment of CF associated infections.[246] Small clinical trials have demonstrated that these therapies are safe and generate significant increases in lung GSH levels, and are associated with positive clinical outcomes including decreased lung inflammation, but their efficacy remains to be proven in larger randomized trials. To this end a number of phase II clinical trials are currently underway in the United States and Europe.

Other antioxidant-based therapies have focused on supplementation with various alimentary factors essential for antioxidant defense that are poorly absorbed by CF patients. These factors include the lipid soluble factors vitamin E, carotenoids, coenzyme Q-10 and assorted fatty acids, as well as the hydrosoluble factors such as vitamin C, selenium, zinc and copper. Although preclinical data using these strategies has been promising, to date little is known about their clinical efficacy.

6.1.6.3 TG2 inhibitors:

The most intellectually satisfying way to restore autophagy in CF airways is to design a therapeutic strategy that specifically corrects the defect in autophagy that arises from dysfunctional CFTR. The potential application of the TG2 inhibitor cystamine in CF patients has recently been reviewed.[453] Cystamine has been shown to restore normal autophagy in CFTR deficient cells and mouse models.[246, 454] In addition cystamine has been shown to restore normal trafficking of ΔF508-CFTR and stabilized expression of the protein at the plasma membrane.[454] The beneficial effects of cystamine treatment

persist for extended periods of time after cystamine treatment has ceased, suggesting that once the physiological effects associated with CFTR dysfunction have been corrected, a self-sustaining homeostasis may be re-established.[454] As a result cystamine represents a promising avenue for treating opportunistic infections and associated physiological defects in the CF airway.

TG2 inhibitors are the subject of intense investigation for their potential utility for CF and neurological diseases like Huntington's disease and Alzheimers disease.[455] Importantly, unlike compounds intended for use in neurological disorders, TG2 inhibitors suitable for use in CF patients will not need to cross the blood brain barrier. As a result, a re-evaluation of TG2 inhibitory compounds abandoned previously for their inability to enter the CNS is warranted.

One common approach used to specifically target TG2 is the chemical modification of natural TG2 substrates like carbobenzyloxy-L-glutaminylglycine (Cbz-gln-gly). Gluten peptide analogues have also been designed as TG2 inhibitors, the most promising of which have been dubbed Z006 and ZDON. These inhibitors bind irreversibly in the active site of the enzyme, with promising results *in vitro*, although their efficacy *in vivo* has not yet been thoroughly examined. Finally, recent screens have identified a host of compounds including LDN 27219, the Janus kinase 3 inhibitor ZM39923, its metabolite ZM449829, tyrphostin 47, and vitamin K3, as potent inhibitors of TG2. Again the *in vivo* efficacy of these compounds remains to be proven. However the continued efforts to design potent and specific TG2 inhibitors could provide exciting opportunities over the coming years to disrupt aberrant BECN1 aggresome formation,

restoring normal autophagy and potentially significantly ameliorating persistent infections and inflammation in CF airways.

6.1.6.4 SUMOylation inhibitors:

TG2 SUMOylation is essential for enhanced enzyme activity and protein levels in the airways of CF patients.[245] Elevated levels of ROS lead to greatly increased expression of PIAS4, the SUMO E3 ligase responsible for TG2 SUMOylation. Disruption of the SUMOylation machinery has been shown to be sufficient to restore TG2 homeostasis in CF cell lines providing a theoretical basis for the use of SUMOylation inhibitors in the treatment of CF associated lung infections. A number of SUMOylation inhibitors have been identified to date, including ginkgolic acid and spectomycin B1, which target SUMO E1 and E2 intermediates respectively. Although the theoretical basis for anti-SUMOylation therapy has been established in many human diseases, the ultimate applicability of such therapies remains to be proven. SUMOylation is a critical regulator of multiple aspects of cell biology including nuclear-cytosolic protein transport, transcription regulation, protein stability and degradation, stress responses and cell cycle progression. Furthermore, SUMOylation has been implicated both in the normal progression of, and the dysregulation of CFTR biogenesis and quality control.[456] Due to these essential roles of SUMOylation in various cellular processes including CFTR processing, direct inhibition of the pathway may be associated with considerable off target effects. An alternative drugable target may instead be PIAS4, the enzyme responsible for aberrant SUMOylation of TG2 in CF airways. Although no specific PIAS4 inhibitors have been identified to date, gene silencing of the enzyme is sufficient to restore TG2 homeostasis *in vitro*. As a result this therapeutic approach to the

treatment of CF associated lung infections, as well as the development of specific PIAS4 inhibitors warrant further consideration.

6.1.6.5 Aggresome inhibitors:

A key event in the disruption of the autophagy pathway in CF airways is the formation of insoluble, HDAC6 positive aggresomes that functionally sequester the BECN1 PI3K complex. Thus, preventing aggresome formation may help to maintain the active pool of BECN1. Evidence for the use of HDAC6 inhibitors to prevent aggresome formation can be found from studies demonstrating that siRNA silencing of HDAC6 abolishes aggresome formation in pancreatic cancer cells.[457] Although first generation HDAC6 inhibitors such as tubacin and tubastatin were unsuitable for clinical trials, the recent description of second generation inhibitors such as N-hydroxy-4-(2-[(2-hydroxyehtyl)(phenyl)amino]-2-oxoethyl)benzamide (HPOB)[458] and ACY-1215[459] paves the way for selective inhibition of aggresome formation *in vivo*.

