The role of endoplasmic reticulum stress and inflammasome activation in inflammatory disease: identifying therapeutic targets in cystic fibrosis and haematological malignancies

by

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ABSTRACT

An increasing number of infectious and inflammatory conditions have identified ER stress and IL-1β as central driving forces in inflammation. In the following studies, we have identified several important pathways that promote the cycle of pulmonary inflammation in cystic fibrosis (CF), including the IL-1β regulation by NF-κB, IL-1β stimulation of neutrophil chemokine production by airway epithelial cells, and inhibition of neutrophil chemokine secretion through ER stress signaling. Based on our observations, we conclude that CF environmental factors such as increased NF-κB priming by chronic infection and the presence of ER stressors can significantly augment inflammatory responses by controlling the maturation of IL-1β. These effects appear to be cell type and cytokine-specific, as downstream induction of CXCL1 and IL-8, key neutrophil chemokines that we found were associated with lung disease severity in CF patients, can also be suppressed by ER stress-mediated inhibition of STAT3 signaling in airway epithelial cells.

Interestingly, we also found that by varying the time of TLR stimulation relative to the induction of ER stress (particularly by proteasome inhibition), we could obtain differential effects on IL-1β production and maturation. We reasoned that these effects could be important in haematological malignancies where proteasome inhibition has recently become a successful first-line treatment. Not only was tumour cell cytotoxicity significantly increased by addition of a TLR adjuvant, IL-1β production could be controlled depending on when the adjuvant was added relative to the proteasome inhibitor. This could be a useful method in which IL-1β-mediated responses can be manipulated while also increasing tumour cell death.

Although additional elaboration of these pathways and evaluation of the clinical feasibility of these approaches is still needed (particularly *in vivo*), we have established the critical first steps in identifying these potential targets and defining their underlying mechanisms.

PREFACE

All work was conducted at the Child & Family Research Institute in association with the BC Children's Hospital and the University of British Columbia. Ethics approval was obtained for collection of blood samples from UBC C&W Research Ethics Board (certificate # H09-01192). Patient samples were obtained through recruitment of patients by A Tang in the BC Children's Hospital CF clinic directed by Dr. Mark Chilvers. Blood samples were obtained with informed written consent from control subjects and CF patients at the BC Children's Hospital. Consent was obtained for children by their parent or legal guardian. Subjects 7 years of age and older were required to provide informed assent as well.

I was the lead investigator for all the work presented in this thesis. All aspects of study design, analysis, and execution were carried out by A Tang and SE Turvey. Flow cytometry data acquisition and generation of Figures 2.3 and 2.4 was performed with the help of AS Sharma. Access to summary P-values from North American and French CF genome-wide association studies was granted by Drs. Lisa Strug and Andrew Sandford and analysis was performed by A Saferali. A Saferali also generated the related tables (Table 3.2, Supplementary Tables 3.1 and 3.2) and Supplementary Figure 3.1 in Chapter 3, and wrote the materials and methods for this section.

The study presented in Chapter 2 of this work was published in *PLoS One* on 23 May 2012. Reproduction of this chapter was permitted as authorship rights were retained upon publication.

> **Tang A, Sharma A, Jen R, Hirscheld AF, Chilvers MA, Lavoie PM, Turvey SE. Inflammasome-medaited IL-1β production in humans with cystic fibrosis. PLoS One, 2012, 7:e37689.**

Certain elements in Chapter 1 of this work were derived from a review published in *European Respiratory Reviews* on 23 September 2014.

Tang AC, Turvey SE, Alves MP, Regamey N, Tümmler B, Hartl D. Current concepts: host-pathogen interactions in cystic fibrosis. European Respiratory Reviews, 2014, 133: 320-32.

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AIM2 – Absent in melanoma 2

AP-1 – Activating protein 1

AMP – Antimicrobial peptide

ASC – Apoptosis-associated speck-like protein containing CARD

ASL – Airway surface liquid

ATF4 – Activating transcription factor 4

ATP – Adenosine triphosphate

BALF – Bronchoalveolar lavage fluid

CF – Cystic fibrosis

CFTR – Cystic fibrosis transmembrane conductance regulator

CLR – C-type lectin receptor

ENaC – Epithelial sodium channel

FEV1 – Forced expiratory volume in 1 second

FLICA – Fluorochrome labelled inhibitors of caspases

fMLP – N-formyl-methionyl-leucyl-phenylalanine

GM-CSF – Granulocyte macrophage colony stimulating factor

GWAS – Genome wide association study

IκBα – Nuclear factor kappa B inhibitor alpha

ICD – Immunogenic cell death

IFN – Interferon

IKK – IκB kinase

IL – Interleukin

IRAK – Interleukin-1 receptor associated kinase 4

IRE1α – Inositol requiring enzyme 1 alpha

IRF – Interferon regulatory factor

LD – Linkage disequilibrium

LPS – Lipopolysaccharide

LRR – Leucine rich repeat

M-CSF – Macrophage colony stimulating factor

MAPK – Mitogen activated protein kinase

MAVS – Mitochondrial antiviral signaling

MHC – Major histocompatibility complex

MOI – Multiplicity of infection

MPL – Monophosphoryl lipid A

NADPH - Nicotinamide adenine dinucleotide phosphate

NE – Neutrophil elastase

PERK - Protein kinase RNA-like endoplasmic reticulum kinase

PMA – Phorobol 12-myristate 13-acetate

NF-κB - Nuclear factor kappa light chain enhancer of activated B cells

NLR – NOD-like receptor

NOD – Nucleotide oligomerization domain

PAMP – Pathogen associated molecular pattern

PBMC – Peripheral blood mononuclear cell

PMN – Polymorphonuclear (cell)

PRR – Pattern recognition receptor

PSGL-1 – P-selectin glycoprotein ligand 1

RIG-I – Retinoic acid inducible gene I

RIP – Receptor interacting protein

RLR – RIG-like receptor

ROS – Reactive oxygen species

RNS – Reactive nitrogen species

SNP – Single nucleotide polymorphism

STAT – Signal transduction and activator of transcription

T3SS – Type III secretion system

TAK – Transforming growth factor activated kinase

TIR – Toll-interleukin-1 receptor

TLR – Toll-like receptor

TNF – Tumour necrosis factor

TRAF – TNF receptor associated factor

TRIF – TIR domain containing adapter inducing interferon β

UPR – Unfolded protein response

UPS – Ubiquitin proteasome system

XBP1- X box binding protein 1

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CHAPTER 1: AN INTRODUCTION TO INFECTION, IMMUNITY, AND HEALTH

Microorganisms are ubiquitous in the environment and constantly challenge host organisms in their pursuit for nutrients and amenable growth conditions (1). To microorganisms, host cells and the mucosal environment provide a good potential source of nutrients including amino acids, fatty acids, sugars, and metal cofactors (2-5). As in the case of many gut bacteria, the majority of colonizing microorganisms are not harmful and can even benefit the host through commensal interactions (6), but a small percentage do display pathogenicity towards human cells and cause illness (7). Although comparatively few in number, these pathogens cause significant morbidity and mortality in humans with respiratory infections alone costing the Canadian health care system almost 3 billion dollars in 2008 (8). Thanks to the proper functioning of the host immune system, only a small minority of colonizing microorganisms will cause illness, and in the developed world, these will cause comparatively little mortality (9). However, it has become very clear that the role of the immune system extends far beyond the control of infection and that dysregulation of immune function can likewise lead to chronic, debilitating diseases (10). It has been increasingly found that various common non-communicable diseases (many of which have large economic burden or high rates of mortality: asthma, inflammatory bowel disease, diabetes, cancer) arise from inflammatory origins or are characterized by inflammatory dysfunction (8, 11). In order to improve health outcomes in these diseases, it is critical that we better understand the immune system and how it maintains the delicate balance of preventing infection while minimizing chronic damage to the host organism.

The host immune system adequately deals with the majority of infections through the innate and the adaptive phases of the immune response. Both phases of the response are critical to successfully fend off microbial insult and are functionally interactive (12): the innate response is characterized by conserved microbial recognition mechanisms and quick initial response time, whereas the adaptive response allows for more unique microbial recognition and the development of memory but has a delayed response time (Table 1.1)(13). This being said, the innate response is critically important not only because it controls microbial invasion and growth during the early stages of infection, but because it is also responsible for shaping the nature of the subsequent adaptive response (14). While many processes and molecules contribute to the innate response, for descriptive purposes, these will be briefly described as soluble and cellular effectors.

Table 1.1: Characteristics of innate and adaptive immunity

	INNATE IMMUNITY	**ADAPTIVE IMMUNITY**
RESPONSE TIME	Immediate, occurs upon infection	Delayed, requires several days (3-4) and functional development by the innate immune system
POPULATION	Granulocytes (neutrophils, eosinophils, basophils, mast cells), monocytes, macrophages, innate lymphoid cells, dendritic cells, NKT cells	T cells, B cells. Activated cells clonally expand into a large effector population
SPECIFICITY	Finite, germ-line encoded repertoire. Recognizes conserved microbial elements	Highly diverse, T cell and B cell receptors only limited by somatic mutation and rearrangement of receptor genes.
MEMORY	Limited to NK cells, otherwise none	Memory can last for years and initiates a quicker response time upon re-stimulation

A comparison of innate and adaptive immunity.

1.1 Soluble effectors of innate immunity

Constitutively produced and induced immune factors are important for protection against colonizing microorganisms by targeting many aspects of microbial survival. Certain soluble host factors, such as natural IgM, the collectins (mannan binding lectin, surfactant proteins A and D), pentraxins (C-reactive protein, serum amyloid P), and ficolins can recognize and bind to carbohydrate epitopes and can be lytic for microbial cells by engaging the complement pathway (15-18). Others including the antimicrobial peptides LL-37 and the defensins, can directly disrupt microbial membranes through hydrophobic or electrostatic interactions (19-23). Many of these factors also have important roles in chemotaxis or may also act as opsonins to enhance phagocytosis by incoming effector cells (21). Yet another method by which the immune system controls microbial growth is by limiting access to nutrients. The antimicrobial proteins lactoferrin and hepcidin accomplish this by either sequestering free iron (24, 25), an essential cofactor in both host and microbial processes (26), or preventing free iron release into the bloodstream during inflammatory responses (27, 28). Although important in preventing infection, maximum efficacy of these soluble factors requires the additional involvement of cellular effectors of the innate response.

1.2 Cellular effectors of innate immunity

Circulating innate immune cells derive from hematopoietic stem cells which have committed to either one of two different lineages: the myeloid lineage, which gives rise to erythrocytes, monocytes, neutrophils, eosinophils, basophils, and mast cells (29); or the lymphocyte lineage, which gives rise to natural killer cells (a source of Th1 cytokines) (30), innate lymphoid cells (a source of Th2 cytokines) (31), and other innate lymphoid cells important in T and B

lymphocyte development (32, 33). These cells may circulate in the bloodstream or take residence in specific tissues or organs where they can perform more specialized functions.