Although HDAC inhibitors may aid in restoring BECN1 homeostasis in the airways of CF patients, there are a number of other factors that need to be taken into account when considering the therapeutic potential of such interventions. First, although specific HDAC6 inhibitors have not yet been tested clinically, other HDAC inhibitors are associated with considerable hematological and gastrointestinal toxicity in cancer patients. Furthermore, HDAC inhibitors have been shown to suppress the innate immune response, both by inhibiting inflammatory responses, and by increasing susceptibility to bacterial and fungal infections.[460] Finally, HDAC6 has been implicated in autophagosome-lysosome fusion, and HDAC6 deficiency leads to autophagosome maturation failure and aggresome accumulation.[461] Hence inhibition of HDAC6 may lead

to secondary disruption of the autophagy pathway, and further increase susceptibility to infection in the airways of CF patients. As a result future investigation will be essential in order to determine the efficacy and safety of HDAC6 inhibitors in the treatment of autophagy defects associated with CF.

6.1.7 Conclusions:

The characterization of etiologic defects in autophagy associated with CFTR mutations has greatly advanced our understanding of immune dysfunction in CF. Although much work remains to be done to fully elucidate the role of autophagy in the control of myriad CF-associated pathogens, early studies suggest that 'autophagy restoration therapy' is worthy of thorough investigation. Proof-of-principle studies have demonstrated rapamycin-induced clearance of CF-associated pathogens and concomitant decreased pathogen induced lung inflammation. However, a complex interplay exists between both pathogenic and non-pathogenic commensal microorganisms in CF airways. For this reason, a systematic survey of the microbiome of normal and CF airways is warranted, and candidate autophagy-inducing drugs will have to be carefully evaluated for their impact on pathogens and benign commensal organisms.

Although the paucity of specific autophagy-inducing drugs limits the clinical potential of this treatment strategy at this time, the nature of the defects leading to impaired autophagy in CF provides a variety of potential targets for therapeutic intervention. Many of the autophagy restoring therapeutic strategies outlined above could be employed in combination with conventional antibiotic therapies to target intracellular and extracellular pathogens simultaneously, accelerating eradication of opportunistic infections in CF airways.

6.2 Co-regulation of NFAT and NFκB pathways

The NFAT and NFκB pathways are classically considered to play distinct roles in innate and adaptive immunity. To this end each transcription factor is regulated through unique canonical signaling pathways. However recent evidence in the literature, combined with the findings presented herein have elucidated considerable crosstalk between the NFAT and NFκB, and suggest cooperation between these pathways during innate immunity.

Regulation of NFκB via calcineurin dependent mechanisms is relatively widely reported in the literature, but remains only partially characterized[462]. During T cell receptor (TCR) signaling calcineurin plays a biologically important role in NFκB activation[190, 463, 464] where it mediates formation of a Carma-1, Bcl-10, Malt-1 complex which destabilizes inhibitory IκB-NFκB[465, 466]. Calcineurin dependent activation of NFκB has also been reported in macrophages[467, 468]. The first report involves a pathway leading to activation of NFκB downstream of CD11b and is dependent upon the regulatory calcineurin B subunit[468]. The other reported calcineurin dependent pathway for NFκB activation in macrophages involves enhanced degradation of IκB through interactions between calcineurin and the 20S proteasome component PSMA6[467].

Conversely, the regulation of NFAT activity by components of the NFκB signaling pathway is poorly characterized. In osteoclasts, which share progenitors with macrophages, calcineurin-independent activation of NFATc family members has been reported to occur via the MAP3K Tpl-2 which is activated during NFκB signaling[368]. Similarly a PKCzeta-Tpl-2 dependent signaling pathway for calcineurin independent activation of NFAT has been reported in T-cells[376, 469-471]. Calcineurin independent

activation of NFAT in skeletal muscle[472] and Foxp3+ regulatory T cells[473] has also been reported, although the later remains controversial[474] and the contribution of the NFκB signaling pathways to the activation of NFAT in these cell types is unknown.

Calcineurin independent activation of NFAT in macrophages, and other cells of the innate immune system remains entirely uncharacterized. Unexpectedly, during our investigation into the role of CnAβ in host defense against *P. aeruginosa* lung infection it was discovered that NFAT is activated following bacterial infection in macrophages in a calcineurin independent fashion. CnAβ-deficiency and pharmacological inhibition of calcineurin with CsA did not impact *P. aeruginosa* induced NFAT activity. Surprisingly, this NFAT activation was instead mediated through IKK activity, and was insensitive to Tpl-2 kinase inhibitor. These findings suggest that NFAT, as well as NFκB are activated directly downstream of the IKK complex in macrophages, and raise the possibility that NFAT activation during innate, and cellular immunity may be mediated through distinct signaling pathways.

However, in spite of the apparent existence of calcineurin-independent signaling pathways *in vitro*, CnAβ-deficiency was associated with partial impairment of NFAT and NFκB activity following *P. aeruginosa* lung infection, suggesting that both calcineurin dependent, and independent signaling mechanisms contribute to NFAT activity *in vivo*. One possibility is that activation of these pathways is cell type specific, as has been proposed previously[165]. Most critically, unlike other immune cells such as dendritic cells[166] and neutrophils[475] where CD14 mediated calcium flux and resultant calcineurin activity appears to be the primary source of NFAT activity during innate immunity, NFAT activity in macrophages may be CD14 independent[166], and is subject to further, or

potentially alternative regulation through the leucine-rich repeat kinase 2 (LRRK2) complex which reportedly inhibits steady state NFAT activity[326].

These distinct signaling pathways may also lead to differential transcriptional activity by NFAT as in spite of strong binding of NFAT to the consensus binding site from the IL-13 promoter employed in our studies, no induction of the classical NFAT-dependent immunoregulatory cytokines (IL-2, IL-3, IL-4, IL-10, IL-12p70, IL-13) was observed following *P. aeruginosa* infection *in vitro* or *in vivo*. Instead, impaired NFAT activity correlated with impaired production of inflammatory mediators including TNF, IL-6, IL-1β and neutrophil chemoattractant MIP-2. These results suggest that NFAT plays primarily an inflammatory role early in *P. aeruginosa* infection.

The theme of interconnectivity between the NFAT and NFκB pathways continued throughout our examination of the role of RCAN1 during *P. aeruginosa* infection. RCAN1 is best characterized as an inhibitor of NFAT signaling, where physical interactions with calcineurin prevent calcium induced phosphatase activity[189]. However RCAN1 has also emerged in the literature as a negative regulator of NFκB signaling where it stabilizes IκB-NFκB interactions through proposed calcineurin-dependent[190], and calcineurin-independent[191, 476] pathways. Our work elucidated the biological significance of these interactions during respiratory tract infections as RCAN1-deficiency resulted in hyper-inflammatory activation of the NFκB and NFAT pathways, as well as a novel dysregulation of the STAT3 pathway resulting in systemic inflammation and greatly enhanced mortality.

The interconnectivity of RCAN1, NFAT and NFκB also forms a feedback switch at the transcriptional level where NFAT[477, 478] and NFκB[478] drive the expression of

RCAN1, which then feeds back to inhibit the activity of these transcription factors, shutting down its own transcription. This circuit ensures not only the timely shut off of inflammatory transcription processes, but also serves to tightly control expression of RCAN1.

When the results of the current studies are placed in context of the literature discussed above a complex signaling network is formed (Figure 6.2.1). The picture which emerges is two intimately connected pathways induced by similar stimuli and mediated by common signaling elements. The ultimate result, as evidenced by our work with the CnAβ-deficient model, is a tight co-regulation of these pathways during innate immune responses resulting in both enhancement of existing inflammatory responses, and in considerable signaling redundancy. This co-regulation of NFAT and NFκB is perhaps not surprising when considered from an evolutionary perspective. Both NFAT and NFκB family members are evolutionarily and structurally related Rel domain containing proteins[150]. As a result, NFAT and NFκB bind to similar consensus DNA sequences[479, 480]. This shared evolutionary history, along with the convergent signaling pathways regulating their activity, and the similarities in their consensus binding sequences, support functional cooperation between the NFκB and NFAT pathways. Indeed NFATc1 and NFATc2 have been shown to interact with NFκB p50 and p65 proteins to drive expression of various genes[480-482]. Cooperativity between NFAT and NFκB in these cases was shown to be regulated through both RCAN1[481], and calcineurin-dependent[482] mechanisms.

6.3 Mast cells contribute to host defense against *P. aeruginosa*

Although considerable work has elucidated roles of mast cells during *P. aeruginosa* infection *in vivo*, little was known regarding the role of these cells during *in vivo* lung infections. Given their localization to interfaces with the respiratory epithelium, mast cells are ideally positioned to mediate early bacterial clearance. This view was supported by previous work in our lab demonstrating that mast cells are capable of phagocytosing and killing *P. aeruginosa* bacteria[248]. Furthermore herein we demonstrate that autophagy plays a critical role in mast cell mediated bacterial clearance, and can be enhanced through pharmacological induction of the process. These *in vitro* results point towards a role for mast cells, along with other tissue resident leukocytes, in early bacterial clearance during *P. aeruginosa* infection. In order to better understand the role of mast cells in host defense against *P. aeruginosa* we employed a mast cell deficient mouse model. Surprisingly, no significant differences in bacterial clearance or animal survival were observed in these mice. Instead, an unexpected role for mast cells in maintaining epithelial integrity was discovered. These findings suggest that the close anatomical relationship between mast cells and epithelial cells in the airways is dispensable for bacterial clearance, but instead contributes to epithelial barrier function. Alternatively, it is possible that the contribution of mast cells to bacterial clearance is important at low doses of bacteria, but is saturated at doses used in our studies.