Macrophages, dendritic cells, and mast cells exist as tissue-resident immune effectors and are able to immediately respond to local inflammatory stimuli. Mast cells in particular respond exceedingly quickly through the exocytosis of cytoplasmic granules containing preformed mediators (including tumour necrosis factor alpha (TNFα) (34), serotonin (35), proteases (36), and histamine (37)). This allows perivascular mast cells to quickly affect permeability of blood vessels, increasing the flow of serum proteins and recruiting additional effector cells to the site of infection (38). Macrophages and dendritic cells are both highly phagocytic cell types that are capable of engulfing and eliminating pathogens in phagolysosomes. These "professional phagocytes" possess a particularly broad repertoire of pattern recognition receptors (PRRs) such as the Toll-like receptors (TLRs) (39-41), making them quite sensitive to microbial stimulation. Of these, dendritic cells are uniquely potent antigen presenting cells, and upon maturation, have a high capacity to present digested microbial peptides on MHC class I, typically intracellularly-derived (also cross-presented (42)), or class II, extracellularly-derived antigens (43, 44) for T cell activation. Although macrophages are considered to have a more limited capacity for antigen presentation, they instead play a critical role in the resolution of inflammation, with involvement in cell proliferation, extracellular matrix deposition/removal, and growth factor production (45). Macrophages can "clean up" dying cells by efferocytosing apoptotic bodies in an immunologically quiescent manner (46-48). Inflammatory and non-inflammatory characteristics of macrophages are reflective of their activation status. Under homeostatic conditions, macrophages adopt an M2 phenotype that favours wound repair (49), but upon sensation of inflammatory stimuli such as lipopolysaccharide (LPS), interferon

gamma (IFNγ), and granulocyte-macrophage colony-stimulating factor (GM-CSF), they adopt an inflammatory M1 phenotype which favours microbial killing through the generation of reactive oxygen and nitrogen species (ROS, RNS) (50, 51). Adoption of the M2 phenotype can result from stimulation with interleukin-4 (IL-4), IL-10, and M-CSF (52). This results in a pro-tumour phenotype that facilitates angiogenesis (53) and has superior ability to perform phagosomal acidification, which may be important for clearance of apoptotic cells (51). Although these subsets have been useful in modeling macrophage phenotypic extremes *in vitro*, it is thought that macrophage phenotypes *in vivo* are more heterogeneous, possessing both M1 and M2 characteristics depending on the mix of signals provided in its local environment (54).

Neutrophils are also highly phagocytic cells. However, unlike macrophages and dendritic cells, they are relatively short-lived (55) and do not reside in tissues (56). Rather, neutrophils circulate in the bloodstream until recruited, in very large numbers, by chemokines (eg. CXCL1 and CXCL8) and other chemoattractants of both host and microbial origin (eg.C5a, fMLP) (57). These chemokines can either be produced locally by the endothelium in response to IL-1β or TNFα (58, 59), or transported from the site of infection and across endothelial cells (chemokine transcytosis) by the Duffy antigen receptor for chemokines (DARC) for presentation to circulating leukocytes (60). Recruited neutrophils express the adhesion molecules PSGL-1 (61) and L-selectin (62, 63) allowing for rolling interactions on the surface of activated endothelium (expressing P- and E-selectins), before firmly adhering and transmigrating at the site of infection via post capillary venules. Neutrophil activation by chemokines/chemoattractants is also important for upregulation of the complement receptor CD11b (64) and antimicrobial activity (65). In particular, the STAT3 pathway has been shown to be important in regulating neutrophil chemotaxis and activation, where conditional knockout of STAT3 in mice resulted in

inhibition of acute neutrophil mobilization in response to granulocyte colony-stimulating factor and also the CXCR2 ligands KC and MIP-2 (66).

Neutrophils are equipped with a vast antimicrobial arsenal consisting of preformed granules of proteases that fuse with phagosomes containing microorganisms. Additionally, phagocytosed pathogens are subject to highly toxic ROS and RNS through NADPH oxidase and iNOS activity within phagosomes (67). Neutrophil extracellular traps (NETs) are a somewhat novel mechanism through which dying neutrophils extrude a network of chromatin covered in antimicrobial peptides and proteases in order to impede microbial growth (68). These functions make neutrophils critically important in the early control of infection, as evidenced by the severity of several neutrophil-related deficiencies including NADPH oxidase deficiency (69) (ie. chronic granulomatous disease) and leukocyte adhesion deficiencies (70).

Aside from the circulating immune cells, various other cell types, and mucosal (eg. airway) epithelial cells in particular, play a critical role in the maintenance of immunity (71). Not only can these cell types secrete multiple antimicrobial factors including lysozyme, defensins, lactoferrin, and surfactant proteins, but they are also essential for the maintenance of barrier function with the environment (71). While also serving as a direct physical barrier, mucosal epithelial cells secrete and are covered by a mucous gel layer which impedes microbial aggregation (72). Ciliated epithelial cells lining the airways drive movement of the mucous gel layer and further prevent microbial adhesion (71, 73). Additionally, epithelial cells express their own repertoire of PRRs (74-78), allowing them to initiate inflammatory responses, secrete inflammatory cytokines and chemokines, and also help instruct recruited immune effector cells through the production of regulatory cytokines IL-33 (79) and thymic stromal lymphopoeitin (TSLP) (80). Their role in the clearance of apoptotic cells was recently shown to be important

in preventing development of an allergic Th2 phenotype through upregulation of IL-33 and transforming growth factor beta (TGFβ) (81).

1.3 Distinguishing friend from foe: pattern recognition receptors

The ability to distinguish foreign cells from the host's own cells forms the basis of an effective and safe immune response. To accomplish this, the innate immune system has evolved an array of various pattern recognition receptors (PRRs) that allows the host to identify foreign cells through the recognition of evolutionarily conserved motifs not found in host cells. These are known as pathogen-associated molecular patterns (PAMPs) and include nucleic acids (dsRNA, ssRNA, and unmethylated CpG DNA) and certain carbohydrates and peptide structures (lipopolysaccharides, lipopeptides, flagellin) found in microorganisms (82, 83).

PRRs can fall into several families including the Toll-like receptors (TLRs), the RIG-like receptors (RLRs), the NOD-like receptors (NLRs), and the C-type lectin receptors (CLRs) (Table 1.2). By far the most well-studied group are the TLRs - type I transmembrane proteins that detect a large diversity of extracellular PAMPs through N-terminal leucine rich repeats (LRRs) (83). Ligand binding and TLR activation involves either homo or heterodimerization of receptors and signalling through cytosolic/C-terminal Toll-interleukin-1 receptor (TIR) homology domains (84). This initiates a signalling cascade involving additional adaptor proteins such as MyD88 (all TLRs except TLR3) and TRIF (only TLR3 and TLR4). Although the molecules involved in signalling through the various TLRs are mostly conserved, differing degrees of expression on specific cell types and the role of compartmentalization can also influence responses. For example, TLRs 1, 2, 4, 5, and 6 are located at the cell surface on the

plasma membrane, while TLRs 3, 7, 8, and 9 are usually restricted to endosomal compartments (83), with only TLR4 being able to employ both MyD88 and TRIF adaptor proteins (85).

Other PRR families, including the RIG-I-like receptors (RLRs) and the Nod-like receptors (NLRs), reside in the cytosol in order to survey the intracellular environment for PAMPs. RLRs are a family of RNA helicases (RIG-I, MDA5, LGP2) (83) that mediate recognition of dsRNA and can induce antiviral type I interferons (IFNs) through the transcription factor IRF3 (86). RIG-I can also mediate activation of NF-κB through association with the MAVS adaptor and subsequent recruitment of TRAF6 (87). The NLRs include the NOD1 and NOD2 proteins which are responsible for the recognition of unique peptidoglycan motifs (88, 89). NLRs are characterized by a NACHT domain, which is important for oligomerization and signalling. Both NOD1 and NOD2 can signal through RIP2 kinase (90) and, like TLRs, recruit the TRAF6 for NF-κB activation (91, 92). The various PRR families and the general mechanism through which they feed into NF-κB activation are shown in Figure 1.1.

Another group of NLRs (NLRP1, NLRP3, NLRC4) form large multimeric complexes known as inflammasomes (93). In the presence of danger signals, inflammasome components oligomerize to mediate caspase-1 activation which then proceeds to process the cytokines IL-1β and IL-18 into their bioactive forms (93). Inflammasomes are typically composed of an NLR, an adaptor protein named ASC, and caspase-1 (94, 95), although some non-NLR inflammasomes have also been described (eg. AIM2 (96)). Unlike NOD1 and NOD2 activation, inflammasome function requires that cells be initially primed by an inflammatory response to generate inactive pro-IL-1β and pro-IL-18 (inflammasome signal 1). Once primed, an additional cellular "danger" signal (inflammasome signal 2: eg. ATP, nigericin, uric acid, bacterial toxins) needs to occur in order to achieve inflammasome activation and caspase-1 activation (Figure 1.2) (93, 97, 98). This is

usually accompanied by a form of inflammatory cell death known as pyroptosis, which is distinct from apoptosis and is mediated by caspase-1 and gasdermin D (99). Caspases are cysteine proteases that are best known for their role in apoptosis although a subset of caspases have been more recently found to mediate inflammatory responses (93). Caspase-1, in particular, has been widely studied for its ability to process pro-IL-1β to its active form. Caspase-1 is initially synthesized as a cytosolic precursor that is subsequently processed into its active p10/p20 subunits by proximity-induced autoproteolysis mediated by its recruitment to inflammasome complexes (100). Although inflammasome activation is typically associated with caspase-1 activation, caspases-4 and -5 have been reported to be involved in non-canonical inflammasome activation in response to cytosolic LPS through a direct interaction (101), where caspase-4 can act on Caspase-1 to mediate processing of IL-1β (102). Caspase-8, like caspase-1 can directly cleave pro-IL-1β to its active form, albeit less efficiently, in response to TLR3 or dectin-1 activation (103, 104). Furthermore, along with RIP3, caspase-8 has a critical role in governing whether cell death proceeds through apoptosis or necroptosis, a caspase-independent form of inflammatory cell death that can involve IL-1β (105).

Table 1.2: Classification of pattern recognition receptors

	Receptor Localization	Target Recognition	NF-κB Signaling
Toll-like Receptors	Cell surface: TLRs 1,2,4,5,6 Endosomal: TLRs 3,7,8,9	Range from lipopolysaccharide, lipoteichoic acid,lipoproteins,flagellin, ss and dsRNA, dsDNA (unmethylatedCpG), zymosan	Ligand recognition occurs through LRRs and dimerization of TIR domains in the cytoplasmic domain initiates signaling. All TLRs except for TLR3 signal through the MyD88 adaptor. Subsequent recruitment of IRAK1-4, TRAF6, and TAK1 lead to IKKβ-mediated NF-κB activation
NOD-like Receptors	Cytosolic: NOD1,2 NLRP1,2,3 NLRC4 NAIP	NOD proteins recognize peptidoglycan components (MDP, DAP). NLRC4 recognizes cytosolic flagellin and typeIII secretion components	NOD proteins signal in association with endosomal membranes. Oligomerization occurs via the central NACHT domain (characteristic of NLRs). Recruits RIP2 kinase, which activates TAK1 and subsequently, IKKβ activation
RIG-I-like Receptors	Cytosolic: RIG-I MDA5 LGP2	Cytosolic dsRNA. RIG-I mediates 5' triphosphate dsRNA. Size-based discrimination of receptor binding: RIG-I (short), mda5 (long)	Activation by dsRNA results in the recruitment of receptors to MAVS via CARD interactions at the mitochondrial membrane. MAVS recruits TRAF6 and TAK1, resulting in activation of IKKβ and NF-κB
C-type lectin Receptors	Cell surface: Dectin-1	β-1, 3 glucans	hemITAM domain dimerization leads to syk and PLCγ2 recruitment. PLCγ2 can control IP_3 and DAG-mediated Ca^{2+} release leading to NF-κB activation

Different classes of PRRs, their ligands, and pathways involved in NF-κB induction.

hemITAM – immunotyrosine-based activation motif (single SH2 domain vs tandem SH2 in ITAM), PLC – Phospholipase C, DAG – Diacylglycerol, IP_3 – Inositol triphosphate.

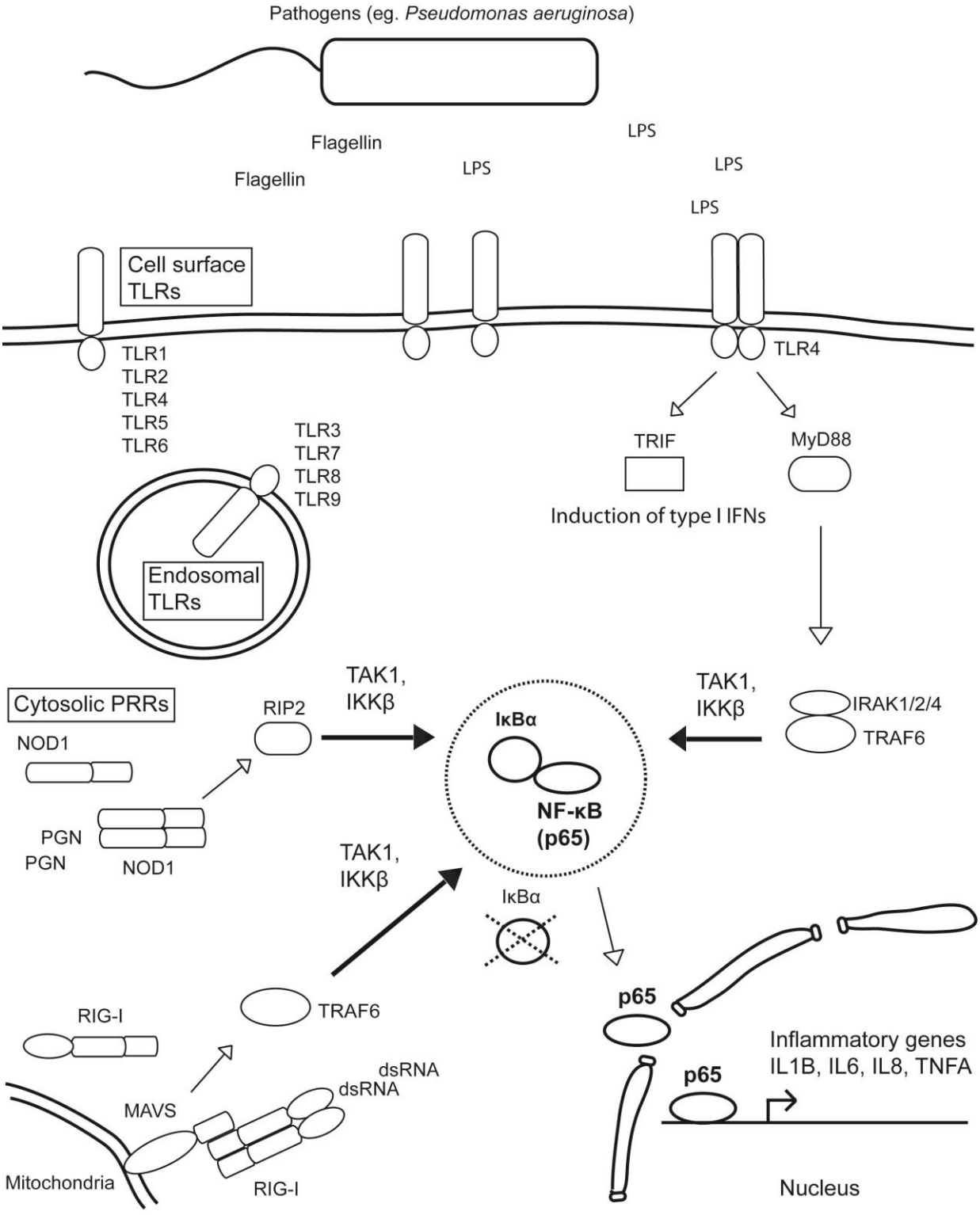

Pathogens (eg. *Pseudomonas aeruginosa*)
Flagellin
Flagellin
LPS
LPS
LPS
LPS
Cell surface TLRs
TLR1
TLR2
TLR4
TLR5
TLR6
TLR4
TLR3
TLR7
TLR8
TLR9
Endosomal TLRs
TRIF
MyD88
Induction of type I IFNs
Cytosolic PRRs
NOD1
PGN
PGN
NOD1
RIP2
TAK1, IKKβ
TAK1, IKKβ
IRAK1/2/4
TRAF6
IκBα
NF-κB (p65)
TAK1, IKKβ
IκBα
RIG-I
TRAF6
dsRNA
dsRNA
MAVS
Mitochondria
RIG-I
p65
p65
Inflammatory genes
IL1B, IL6, IL8, TNFA
Nucleus

Figure 1.1: Stimulation of various pattern recognition receptor pathways elicit inflammatory cytokine production through NF-κB

PRRs mediate NF-κB responses through various sensors including cell surface and endosomal TLRs, cytosolic NLRs (eg. NOD1), and cytosolic RLRs (eg. RIG-I). TLRs can mediate canonical NF-κB activation by signaling through the MyD88 adaptor protein which recruits TRAF6 through IRAK1, 2, and 4. This leads to TAK1-mediated activation of IKKβ, culminating in phosphorylation and degradation of IκBα, allowing nuclear translocation of p65 and transcription of NF-κB target genes. Upon recognition of cytosolic PGN, NOD1 oligomerizes and activates the kinase RIP2 through CARD-CARD interactions which can then proceed to activate TAK1, IKKβ, and NF-κB. RIG-I recognizes cytosolic dsRNA and activates NF-κB activation through interactions with the adaptor protein MAVS at the mitochondrial membrane. MAVS recruits TRAF6, ultimately resulting in phosphorylation of TAK1 and IKKβ.

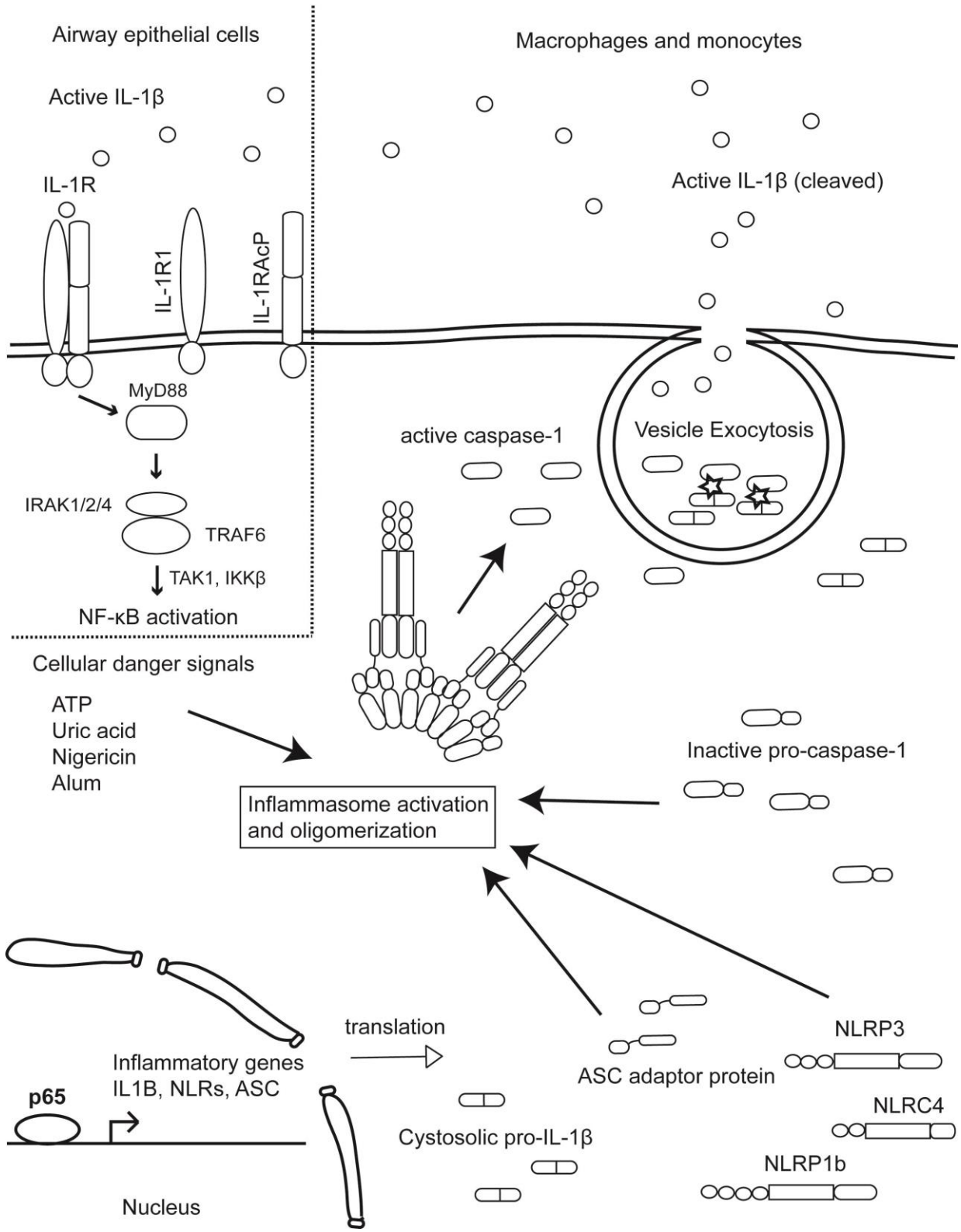

Airway epithelial cells
Macrophages and monocytes
Active IL-1β
IL-1R
IL-1R1
IL-1RAcP
Active IL-1β (cleaved)
MyD88
IRAK1/2/4
TRAF6
TAK1, IKKβ
NF-κB activation
active caspase-1
Vesicle Exocytosis
Cellular danger signals
ATP
Uric acid
Nigericin
Alum
Inactive pro-caspase-1
Inflammasome activation
and oligomerization
translation
Inflammatory genes
IL1B, NLRs, ASC
p65
ASC adaptor protein
NLRP3
NLRC4
Cytosolic pro-IL-1β
NLRP1b
Nucleus

Figure 1.2: NLR Inflammasome activation mediates IL-1β maturation and secretion, propagating inflammatory signalling to airway epithelial cells

NF-κB can drive transcription of genes involved in inflammasome activation and IL-1β processing. Once cytosolic pro-IL-1β is produced, cells can responds to danger signals including ATP and uric acid to induce assembly of large inflammasome complexes, consisting of an NLR, the adaptor protein ASC, and pro-caspase-1. This mediates autocatalysis of pro-caspase-1 which proceeds to cleave pro-IL-1β into its active form and also mediates its secretion via an unconventional secretion pathway. Secreted IL-1β can then proceed to activate other cell types, such as airway epithelial cells through the IL-1 receptor, signaling through a MyD88, TRAF6, and TAK1-dependent pathway (like TLRs) to activate NF-κB.