6.4 Limitations

6.4.1 Limitations of statistical methods

The biological sciences often deal with relatively small data sets when performing statistical analysis, with three biological replicates representing the widely accepted minimum number of experiments. Technical replicates are often employed to minimize

variability, but do not add to the statistical power of the experiment. This can create issues when performing tests for normality and equality of variance, which often fail with small numbers of samples. Hence it has been assumed herein that all data sets analyzed displayed both normal distributions, and equal variance, allowing for the use of parametric statistics.

It is important to note however that statistical significance is used in these studies only to support biological significance. For instance under the correct circumstances a 2% difference in CFU could reach statistical significance, but it is unlikely to impact any biological function such as lung injury, or mortality. Hence a result can be statistically significant, while biologically insignificant. Statistics herein are used to support observed biologically significant differences, such as lung injury, bacterial clearance, or mortality. Hence the assumptions of normality and equal variance, even if not strictly true, are unlikely to impact the ultimate interpretation of data.

6.4.2 Limitations of *P. aeruginosa* lung infection model

Pseudomonas aeruginosa is a bacterium which is efficiently cleared from the lungs of healthy humans, and mice. Lung infections usually arise clinically when there is dysfunction of the innate inflammatory response, or when disruption of anatomical barriers to infection occurs. However spontaneous lung infection with *P. aeruginosa* does not occur in mice, raising the need for an artificial infection model. In the current studies, an acute lung infection model was employed where mice were infected intranasally with *P. aeruginosa* bacteria. Although extremely informative when examining the early innate immune responses against the bacteria, this model has several significant drawbacks which must be considered. First the number of bacteria used in many studies is extremely

high, involving administration of as many as 1×10^9 bacteria. These numbers far exceed doses normally encountered in the environment, but are necessary because the bacteria are rapidly cleared from the lungs. Even at these extreme doses, few viable bacteria are recovered 48 hours post infection. Hence in order to examine an active immune response, or assess bacterial clearance, extremely high doses of bacteria are required.

The second drawback of this model is that different strains of bacteria yield very different results. These studies predominantly used two strains of bacteria: the CF lung isolate 8821 and the environmental isolate PAK. These strains differ in their phenotypes as 8821 is a mucoid, sessile strain, which does not express flagella and has truncated LPS, while PAK is a planktonic, motile strain which expresses full length LPS and flagella. As a result of these differences, PAK is much more potently inflammatory, and lethal at relatively low doses, while 8821 caused no mortality, save in the presence of chloroquine. As a result of these differences, PAK was used to gain critical insight regarding survival outcomes, but yielded poor CFU recovery as only low doses were tolerated. Conversely 8821 was well tolerated allowing analysis of bacterial clearance, but did not cause mortality. Hence both strains were used in all studies which can complicate interpretation of the results. To overcome this complication both 8821 and PAK were employed wherever possible in order to demonstrate observed effects were generalizable across multiple strains.

The final difficulty encountered with the acute *P. aeruginosa* lung infection models involves interpreting the results of survival studies. As stated previously, mortality was only observed with the strain PAK, which has full length LPS and flagella. This observation, combined with the relative rapidity of the mortality observed (24-48

hours, shorter for RCAN1-deficient animals) suggests that observed mortality may be a result of endotoxemia, and not *P. aeruginosa* specific mechanisms.

6.4.3 Limitations of $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mouse model

$Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice have a spontaneous mutation in the upstream regulatory elements of the *c-kit* gene[483] which renders them almost completely devoid of mature mast cells at many anatomical sites, including the lungs[484], while avoiding deficiencies such as anemia and infertility observed in other mast cell-deficient mice harboring mutations in the coding region of the *c-kit* gene[485]. This feature, combined with the ability to reconstitute these mice with bone marrow-derived mast cells (BMMCs)[484] makes them a valuable tool for studying mast cell biology. However it is important to consider that in addition to mast-cell deficiency, other phenotypic abnormalities have been reported in this strain. Most critically, $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice have been reported to have splenomeglia, with expanded myeloid populations, as well as neutrophilia and thrombocytosis, although the extent of these phenotypes can vary greatly between animals[486]. However it is unlikely that these defects were responsible for increased susceptibility to acute lung injury in these mice as reconstitution with mast cells was able to fully reverse the observed phenotype, strongly implicating mast-cell deficiency as the underlying cause.

6.5 Future Directions

6.5.1 Preclinical testing of 'autophagy restorative therapy' in CF animal models

Initial results presented herein, and those discussed previously for other common CF associated pathogens provides a strong theoretical basis for the use of autophagy restorative therapy in the treatment of lung infections in CF patients. However before

human trials can be considered, substantial pre-clinical safety and efficacy testing will be required. The most significant obstacle to these tests is the lack of a suitable animal model which satisfactorily replicates pathophysiological and immunological features of the CF lung. Multiple CF mouse models have been generated over the past decades. However these mice do not acquire spontaneous lung infections, and display much milder lung pathology than that observed in CF patients. These differences may be due in part to anatomical differences in the respiratory tract between mice and humans, and the expression of a second Cl^- channel in mice which is not present in humans, and can partially compensate for deficiencies in CFTR (reviewed in[487])

As a result of the short comings of CFTR-deficient mice as a model of the human disease, a number of other CF animal models have recently been developed. Among the most promising are the CF pig and CF ferret models[488]. Both of these models develop spontaneous colonization of the respiratory tract similar to that seen in humans. However these models are not without their drawbacks as both CF pigs and ferrets develop sever intestinal blockages within the first weeks of life which are fatal without surgical intervention. Furthermore respiratory tract infections in CF ferrets are so severe that they are fatal without aggressive antibiotic therapy at weaning, complicating their use a model of CF airway infections[489]. More similar to humans, the airways of CF pigs which survive to the age of 6 months do become colonized with a variety of bacterial and fungal pathogens. However the microbiome in the lungs of these animals is significantly different than that observed in humans, and does not include *P. aeruginosa*[490].

As a model which effectively recapitulates spontaneous colonization of the CF airways has yet to be described, alternative infection models must be considered.

Perhaps one of the most attractive options involves administration of *P. aeruginosa* bacteria in the drinking water of CFTR-deficient mice. This approach results in a low level chronic colonization of the respiratory tract of CFTR-deficient, but not wild-type mice[491]. This model could be used to test both the prophylactic and therapeutic potential of various interventions, or combinations of interventions discussed above.

Alternatively, administration of *P. aeruginosa* encased in agar or alginate can cause chronic inflammatory responses observed in CF airways due to the slow and continuous release of bacteria. This approach has been shown previously to elicit prolonged hyperinflammatory responses in the lungs of CFTR-deficient mice[492]. This model could be used to test the efficacy of various autophagy restorative therapies against established bacterial lung infections as it mimics *P. aeruginosa* biofilms. Promisingly, this approach could also be used to deliver a tightly control microbiome into the lungs which would better represent clinical scenarios.

6.5.2 Identity of mast cell secreted factors which oppose lung injury

Studies presented herein demonstrate a previously unappreciated and biologically important role for mast cells in opposing bacteria induced acute lung injury during *P. aeruginosa* lung infection. The absence of mast cells resulted in increased epithelial permeability, an increased accumulation of neutrophils within the parenchyma of the lung, and significant dissemination of bacteria from the site of infection. These findings could provide novel insight and therapeutic options in diseases such as cystic fibrosis associated *P.* aeruginosa infection which is characterized by chronic lung damage and changes in the numbers, distribution, and phenotypes of mast cells throughout the airways[493]. However the first step towards any therapeutic intervention will be the

identification of the mast cell secreted factor or factors responsible for the observed effects. Initial studies have found minimal difference in cytokines known to influence acute lung injury, such as IL-6, IL-10 and TGF-β1 in either *in vitro* or *in vivo* models (data not shown). However lung injury is extremely complex and alternative mechanism should be considered. One such mechanism is the role of mast cell derived IL-4 and IL-13 in *P. aeruginosa* induced lung damage. These immunoregulatory cytokines are produced by mast cells early in infection, and IL-4 and IL-13 deficient mice both display greatly increased lung damage in response to various stimuli, which is characterized by elevated levels of TNF, and increased caspase activity[494-497]. Furthermore, administration of recombinant IL-4 and IL-13 have both been shown to reduce immune complex induced lung injury, and associated TNF production *in vivo*[498]. The role of these factors could be tested first by looking for deficiencies in the levels of these cytokines both *in vivo* in the BALF and lung supernatants of $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice, and *in vitro* in the co-culture system. Confirmation of the role of these factors could also be obtained *in vivo* through reconstituting $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice with either wild-type BMMCs, or BMMCs isolated IL-4 or IL-13-deficient animals. If these factors are responsible for the observed resistance to ALI, then reconstitution with IL-4 or IL-13 deficient mast cells should not correct the defects observed in $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice.

6.5.3 The roles of xenophagy vs. LAP during *P. aeruginosa* lung infection

Although a role for autophagy in host defense against *P. aeruginosa* has been established, the outstanding question of how exactly the pathway mediates bacterial killing remains to be addressed. There are two distinct, but not exclusive, autophagy related pathways which may contribute to killing of the bacteria inside host cells. First, if

the bacteria were to gain access to the cytosol, or if it were to reside within damaged phagosomes, it could be targeted to autophagosomse for degradation through xenophagy. One possibility is that when *P. aeruginosa* persists in the endosomal pathway by inhibiting Rab5 activity it may induce membrane damage, targeting the bacteria containing vesicle to the autophagy pathway through a Galectin-8 dependent mechanism. However bacteria were rarely observed in distinct autophagosomes in EM samples, raising the possibility that direct killing by xenophagy may not represent a central mechanism for bacterial clearance.