1.4 NF- κB as a central regulator of inflammation

Signaling through PRRs ultimately results in the activation of many inflammatory pathways including the MAP kinases, STATs, and IRFs. Of these, the NF-κB pathway has been one of the most thoroughly studied, and is considered a central pathway in inflammation. Under non-inflammatory conditions, NF-κB is bound in an inactive state in the cytosol by the protein IκBα (106). Signaling through TLRs through the MyD88 pathway results in recruitment of the serine/threonine kinases IRAK4, IRAK1, IRAK2, (107) and the E3 ubiquitin ligases TRAF2 and TRAF6 (108) to the intracellular receptor complex. TRAF6 proceeds to ubiquitinate/activate TAK1 kinase on lysine 63 (109) and active TAK1 phosphorylates the IκB kinase complex (IKK). IKKβ in turn phosphorylates IκBα, mediating activation of NF-κB pathway of inflammatory responses. IκBα release of NF-κB into the nucleus allows it to mediate the transcription of many pro-inflammatory genes. These include many cytokines, chemokines, and adhesion molecules important for intercellular communication and trafficking during inflammation (*IL1B, TNF, IL6, CXCL1, CXCL8, ICAM1, VCAM1*) (110). Although NF-

κB is typically described as a single pathway of activation, it is actually composed of five different family members: p50, p52, RelA (p65), cREL, and RelB. Different combinations of dimers, homo and hetero, will bind to variable κB binding sites in the nucleus and mediate different transcriptional outcomes (111). The mechanism shown in Figure 1.1 describes canonical NF-κB signalling by IKKβ and the p65/p50 subunits of NF-κB. Non-canonical signalling may occur through a different mechanism that is dependent on RelB/p52 activation involving activation of NF-κB inducing kinase (NIK) and IKKα, and mediates biological effects distinct from the canonical pathway (112).

1.5 Orchestration of the innate immune response: immunological communication through cytokines and chemokines

Once activated by microbial stimuli, cells establish a complex communication network through secretion of small (generally less than 20 kDa) signaling proteins known as cytokines which act in an autocrine or paracrine fashion (57). Cytokines can have many effects ranging from both pro and anti-inflammatory functions, chemotaxis, proliferation, as well as effects on cellular differentiation. IL-1β and TNFα are examples of pro-inflammatory cytokines by virtue of their ability to stimulate inflammatory responses (eg. through NF-κB), and essentially serve to propagate an existing inflammatory response in other cells. In contrast, IL-10 is an anti-inflammatory cytokine, and is secreted by several regulatory cell types (eg. T regulatory (113) and myeloid derived suppressor cells (114)) to inhibit the expression of inflammatory transcripts through NF-κB (115). Still other cytokines like TGFβ and IL-6 may have dual pro- and anti-inflammatory functions depending on the context of stimulation and evaluation criteria (115).

IL-1β, the primary pro-inflammatory cytokine of interest in this thesis, undergoes complex regulation and unconventional secretion. These mechanisms are explained in the background section of Chapter 2.

Other inflammatory proteins, such as chemokines, possess strong chemoattractive properties for the recruitment of effector cells. Chemokines are categorized based on the positioning of their first two cysteine residues (ie. XC, CC, CXC, CX3C) and function by binding to G-protein coupled receptors (GPCRs) on their target cells (57). Subsequent migration occurs according to the direction of the increasing chemokine gradient. In neutrophils, signaling through the chemokine receptors CXCR1 and 2 activate pathways involved in cell motility including the Rho, Rac, and ERK1/2 pathways (116, 117). This allows for expression of intercellular adhesion molecules and the reorganization of intracellular actin networks for migration (57). CXCL1 and IL-8 (also known as CXCL8) in particular are extremely potent in recruiting circulating neutrophils to sites of infection, and like many other chemokines, can show some degree of redundancy by sharing the same receptor (eg. CXCR2) (118).

The quantity and nature of the cytokines secreted into the local environment has a profound effect on directing adaptive immune response development through T helper (Th) cell differentiation. T helper cell development is mediated by the local cytokine milieu, in which levels of IL-12, IFNγ, IL-4, IL-2, IL-6, TGFβ, and IL-21 (119), help drive differentiation towards Th1, Th2, Th17, and regulatory T cell (Treg) subsets. This can also dictate responsiveness of effector memory cells upon future inflammatory challenge (119, 120). Taken together, it is clear that appropriate signalling between cells is imperative for effective inflammatory resolution and immune development that causes minimal damage to the host.

1.6 When immune dysfunction occurs: cystic fibrosis as a complex immunodeficiency

Manifestation of innate immune deficiencies in humans can differ in severity depending on the specific mutation and the gene in question. As one would expect, reported mutations in complement, *IRAK4*, *MYD88*, *NEMO*, and other innate immune signaling molecules all lead to deficiencies that result in recurrent infections(121), albeit only by characteristic subsets of microorganisms (primarily *Streptococcus pneumoniae* and *Staphylococcus aureus*) (122-125). Deficiencies in the dsRNA sensor *TLR3* and *UNC93B1*, a gene involved in the trafficking of endosomal TLRs, would be predicted to give rise to viral susceptibility and are indeed characterized by recurring herpes simplex virus infection (126, 127). Conversely, there are other genetic diseases like Cystic Fibrosis (CF), whose aetiology does not directly lie in immune dysfunction, yet displays a severe inability to resolve infections (128). In fact, CF patients exhibit a very broad range of infecting microorganisms (perhaps more so than the immunodeficiencies stated above) (129), implying that the primary defect in CF has broad consequences for the CF lung environment that render deficient many aspects of the immune response.

Cystic Fibrosis is a monogenic disease that results from functional defects in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), an anion channel known to be important for the transport of chloride ions, but also other anionic molecules including bicarbonate and glutathione (128). Although CF airways appear normal at birth, patients soon become infected by a characteristic set of infections at different ages. These include *Haemophilus influenzae*, *Staphylococcus aureus*, *Burkholderia cenocepacia*, *Pseudomonas aeruginosa*,

Stenotrophomonas maltophilia, *Achromobacter xylosoxidans*, *Candida albicans*, non-tuberculous mycobacteria, and *Aspergillus fumigates* (129). *P. aeruginosa*, in particular, will have become one of the most dominant CF pathogens by adulthood, colonizing up to 80% of CF patient airways (129). Although it was initially thought that the majority of lung disease was caused by a few key pathogens (including *S. aureus* and *P. aeruginosa*) (130), this understanding was based on clinical identification methods requiring bacterial culture but with the advent of novel methods in molecular microbiology including polymerase chain reaction (PCR) (131) and sequencing (132, 133), many new potential players in CF lung exacerbations have and continue to emerge, including an underappreciated role for viral and fungal infections in CF (134).

Due to the inability of the CF immune response to successfully clear chronic infection by *P.aeruginosa,* clonal bacterial populations undergo phenotypic conversions in morphotype, motility, virulence, antimicrobial susceptibility, production of adhesion molecules, and exopolysaccharides (135). Many of these changes can contribute to subversion of the immune response by diminishing the expression of virulence factors. These adaptations include the loss of flagella and resistance to phagocytosis (136-138), mutations in type III secretion system components (139, 140), and changes to LPS structure (141, 142). Phenotypic switching to mucoid colonies (143), formation of biofilms (144), and acquisition of antibiotic resistance (145) may also all occur, making clearance of *P. aeruginosa* even more problematic in CF.

Many hypotheses have been put forth to explain why the loss of CFTR function might lead to such profound immunodeficiency. One of the leading explanations is that dehydration of the airway surface liquid (ASL) results in thickened mucous which resists mucociliary clearance by the ciliated epithelium (128). This provides a nutrient-rich and more stable environment where

microorganisms can persist. Mucous from CF patients is found to have elevated levels of free amino acids (146, 147) and iron (148, 149), which should typically be sequestered by molecules including transferrin, lactoferrin, and lipocalin-2 (150). Iron is used as an essential cofactor for microbial enzymes and its levels have been associated with increased amounts of the inflammatory cytokines IL-1β and TNFα (149), and also with biofilm formation (151). Although previously attributed to excessive Na^+ reabsorption through loss of epithelial sodium channel (ENaC) regulation by CFTR (152, 153), new data suggests that this is more strictly due to loss of Cl^- secretion as Na^+ conductance was relatively unaffected in porcine and human airway epithelia (154). However, ENaC involvement may still contribute to disease severity to some degree as its overexpression does give rise to a CF-like phenotype in mice (155) and administration of hypertonic saline in addition to amiloride (an inhibitor of ENaC) to patient lungs improves forced vital capacity as well as respiratory symptoms (156).

Newer studies have found that the pH of the ASL is acidic (157), most likely due to the reduction of bicarbonate secretion through non-functional CFTR (158). This acidic ASL environment inhibits the ability of secreted antimicrobial factors (eg. lactoferrin and lysozyme) in the ASL to kill microorganisms as indicated by the finding that antimicrobial activity can be restored with the addition of bicarbonate (159). Antimicrobial function of lactoferrin and lysozyme were found to be greatly reduced at low pH, despite being found in similar concentrations in the ASL of CF patients when compared to healthy controls (159). β-defensin dysfunction has also been attributed to inactivation by abnormal ASL composition (160, 161). Although this was initially attributed to high salt concentrations in CF ASL, salt-independent functional attenuation was also observed in subsequent studies (162). Low pH also has significant effects on mucous viscosity and mucin aggregation during release from secretory

granules (163). Again, rescue of bicarbonate secretion through CFTR was able to normalize mucous secretion, viscosity, and structure, allowing normalized clearance by ciliated cells (164, 165).