The alternative explanation for the role of autophagy in bacterial clearance during *P. aeruginosa* infection involves LAP, which has been shown to enhance classical phagocytic killing. As with xenophagy, LAP requires the beclin-1 PI3K complex, as well as LC3, Atg5 and Atg7. LAP is also reportedly subject to regulation by the mTOR complex. Hence pharmacological and genetic approaches used to manipulate the autophagy pathway in our studies would impact both xenophagy, and LAP pathways. Future work employing knockdown or knockout of essential autophagy proteins which are not required for LAP, such as Atg9, may be able to better elucidate the relative contributions of these two pathways to bacterial clearance during *P. aeruginosa* infection.

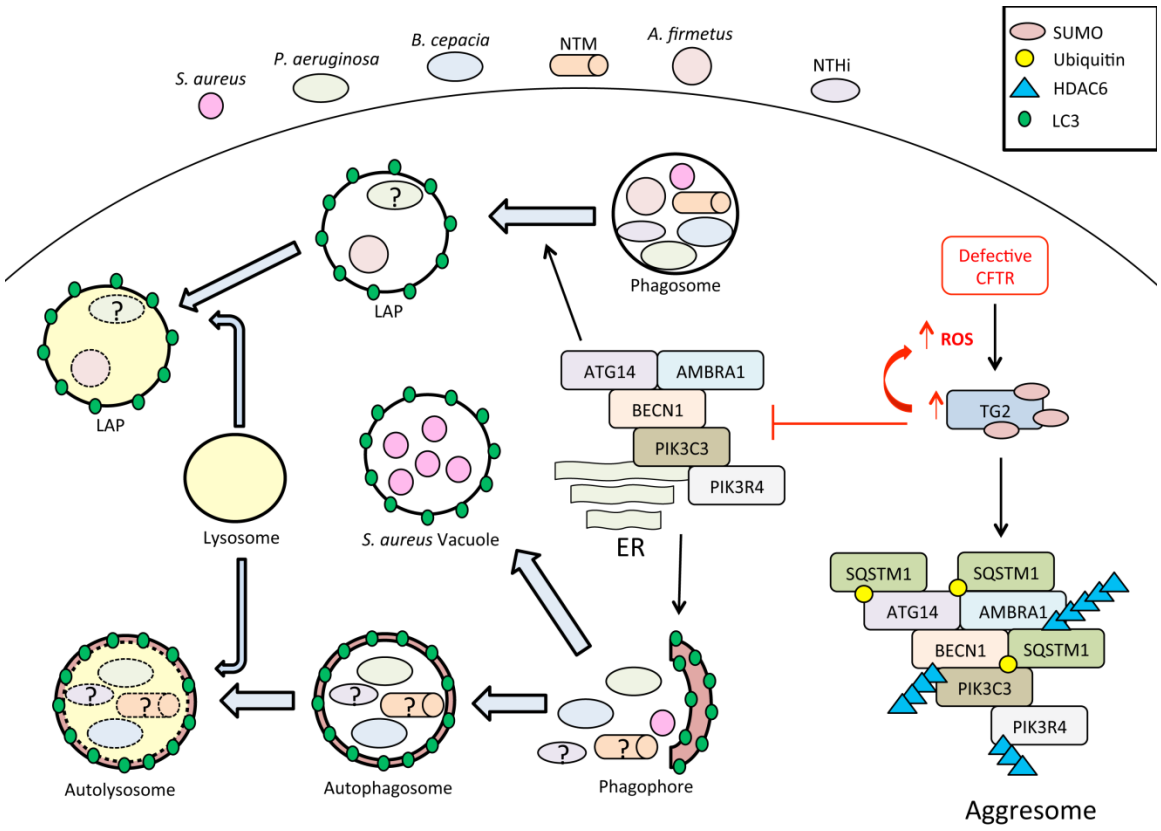
S. aureus
P. aeruginosa
B. cepacia
NTM
A. firmetus
NTHi
SUMO
Ubiquitin
HDAC6
LC3
Phagosome
LAP
LAP
Lysosome
S. aureus Vacuole
ATG14
AMBRA1
BECN1
PIK3C3
PIK3R4
ER
Defective CFTR
ROS
TG2
SQSTM1
SQSTM1
ATG14
AMBRA1
BECN1
SQSTM1
PIK3C3
PIK3R4
Aggresome
Phagophore
Autophagosome
Autolysosome

Figure 6.1.1: Defective CFTR disrupts BECN1 PtdIns3K activity and impairs clearance of CF associated pathogens: Mutations in the CFTR/cystic fibrosis transmembrane conductance regulator drive increased intracellular levels of reactive oxygen species (ROS) and calcium (Ca^{2+}) leading to increased small ubiquitin like modification (SUMO)ylation of TG2/tissue transglutaminase 2. This SUMOylation prevents ubiquitination and proteasomal degradation of the protein, leading to greatly enhanced TG2 activity, which in turns feeds back to drive ROS production. Increased TG2 also promotes crosslinking of the BECN1/beclin-1 phosphatidylinositol 3 kinase (PtdIns3K) complex leading the generation of BECN1, SQSTM1, HDAC6/histone deacetylase 6 and ubiquitin positive aggresomes. Accumulation of BECN1 in aggresomes results in a functional sequestration of the PtdIns3K complex that prevents ER translocation necessary for the initiation of autophagy, or phagosome localization necessary for LC3-associated phagocytosis (LAP).

Upon entering the cell, *P. aeruginosa* can become targeted to the autophagy pathway through yet uncharacterized mechanisms. LAP may also play a role in the clearance of intracellular *P. aeruginosa* bacteria. Following phagocytosis *B. cepacia, H. influenzae* and NTM persist within the phagocytic/endocytic pathway where they actively inhibit lysosomal fusion with bacteria containing vesicles. In healthy macrophages, *B. cepacia* containing vacuoles are targeted to the autophagy pathway for degradation. A similar mechanism is involved in the clearance of *M. tubercuolosis*, which employs the same intracellular life cycle as NTMs. However whether or not NTMs are specifically targeted for degradation by the autophagy pathway remains undefined. Similarly, the role of autophagy in the clearance of intracellular *H. influenzae* remains unknown. Following phagocytosis, the degradation of *A. fumigatus* spores requires LAP for effective lysosomal degradation. Unlike the other common CF associated pathogens, *S. aureus* escapes from the phagosome upon entering the cell. Cytosolic bacteria, or bacteria contained within damaged phagosomes are subsequently targeted to the autophagy pathway where they inhibit lysosomal fusion, creating a replicative niche for the bacteria.

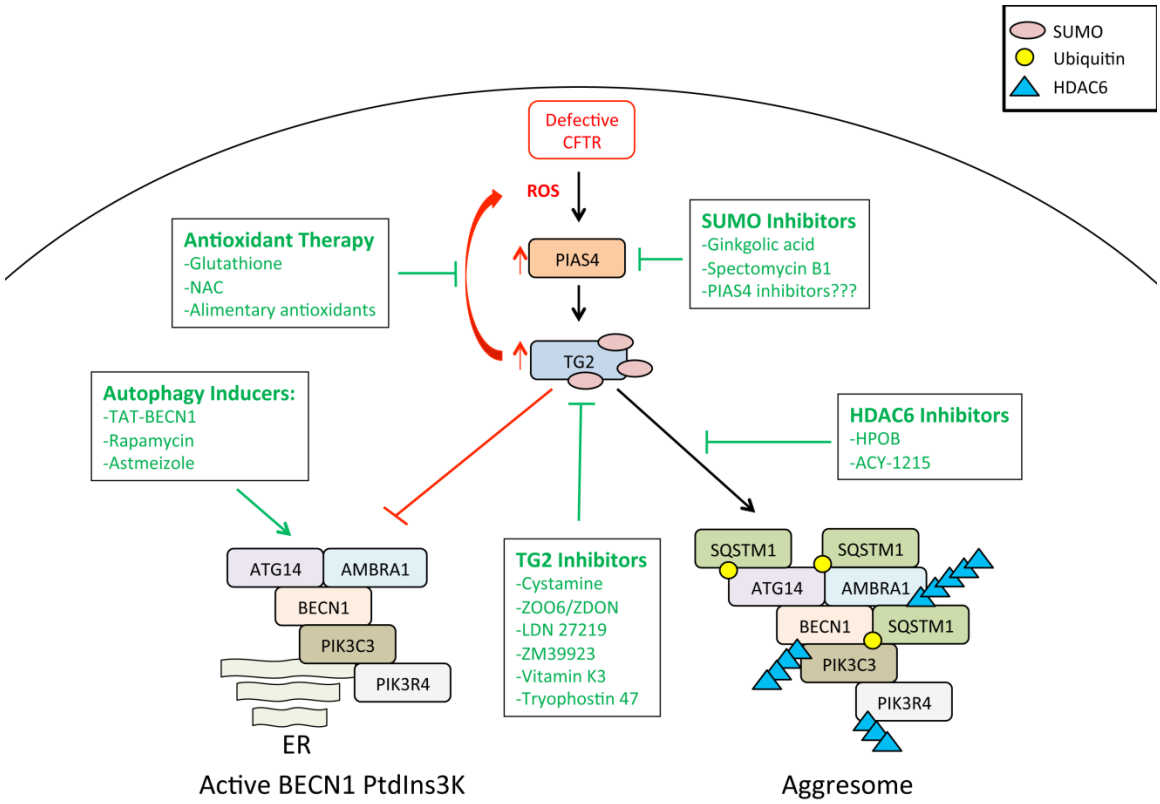

Figure 6.1.2: Autophagy restoration therapy for CF associated lung infections: Mutations in CFTR/cystic fibrosis transmembrane conductance regulator result in uncontrolled production of ROS and activation of PIAS4/protein inhibitor of activated STAT, 4 which leads to SUMOylation of TG2/tissue transglutaminase 2. This post-translational modification results in increased protein stability and activity which drives functional sequestration of BECN1/beclin-1 in HDAC6/histone deacetylase-6 positive aggresomes resulting in defective