Loss of CFTR in neutrophils has also been implicated in affecting production of reactive oxygen species (166, 167). Although this is somewhat controversial (168), increased markers of oxidation in CF lungs are frequently observed (169-171). Increased reactive oxygen species in the CF lung may oxidize proteins, causing loss of protein function and correlates with the loss of lung function (171). Loss of CFTR can also directly impact secretion of thiocyanate and its subsequent oxidation to hypothiocyanite, a potent antimicrobial, contributing to inefficiencies in microbial killing (172, 173). Thiocyanate levels were found to correlate with better lung function in CF patients, although levels were found to be only slightly reduced in CF pigs and not different in the BALF of CF patients and controls (174). Thiocyanate can also serve as an antioxidant, protecting against hypochlorite generation and excessive inflammation (172). Reduced glutathione (GSH), one of the primary antioxidants in the lung, also undergoes transport into the airways by CFTR (175) and is consequently found to be decreased in bronchoalveolar lavage fluid (BALF) from CF airways and epithelial cells (176).

The CF lung environment can also inhibit the efficacy of cellular components of the immune system. Neutrophils, which are recruited to the lung by the chemokines CXCL1 and IL-8, have impeded motility due to the thickened CF airway mucous (177). Moreover, pathogens such as *Staphylococcus aureus* and *P. aeruginosa* may effectively resist phagocytosis by neutrophils through biofilm formation (178, 179) and persist in the airways. Lung damage may be further affected by secretion of neutrophil elastase (NE), proteinase 3, cathepsin G, and matrix metalloproteinases which can degrade the airway mucin barrier (180) and host tissue

components (181). NE can cleave and inactivate chemokine receptors such as CXCR1 (182), further impairing immune function in the CF airways, as well as process pro-IL-1β into its active form (183). Interestingly, neutrophil apoptosis itself may be a signal for inflammatory resolution. Normally, macrophages that take up apoptotic neutrophils cease production of inflammatory cytokines and lipid mediators and instead begin to secrete anti-inflammatory mediators including IL-10 and TGFβ (54). However, neutrophil apoptosis and clearance has been suggested to be altered in CF, and seem to undergo secondary necrosis in the presence of pathogens (184), thereby preventing proper resolution of the inflammatory response. These immune abnormalities in CF can be further extended to T cells, where the local environment seems to favour a Th2/Th17 phenotype via production of the cytokines IL-1β, 4, 5, 13, 17 (185, 186). This phenotype is thought to be a risk factor for infection as opposed to a more protective Th1 phenotype against *P. aeruginosa* (187).

It has also been suggested that the CFTR may directly interact with pathogens, serving as a receptor for intestinal epithelial uptake of *Salmonella typhi* (188) and airway epithelial uptake of *P. aeruginosa* (189). Inhibition of binding either through the expression of F508del-CFTR or by pre-treatment of the micro-organism with purified CFTR peptide leads to increased bacterial survival, altered inflammatory cytokine production, and cell death (189).

Ultimately, multiple factors promote persistent and recurring infection, and subsequently, sustained inflammatory cycles that eventually cause structural damage and progressive deterioration of lung function. Novel therapies targeting the basic defect in CF have increasingly yielded improvements in lung function and complementary strategies simultaneously targeting both the basic defect and inflammation is a very promising approach for improving health outcomes in CF.

1.7 Anti-inflammatory strategies in the treatment of CF lung disease

Although inflammation is an essential component of effective immune responses, in CF it is chronic, exaggerated, and destructive, making it an attractive therapeutic target to improve lung function. There are many possible anti-inflammatory targets in CF, but due to the complex environment, a concerted effort addressing various aspects of both inflammation and infection is required. Chronic infection and inflammation are hallmarks of CF lung disease, characterized by heavy cellular infiltration by neutrophils and macrophages. Many studies indicate significant increases in the inflammatory mediators IL-1β, IL-6, and IL-8 recovered from the BALF, nasal lavage fluid, or sputum from CF patients (190-192). These mediators are largely responsible for the excessive recruitment of leukocytes into the lung. The recruited cells ultimately appear incapable of clearing infections and cause additional damage by releasing active proteases and generating free radicals. While chronic infection is certainly a key factor in the sustained inflammation observed in CF, there is also evidence that inflammatory responses might be intrinsically dysregulated in CF. It has been reported that CFTR is a negative regulator of NF-κB activation, and that CFTR function helps to suppress inflammatory responses (193). Endoplasmic reticulum (ER) stress is another possible contributing factor that has been increasingly found to be important in many inflammatory diseases. Although close to 70% of CF patients possess at least one copy of the F508del *CFTR* mutation (194), which may contribute to ER stress directly (195), there are multiple sources of ER stress that also occur extracellularly in the CF lung environment that are predicted to exacerbate inflammation (196).

Further evidence linking inflammation and CF lung function can be garnered from several genome-wide association studies (GWAS) (197). These human studies have identified several innate immune genes affecting lung function in CF. MBL2 is a secreted innate effector which binds to carbohydrate moieties on microorganisms (198). Interestingly, although MBL2 deficiency is predicted to predispose individuals to severe respiratory tract infections (199), its high prevalence in the human population (200) may indicate that its functional importance is diminished except in certain populations with immune dysfunction. Though findings have been inconsistent, meta-analysis of existing MBL2 studies has found that overall, patients deficient in MBL2 have been associated with worse lung function, earlier acquisition of *P. aeruginosa*, and increased incidence of death or need for lung transplantation (201). This has been especially observed in the presence of polymorphisms in the cytokine TGFβ, where MBL2 and TGFβ exhibit gene-gene interactions to associate with lung function decline and age of *P. aeruginosa* acquisition (202). The cytokine TGFβ is secreted by airway epithelial cells upon stimulation and itself plays an important role in T cell development of a Th17 or regulatory phenotype (203, 204). TGFβ also has a pro-fibrogenic role in the airways (205, 206) and may mediate structural changes in the CF lung that ultimately impair airway clearance. Finally, polymorphisms in *IFRD1* have also been associated with disease in CF patients, potentially resulting from effects on neutrophil oxidative index and TNFα production (207).

Neutrophilic inflammation is considered a hallmark of CF lung disease, and may itself result in significant lung damage by releasing reactive oxygen/nitrogen species and proteases (208). Genetic evidence for this can be derived from polymorphisms in *CXCL8*, a neutrophil chemokine, which have been associated with lung disease severity in CF (209). CF patients from a German cohort who possessed a specific haplotype for *CXCR1* and *CXCR*2, the cellular

receptors for IL-8, had decreased expression of CXCR2, reactive oxygen species generation, and bacterial killing by neutrophils (210). This supports a previous study where CXCR1 cleavage by proteases already present in early CF lung disease, led to reduced bactericidal activity in neutrophils (182). Polymorphisms in the inflammatory genes *IL1B*, *TLR9*, *TNFα*, *CD95*, *STAT3*, and *TNFR* have also been identified to impact Cl^- transport across nasal and intestinal epithelia, in a CF twin/sibling cohort with extreme disease phenotypes (182). These associations provide evidence that inflammatory genes can modify the basic defect in CF, and therefore potentially affect disease severity.

Recently, the largest CF GWAS to date (n = 6, 365) was established through meta-analysis of North American and European cohorts. This study identified five genetic loci in *MUC4/MUC20*, *SLC9A3*, HLA class II genes, *AGTR2/SLC6A14*, and *EHF/APIP* in which polymorphisms were significantly associated with changes in lung function (211). Several genes identified through CF GWAS are known to be involved in inflammation and host defence, these include IFRD1 (207) which has demonstrated important roles in antimicrobial activity mediated by neutrophils, as well as HLA class II (211) which is important in presentation of foreign peptides to the immune system, and the mucin MUC4 (211) whose abnormal expression has been associated with inflammatory diseases including Chron's disease (212).

1.8 Current anti-inflammatory therapies in CF

Until recently, CF therapies were centered on the use of antibiotics and physiotherapy to fight infections and clear the airways. But now, the landscape of CF treatment strategies has been transformed by the successful introduction of the CFTR potentiator, Ivacaftor, which has

resulted in significant increases in lung function and weight gain in patients (213, 214). Unfortunately, Ivacaftor is only effective in a small subset of patients as less than 3% of patients worldwide possesses the G551D mutation (194). Chemical correctors that rescue F508del CFTR localization (ie. Lumacaftor) in combination with Ivacaftor have yielded some improvements in lung function and have reduced exacerbations (215). However, this could only be observed in patients homozygous for the mutation and heterozygotes did not experience improvement in forced expiratory volume (FEV1) (216). Therefore, anti-inflammatory strategies that supplement chemical correctors and potentiators are still expected to be helpful in improving lung function. Some antibiotics, such as the macrolide azithromycin, have shown efficacy in fighting both microbial growth and the inflammatory component of CF lung disease, and has become a mainstay of CF treatment regimens (217). Its anti-inflammatory effects have been shown to occur through multiple mechanisms including the breakdown of biofilms (218) by inhibition of quorum sensing (219), inhibition of inflammatory responses through NF-κB and AP-1 (220), and even restoration of chloride efflux in CF cells (221, 222). Other therapies including Dornase alfa (recombinant deoxyribonuclease I) and hypertonic saline reduce mucous viscosity and improve clearance also help to break the cycle of infection and inflammation in the airways (223-225).

Clinical trials using corticosteroids (prednisone) and high-dose ibuprofen have proven that anti-inflammatory therapies can improve clinical outcomes or delay lung function decline (226, 227). However, long-term exposure to systemic corticosteroids leads to high rates of adverse effects including stunted growth (228), muscle weakness (229), brittle bones (229, 230), while inhaled corticosteroids were not found to provide any benefit (231). High-dose ibuprofen was effective in slowing decline of lung function and body weight, especially in children (232, 233).

Although found to be well-tolerated, concerns over the development of adverse side effects including renal and gastrointestinal toxicity that may have limited its use in the clinic (233, 234). Many specific anti-inflammatory medications have been in development to help reduce non-specific effects, targeting anything from chemokines to oxidative stress. However, the majority have been plagued by an inability to show efficacy in clinical trials. Supplementation of low antioxidant levels with inhaled glutathione did not show any benefit in the reduction of oxidative stress markers, inflammation, or lung function (235). Additionally, a clinical trial involving long-term (~6 months) use of oral N-acetylcysteine, the precursor to cysteine and glutathione, showed stabilization of lung function but again had no effect on inflammatory markers (236). Treatment of patients with a leukotriene B4 receptor (LTB_4) antagonist had to be terminated early due to a significant increase in pulmonary exacerbations (237) and additional examination in murine models found that its application increased numbers of *P. aeruginosa* in the lungs (237). Given the Th2/Th17 biased nature of the CF inflammatory response (185), a clinical trial was conducted involving recombinant IFNγ administration as an aerosol to induce a protective Th1 respsonse in the lungs. However, this also did not provide any benefit in reducing inflammatory markers or microbial growth, and may have even increased the number of hospitalizations resulting from pulmonary exacerbations (238). Anti-protease therapies including inhalational α1-antitrypsin and secretory leukoprotease inhibitor have demonstrated some efficacy in small studies and investigations into efficacy are still ongoing (227).