autophagy. Aberrant TG2 activity also perpetuates ROS production resulting in a feedback loop ensuring further activation of TG2. Therapeutic interventions aimed at restoring autophagy in the airways of CF patients can target multiple facets of this pathway including: 1) Antioxidant therapy to reduce ROS production and disrupt feedback activation of TG2. 2) Direct autophagy induction to force available BECN1 into active PtdIns3K complexes. 3) TG2 inhibitors to prevent crosslinking and functional sequestration of BECN1. 4) SUMO or PIAS4 inhibitors to decrease stability and activity of TG2. 5) Aggresome/HDAC6 inhibitors to prevent the formation of BECN1 containing aggresomes.

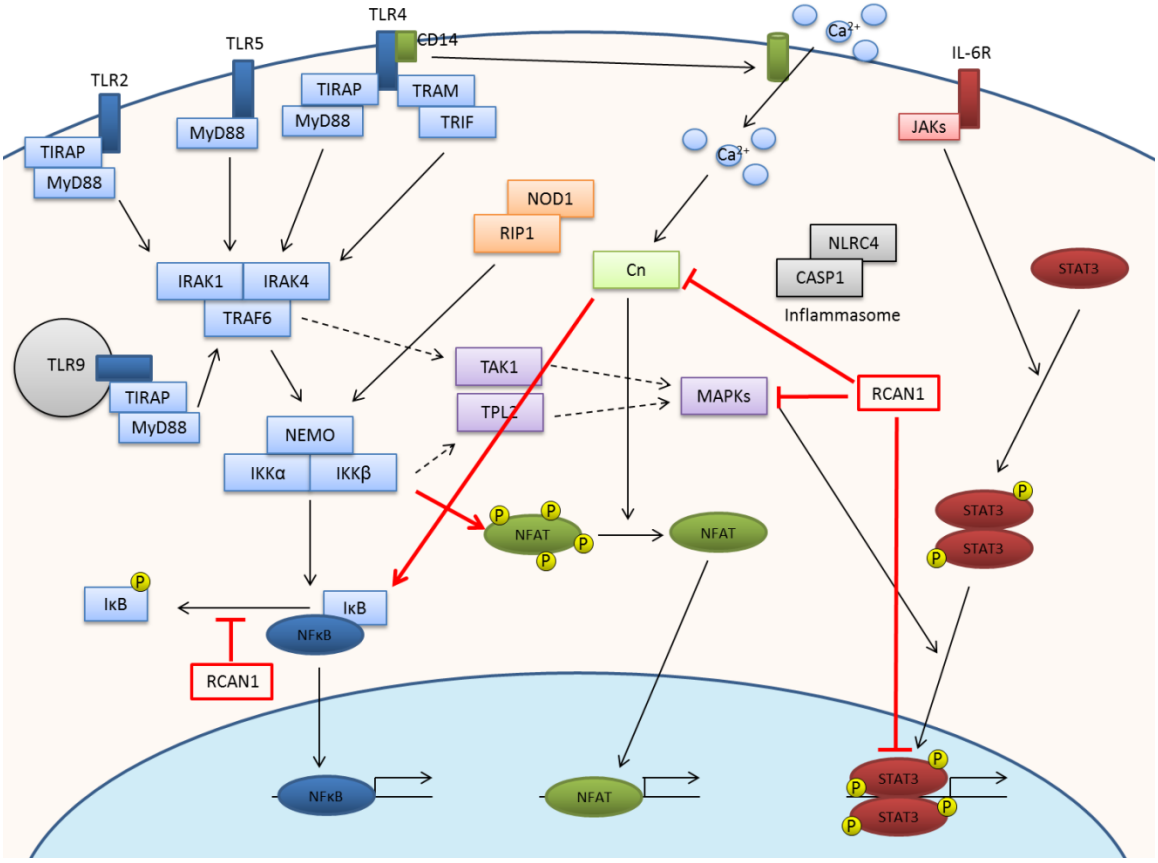

Figure 6.2.1: Crosstalk between NFκB and NFAT signaling pathways: Canonical activation of NFκB is triggered through TRIF and MyD88 dependent TLR signaling pathways leading to activation of TRAF6 and the IKK complex which phosphorylates IκB and frees NFκB for nuclear translocation and transcriptional activity. Canonical activation of the calcineurin-NFAT pathway occurs through TLR4 associated CD14 which induces an influx of extracellular calcium leading to activation of calcineurin and dephosphorylation of NFAT which exposes a nuclear localization sequence. NFAT and NFκB signaling drive expression of RCAN1 which negatively regulates both NFAT activation through interactions with calcineurin, and NFκB signaling through stabilization of NFκB-IκB interactions. RCAN1 also suppresses MAPK activity leading to dysregulation of the STAT3 pathway. Crosstalk between the NFAT and NFκB pathways is mediated through IKK dependent activation of NFAT and calcineurin dependent activation of NFκB.

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APPENDIX A

December 18, 2013

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