Given the important contribution of neutrophils to lung damage in CF, an appealing strategy involves targeting neutrophilic inflammation through CXCR2 antagonism, where CXCR2 is a shared neutrophil receptor for the chemokines CXCL1 and IL-8. Although a trial involving

treatment with an oral CXCR2 antagonist did not produce any changes in FEV1 or forced vital capacity (FVC) over two months, they did observe a small decrease in sputum inflammatory biomarkers (neutrophils, elastase, and free DNA) (239). An additional study examining IL-8 inhibition in CF sputum showed promise but did not proceed into clinical trials, citing various other chemotactic contributions in CF sputum in addition to IL-8 (C5a, LTB_4, platelet activating factor) (240). From these studies, it appears that a combination therapy targeting complementary aspects of neutrophil chemotaxis may ultimately be required to achieve attenuation of the neutrophil-dominated response.

Researching the basic inflammatory mechanisms of CF may open many new therapeutic options that may not be limited to CF alone, but to a spectrum of chronic diseases including COPD and asthma. Research in this field has been extremely informative for the development of novel anti-inflammatory strategies and shows much promise in improving quality of life for CF patients and patients of other chronic diseases.

1.9 Beyond CF: targeting inflammation in cancer

Like in CF, the relationship between inflammation in cancer development and immunity is complex. A strong contribution of infection and inflammation to cancer development has been established from the high frequency of cancer among patients with inflammatory bowel disease, chronic *Helicobacter pylori* infection, and chronic hepatitis (241). In CF, there is surprisingly no increased risk of lung cancer development, but rather an increase in the risk of digestive tract cancers as well as testicular cancer and lymphoid leukemia (242-244). On the other hand, some infections have also been previously associated with tumour regression (245-247) and several TLR agonists have been successfully employed in the clinic including the TLR7/8 agonist

imiquimod and the TLR2/4 agonist Bacillus Calmette-Guérin (82). It appears that the inflammatory response can play important roles in both promoting and opposing tumour development, whereby chronic, low level TLR activation may promote tumour formation but strong TLR activation may prevent it (82).

One of the primary challenges in the control of tumour growth by the immune system is the resistance to apoptosis of tumour through normal intrinsic cellular mechanisms and the over-expression of inhibitory molecules by tumour cells that mediate evasion of immunosurveilance (248). To overcome this, it is possible that given the correct inflammatory environment, immune responses to tumour cells can be initiated by inducing cell death that also activates the immune system through damage-associated molecular patterns (DAMPs) (ie. immunogenic cell death (ICD)). These include molecules such as HMGB1, ATP, calreticulin, and nucleic acids, which are typically sequestered in healthy cells but can become exposed in tumour cells as well as during cell injury (249). Activation of dendritic cells by tumour DAMPs can subsequently promote cytotoxic T cell responses against tumours (249, 250), while activation of the production of IFNγ by NK cells and other cell types can also induce anti-tumour M1 macrophage responses (54). However, DAMP activation of PRRs can also have opposing effects on tumour immunity. TLR activation triggers production of inflammatory cytokines, including IL-1β, TNFα, and IL-6 which can act on tumour cells to promote survival and proliferation through NF-κB and STAT3 activation (251). IL-1β and TNFα can also promote tumour spread through their ability to promote vascularization and upregulation of adhesion molecules that may aid in tumour dissemination (252). Indeed, serum IL-1β and IL-6 levels typically correlate with worse cancer prognosis and studies that have targeted IL-1β have found positive effects on slowing cancer progression and mestastasis (253, 254).

Although supplemental TLR activation has been long considered for use in augmenting efficacy of cancer treatments, there is currently no clear consensus in the literature as to whether TLR activation strategies are beneficial in cancer and effects are highly dependent on the specific TLR in question and the type of cancer (82). In light of the high similarity in signaling between different TLRs, studies examining TLR4 activation, which employs both MyD88 and TRIF adaptors (85), have mostly described cancer-promoting effects. However, TLR3 activation, which only recruits the TRIF adaptor (85), has been mostly observed to produce anti-tumour effects (82). Activating mutations in MyD88 have a tumour promoting role (255) and knockout of either MyD88 or TLR4 yield reductions in chemically-induced colon and skin cancer development (256, 257). One TLR4 agonist of particular interest is monophosphoryl lipid A (MPL), which seems to preferentially signal through the TRIF pathway (258) and may also help to minimize the tumour-promoting effects of the MyD88 pathway (255). MPL still signals through cell surface TLR4 as opposed to endosomal TLR3, but is significantly less toxic than LPS, making it more amenable for use *in vivo* (258).

Overall, it does not appear that TLR monotherapy is sufficient to successfully combat most cancers, and in many cases may even have detrimental pro-inflammatory effects. However, combination therapies that employ TLR signaling to appropriately stimulate the adaptive immune response while also directly increasing tumour cytotoxicity may be a useful strategy in developing a functional immune response against tumour antigens.

1.10 Targeting the ubiquitin-proteasome system as a cancer treatment strategy

Despite the relative lack of effective TLR monotherapies in cancer, a particularly interesting strategy could see the use of TLR adjuvants in combination with inhibitors of the ubiquitin-proteasome system (UPS) as a method to induce immunogenic tumour cell death. Using this strategy, tumour cells undergoing UPS-mediated cell death could do so in an increasingly inflammatory environment which may facilitate development of an anti-tumour immune response. Targeting of the ubiquitin-proteasome system (UPS) in the form of the proteasome inhibitor bortezomib has already been quite successful in fighting hematologic, particularly B-cell, malignancies, such as multiple myeloma (259) and non-Hodgkin's lymphomas (eg. mantle cell lymphoma (260) and Waldenström's macroglobulinemia (261)).

The UPS is the regulatory system involved in normal protein turnover in cells. It is composed of a network of three types of enzymes: E1 (ubiquitin activating enzymes), E2 (ubiquitin conjugating enzymes), and E3 (ubiquitin ligases), which are responsible for covalent modification of proteins with ubiquitin, a 76 amino acid protein which targets proteins for degradation when attached to specific lysine residues (262). Proteins that have been polyubiquitinated can now be recognized by the 19S regulatory cap subunit before being degraded by the catalytic core (263).

Some common characteristics of tumour cells are the loss of cell cycle regulation (sustained proliferation), increased error rate of DNA replication, activation of invasion and angiogenesis as well as suppression of death signals (248). As a consequence, transformed cells tend to accumulate large quantities of protein, and to deal with this increased load, tumour cells

increase expression and activity of proteasomes (264, 265). At the same time, this increases basal levels of ER stress and makes these tumour cells more vulnerable to proteasome inhibitor-induced cell death. These characteristics make multiple myeloma cells an excellent target of proteasome inhibition, where the highly proliferative cell type (B cell precursor) must also deals with high protein loads in the ER, making them susceptible to proteasome inhibition and ER stress-induced death mechanisms (266).

Proteasome inhibition can display anti-tumour activity through a range of mechanisms: upregulation of pro-apoptotic proteins (267) and suppression of anti-apoptotic proteins (268), and inhibition of NF-κB (a survival factor) (269). Proteasome inhibition can also limit spread of the tumours through inhibition of cell cycle progression (270), inhibition of angiogenesis (271), and downregulation of adhesion molecules needed for migration (272). Apoptosis induction by proteasome inhibition has been observed to involve both the intrinsic pathway mediated by caspase-9 (273) and the extrinsic pathway mediated by caspase-8 (274). Bortezomib treatment results in the accumulation of pro-apoptotic Bax and Bak at the mitochondria (275) and the subsequent release of cytochrome c along with other pro-apoptotic markers (276) leading to apoptosome formation and caspase-9 activation (277). Bortezomib treatment also activates caspase-8 in a manner dependent on the induction of autophagy (274) and may also sensitize cells to TRAIL-dependent apoptosis signals (278). Additionally, death in cells undergoing proteasome inhibition could be modulated through supplementation with additional amino acids (primarily cysteine) (279), inhibition of the ER stress transcription factor XBP1s (280), and may also involve caspase-independent mechanisms (281).

Overall, targeting of the UPS provides a more specific means by which to target tumour cells due to their sensitivity to alterations in proteostasis. While this is true for haematological

malignancies such as multiple myeloma, solid tumours, which may not be as susceptible to ER stress, may require additional treatment strategies. This is a gap that could potentially be filled by the use of TLR adjuvants which could be used not only to sensitize cells to ER stress-mediated cell death, but to also help generate an anti-tumour immune response by an appropriate inflammatory milieu.

1.11 Summary of thesis objectives

The innate immune system represents powerful a mechanism through which the body maintains homeostasis in light of constant microbial challenge. However, ineffective regulation of innate immune responses may likewise lead to excessive damage to the host and the eventual development of adverse cellular events including tumorigenesis and auto-inflammatory disorders. Understanding the mechanisms involved in achieving an effective but measured innate immune response will allow us to identify key processes that can be manipulated to improve therapeutic options in a wide range of diseases, ranging from cystic fibrosis to cancer.

One of our main objectives was to conduct basic research in a way that could have strong clinical implications or translatability. As such, we established our disease models using cell lines in order to better control for inter-individual variation, but have emphasized replication in primary samples throughout our studies, either through the use of patient cells or by interrogating patient data from CF genome-wide association studies. In the first study presented, our objective was to define the origins of increased amounts of IL-1β typically observed in the lungs of CF patients. In the second study, our objective was to define a mechanism through which IL-1β could affect neutrophilic inflammation via airway epithelial cells under conditions of ER stress. Finally, in the third study, our objective was to expound on

the relationship between ER stressors and IL-1β production, specifically through the use of proteasome inhibitors and its potential effect on cancer therapies. We hope that these studies will provide valuable mechanistic insight into how inflammation and innate immune system can be successfully manipulated in various inflammatory diseases.

CHAPTER 2: INFLAMMASOME ACTIVATION STATUS AND IL-1B PRODUCTION IN PATIENTS WITH CF

2.1 Rationale: CF is characterized by elevated levels of pro-inflammatory cytokines in the airways including increased levels of IL-1β (185, 190, 191). IL-1β plays an especially important role in the induction of other pro-inflammatory cytokines and chemokines, including IL-6 (282), IL-8 (283), and CXCL1 (284), consequently amplifying the inflammatory response through neutrophil recruitment (285, 286). *IL1B* polymorphisms have also been previously associated with disease severity (287), and manifestation of the basic defect (chloride conductance) in CF (288). As a major pathway of IL-1β maturation is mediated by the inflammasomes, their activation may have an important role in driving excess inflammation in CF. The purpose of this study was to determine whether inflammasome activation is an intrinsically dysregulated process in CF, and specifically whether dysregulation of CFTR activity leads to increases in IL-1β production.

2.2 Background: IL-1β is a major inflammatory mediator and pyrogen. Its physiological effects are potentially important to the pathogenesis of lung exacerbations in CF, including the recruitment of inflammatory effector cells, the induction of other pro-inflammatory cytokines such as IL-6, IL-8, and CXCL1 (283, 284), as well as the developing of T cell responses towards an inflammatory Th17 phenotype (289). IL-1β is initially synthesized in the cytosol as a biologically inactive precursor (pro-IL-1β) in response to PRR activation. This 33 kDa pre-protein is subsequently converted into its active form by cytosolic multimeric protein complexes named "inflammasomes." Inflammasomes oligomerize in response to cellular danger signals (ATP, uric acid, and pathogen components) and mediate the auto-activation of

caspase-1 (290) through association with the protein sensor (NLR, RIG-I, AIM2, etc) and the adaptor protein ASC (93, 95). Active caspase-1 then proceeds to cleave pro-IL-1β (291) into its biologically active form for secretion (Figure 1.2). IL-1β is relatively unique compared to other cytokines. It does not possess a signal peptide that allows for conventional secretion through the ER and Golgi (292). Rather, it appears to depend on a vesicular secretion mechanism that can involve caspase-1 and pyroptotic cell death (293) and mediators of autophagy (294). A related cytokine, IL-18, has been found to be processed in the same manner and its active form has important roles in the induction of IFN-γ and activation of Th1 cells (295). Alternatively, IL-1β can also be activated by other proteases including caspase-8, neutrophil elastase, proteinase-3, and cathepsin G, although at somewhat differing cleavage sites (296). Secreted active IL-1β signals through the IL-1 Receptor (IL-1R) which shares the same signalling machinery as most TLRs (ie. MyD88, TRAF6) (297). This initiates pro-inflammatory signalling in nearby cells to activate NF-κB which can proceed to secrete their own inflammatory mediators (297).

There are a multitude of PAMPs and DAMPs that can prime and activate the inflammasome in the CF lungs, including Burkholderia species (298), *P. aeruginosa* (299), and *S. aureus* (300). Cellular danger signals including ATP and uric acid from dying host cells can also trigger inflammasome activation in the presence of the PAMP-rich CF lung environment. *P. aeruginosa*, one of the most common and clinically relevant pathogens among CF patients, activates the NLRC4 inflammasome through its type III secretion system (301, 302). Inflammasome responses depend on NF-κB signaling, where NF-κB is important in both the upregulation of specific inflammasome components, as well as IL-1β expression (303-305). Therefore, we hypothesize that the degree of inflammasome activation and IL-1β production

will be higher in CF cells, and this will be caused either by increased extracellular priming of TLRs or by intrinsically increased NF-κB activity in CFTR-deficient cells.

Aim: To determine whether CF cells innately produce increased amounts of IL-1β through dysregulation of CFTR activity

2.3 Materials and methods

Ethics Statement

Blood samples were obtained with informed written consent from control subjects and CF patients at the BC Children's Hospital. Consent was obtained for children by their parent or legal guardian. Subjects 7 years of age and older were required to provide informed assent as well. Protocols were approved by the Clinical Research Ethics Board (H09-01192).

Cell Culture

CF (IB3-1 and CuFi-1) and control (S9 and NuLi-1) cells were obtained from the American Type Culture Collection.IB3-1 cells were derived from a patient expressing the ΔF508 and W1282X mutations and CuFi-1 were derived from a ΔF508 homozygous patient. S9 cells are IB3-1 cells that have been transfected with CFTR using an adeno-associated viral vector and NuLi-1 cells were derived from a patient possessing a wild-type CFTR genotype. THP1-XBlue cells stably express a secreted embryonic alkaline phosphatase (SEAP) reporter inducible by NF-κB and AP-1 (Invivogen). Cells were cultured as recommended by their respective suppliers using standard protocols. S9 and IB3-1 cells were cultured in basal LHC-8 (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS), 2mM L-glutamine, and 1mM sodium pyruvate. NuLi-1/CuFi-1 cells were cultured in BEBM serum-free medium

(Lonza) with supplement bullet kit (EGF, hydrocortisone, bovine pituitary extract, transferrin, bovine insulin, triiodothyronine, epinephrine, retinoic acid), 2 mM L-glutamine, and 1mM sodium pyruvate. PBMCs from CF patients and controls were cultured in RPMI-1640 (Hyclone) supplemented with 10% FBS, 2 mM L-glutamine, and 1 mM sodium pyruvate (complete RPMI). THP1-XBlue cells were cultured in complete RPMI with the addition of zeocin (100 μg/ml) to select for cells expressing the SEAP NF-κB/AP-1 reporter. Prior to stimulation, bronchial epithelial cell lines were plated in coated 96-well plates (BD Biosciences) at 3 x 10^4 cells/well unless indicated, and allowed to adhere overnight. Plates for S9 and IB3-1 stimulations were coated in a mixture of bovine serum albumin (10 μg/cm^2), fibronectin (1 μg/cm^2), and bovine collage type I (3.3 μg/ cm^2) (BD Biosciences). Plates for NuLi-1 and CuFi-1 were coated with collagen type IV (6 μg/ cm^2) (Sigma Aldrich). PBMCs were plated in 96-well plates at a density of 1.5 x 10^5 cells/well in 200 μl (7.5 x 10^5 cells/mL). THP-1 reporter cells were differentiated into a macrophage-like phenotype using 50 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich) for 24 hours at a density of 1 x 10^5 cells/well in 200 μl (5 x 10^5 cells/ml). Cells were washed with PBS and allowed to rest a further 42 hours prior to stimulation.

CF and control subject PBMCs

The diagnosis of CF was established by typical clinical features, increased sweat chloride concentrations (> 60 mM), and detection of CF-inducing mutations. All patients with CF were clinically stable at the time of blood donation, and any subjects who were receiving systemic corticosteroids were excluded due to potential immunomodulatory activity. Control samples were provided by healthy adult volunteers. In previously published work we have demonstrated that TLR-mediated inflammatory responses are stable in humans from birth to 60 years old

(306), and therefore did not age-match the CF patients and control subjects. Peripheral blood was collected in sodium heparin tubes (BD Biosciences) and PBMCs were isolated using density gradient centrifugation on Ficoll-Paque^{TM} Plus (GE Healthcare). The layer containing PBMCs was isolated, washed twice in PBS and resuspended in complete RPMI. Cells were enumerated by trypan blue exclusion using the Countess automated cell counter (Invitrogen). For derivation of macrophages from monocytes, monocytes were allowed to adhere to plastic for 2 hours in RPMI 1640 after which non-adherent cells were removed. Monocytes were allowed to differentiate in RPMI 1640 supplemented with 10% human AB serum for 10 days.

Cell stimulation and cytokine quantification

Bronchial epithelial cells were plated and allowed to adhere overnight prior to stimulation. Bronchial epithelial cells were rested or primed with LPS for 5 hours and stimulated with live *P. aeruginosa* PAO1 or ATP for the times indicated. PBMCs and THP-1 reporter cells were either rested or primed with LPS (Invivogen) or heat-killed PAO1 overnight (16 hours). The next day the cells were challenged with live PAO1, PAO1Δ*exsA*, ATP (Invivogen), or Poly(dA:dT) (Sigma Aldrich) for the times indicated (see Fig. 1). For stimulations with Poly(dA:dT), lipofectamine LTX was used at a 1:1 (w:v) ratio of μg of DNA to μl of lipofectamine and was mixed 30 minutes prior to stimulation. The NF-κB inhibitor Bay11-7082 (Invivogen) was added to cultures 1 hour prior to TLR priming. If no priming was involved, inhibitor was added 1 hour prior to inflammasome stimulation. The CFTR inhibitor $CFTR_{inh}172$ (Sigma Aldrich) was added to cultures 18 hours prior to inflammasome stimulation. The caspase-1 inhibitor z-YVAD-fmk (Biovision) was added to cultures 1 hour prior to inflammasome stimulation. Supernatants were collected and stored at -20˚C. Cytokines released into supernatants from PBMCs stimulated with inflammasome activators were quantified using

sandwich ELISA (eBioscience). Overnight priming (16 hours) of cells was done to increase the amount of pre-formed pro-IL-1β prior to inflammasome stimulation and to help standardize inflammasome stimulation and harvest times since patient sample collection times could vary widely throughout the day.

CFTR activity assay

N-(Ethoxycarbonylmethyl)-6-Methoxyquinolinium Bromide (MQAE) is an intracellular dye that is quenched by chloride ions. MQAE (5 mM) was added 1 hour prior to stimulation in a buffer consisting of 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 5 mM glucose, and 5 mM HEPES. To make a chloride-free buffer, $NaNO_3$, KNO_3, $MgSO_4$, and $CaSO_4$ were used in place of the aforementioned chloride-containing salts in equimolar amounts. Cells were washed in chloride-containing buffer prior to replacement with chloride-free buffer containing forskolin. If CFTR inhibitor was used, this was added at the time of MQAE incubation and also at time of buffer replacement. Cells were monitored for changes in fluorescence over 6 hours, reading at 350/460 nm excitation/emission.

Immunoblotting

$1x10^6$ cells were seeded in 12-well plates, stimulated as indicated, and lysed in RIPA buffer supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Scientific). Protein concentrations were determined by Bradford assay (Thermo Scientific). Lysates were resolved by electrophoreses on 10% SDS-polyacrylamide gels and transferred onto PVDF membranes (Millipore). Blots were blocked for 1 hour at room temperature and probed overnight at 4˚C for pro-IL-1β (Santa Cruz), IκBα (Cell Signaling), or β-actin (Cell Signaling). Blots were subsequently probed with fluorescently-labeled secondary antibodies, IRDye 680 or 800CW (LI-COR Biosciences) for 1 hour. Both blocking and probing steps were carried out in

tris-buffered saline (G Biosciences) containing 5% bovine serum albumin and 0.1% TWEEN 20 (Calbiochem). Blots were imaged on a LI-COR Odyssey infrared imaging system (LI-COR Biosciences) and quantified using the included analysis software.

Quantification of caspase-1 activity

Bronchial epithelial cells were plated in 6-well plates at 5 x 10^5 cells/well overnight. Cells were primed with LPS for 5 hours and stimulated with ATP for 1 hour or stimulated with live PAO1for 3 hours. PBMCs were stimulated the same day as blood donation. PBMCs were seeded in a 96-well plate at a density of 4.5 x 10^5 cells/well (2.5 x 10^6 cells/ml), primed with LPS for 5 hours and then stimulated with ATP for 1 hour or Poly(dA:dT) for 3 hours or stimulated with live PAO1 for 3 hours. Caspase-1 activity was measured using FLICA (Immunochemistry Technologies), a cell-permeable fluorescent probe (FAM-YVAD-fmk) that binds active caspase-1. Cells were incubated 1 hour with FLICA at 37°C and stained with PE-Cy7-conjugated anti-CD14 antibodies (eBioscience) to identify monocytes. The gating strategy consisted of including live cells that were CD14 positive which were subsequently analyzed for the frequency of FLICA-positive cells.

NF-κB/AP-1 Activity Assay

Supernatants from THP-1 reporter cells were incubated with Quanti-Blue substrate (Invivogen) at 37˚C and allowed to develop for 16-18 hours. Quanti-Blue contains a substrate for alkaline phosphatase and changes in the amount of NF-κB/AP-1 activity were quantified by optical density(λ = 655) measured using a SpectraMax 384 Plus plate reader and SoftMax Pro software (Molecular Devices).

Bacterial strains

P. aeruginosa laboratory strains PAO1 and the PAO1Δ*exsA* mutant were obtained from Dr. Robert Hancock. *P. aeruginosa* strains PAO1 and PAO1Δ*exsA* were grown from overnight cultures in Luria Bertani (LB) broth and LB + streptomycin (150 μg/ml) until mid-logarithmic phase. Cells were washed once in PBS and resuspended in PBS to an optical density of 0.5 (λ = 600 nm). To prepare heat-killed bacteria, live PAO1 was resuspended in PBS to an optical density of 0.5 and heated at 60° C for 1 hour. For stimulations, live PAO1 was resuspended to an optical density of 0.5 in PBS and further diluted in culture medium prior to stimulation to achieve the desired multiplicity of infection. Heat-killed PAO1 was added in a volume equivalent to that used to achieve an MOI of 1 for live PAO1.

Statistics

All graphs display the mean $\pm$ SEM and were generated with Prism 5 (Graphpad). Statistical significance was determined by performing one or two-way ANOVA and the Bonferroni, Dunnett's, or Kruskal-Wallis post-test where applicable. *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

2.4 Results

Airway epithelial cells do not produce significant amounts of IL-1β in response to inflammasome stimulation

The inflammasomes and their respective activators examined in this study are listed in Table 2.1 and cells were stimulated in accordance with the schedule in Figure 2.1. In CF, airway epithelial cells have been shown to possess a hyper-inflammatory phenotype and produce an

exaggerated pro-inflammatory cytokine response (307, 308). To determine if airway epithelial cells contribute to the increased IL-1β production in patients with CF, CF (IB3-1, CuFi-1) and control (S9, NuLi-1) bronchial epithelial cell lines were stimulated with the inflammasome inducers *P. aeruginosa* strain PAO1 (PAO1) and LPS followed by ATP. IL-1β levels in cell culture supernatants were not greatly increased in either the CF or control cell lines (Fig. 2.2A-D), although a small increase in IL-1β production was detected in NuLi-1 and CuFi-1 cells, but not in S9/IB3-1 cells by 24 hours. Despite the lack of IL-1β production, these airway cells were highly responsive to recombinant IL-1β, producing large quantities of IL-8 over 24 hours (Fig. 2.2A-D inserts).

Table 2.1: Inflammasome activators used in this study

Inflammasome	Inflammasome activator
NLRP3	ATP (Concentration: 5mM)
NLRC4	*Pseudomonas aeruginosa* strain PAO1. (Multiplicity of Infection (MOI) = 1)
AIM2	Poly(dA:dT) with transfection (Concentration: 1 μg/ml)

The inflammasome activators used in this study and the corresponding inflammasomes which they are expected to activate.

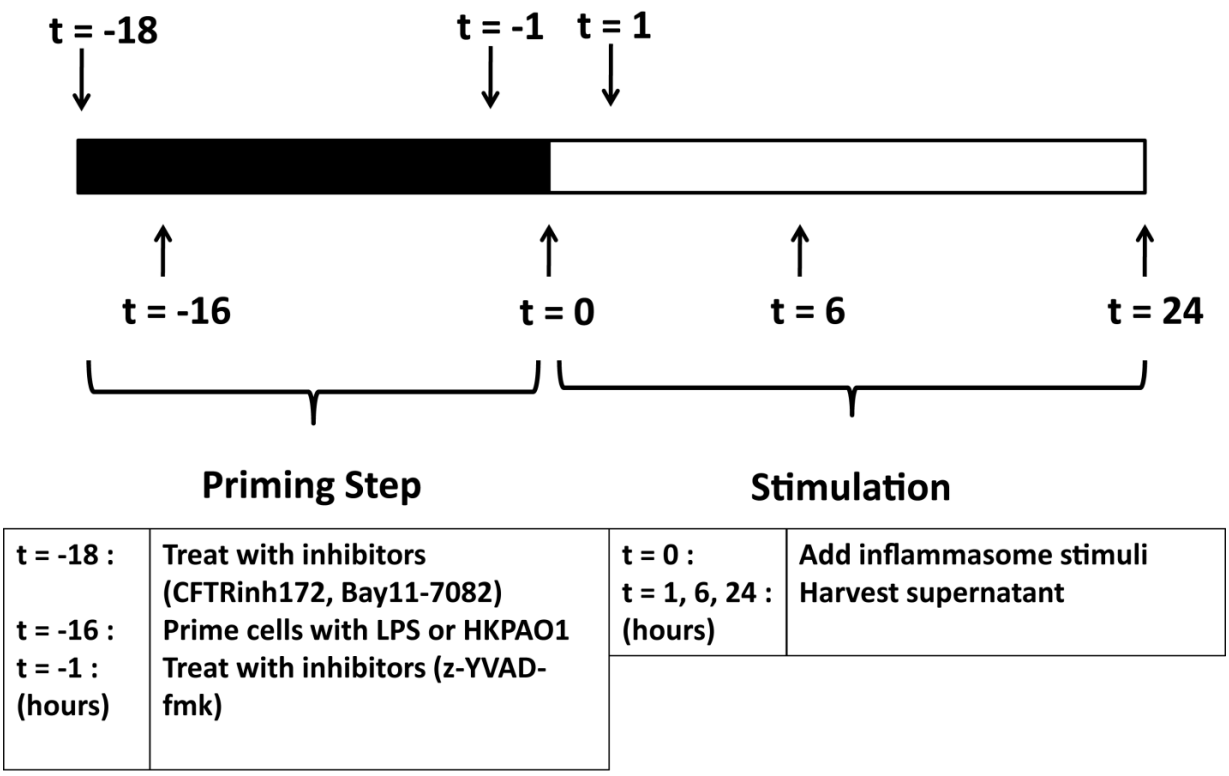


Figure 2.1: Cell stimulation and inhibitor schedule

This schedule outlines the timing of inhibitor addition and priming in relation to inflammasome stimulation (t = 0) for THP-1 reporter and PBMC cytokine quantification experiments. Inhibitor treatments and stimulations were carried out as described in the Materials and Methods section.

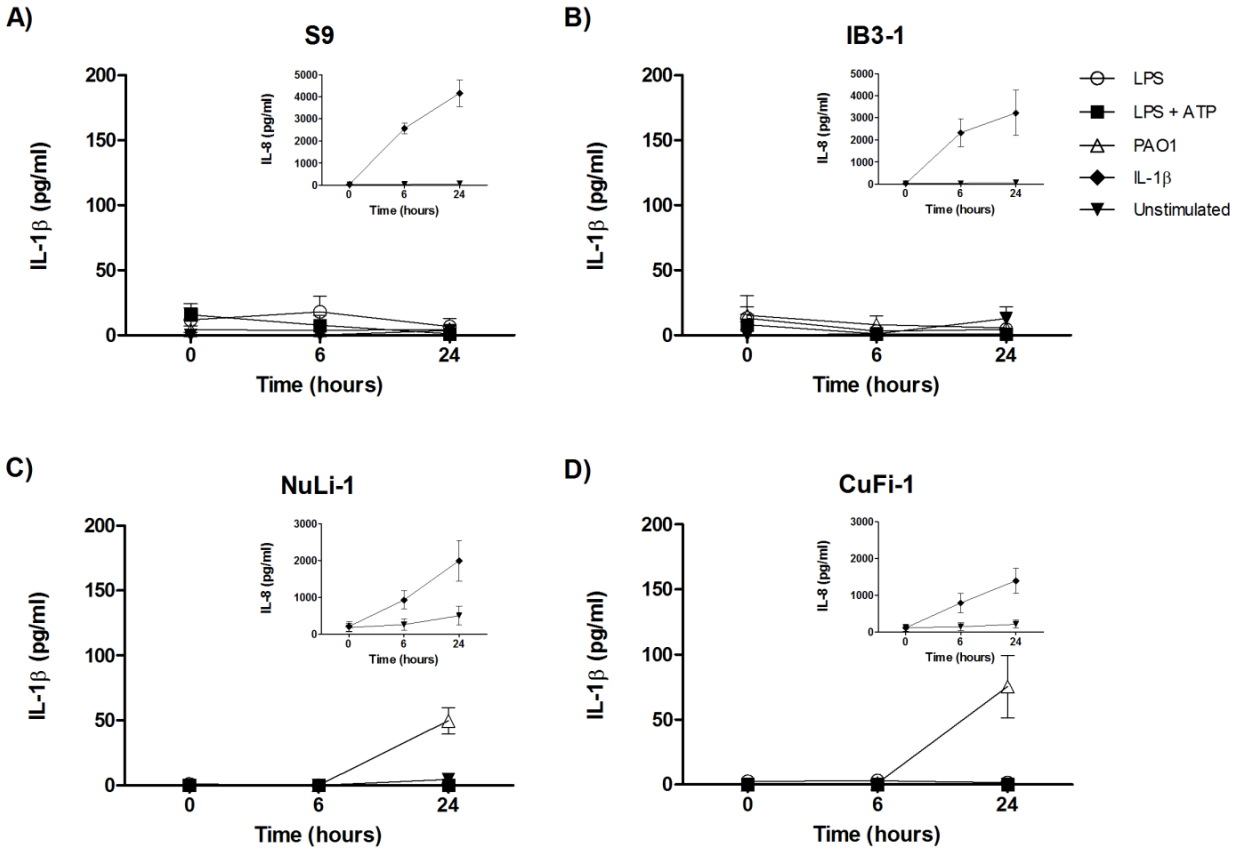


Figure 2.2: Airway epithelial cells do not produce significant amounts of IL-1β in response to inflammasome stimulation

Control cell lines ((A) S9, (C) NuLi-1) and CF cell lines ((B) IB3-1, and (D) CuFi-1) were stimulated with *P. aeruginosa* (MOI = 10), ATP (5 mM), or IL-1β (10 ng/ml), for the indicated times (n = 3). Cells that were primed with LPS (100 ng/ml) were done so for 4 hours prior to stimulation with ATP. Cell culture supernatants were assayed for IL-1β and IL-8 production by ELISA. Insert shows IL-8 secretion in response to stimulation with IL-1β (10 ng/ml).

Airway epithelial cells do not significantly upregulate caspase-1 activity in response to inflammasome stimulation

To examine if inflammasome activation occurs in these airway cells, caspase-1 activity was quantified by flow cytometry using a FLICA probe. There was no significant increase in the percentage of FLICA-positive cells upon stimulation with live PAO1 or LPS + ATP at the times examined (Fig. 2.3A-B). Because previous studies have also indicated a role for caspase-1 in the activation of NF-κB through TLR signalling (309), we examined whether chemical inhibition of caspase-1 altered NF-κB-dependent IL-6 production in response to *P. aeruginosa*. However, we did not find that treatment with the caspase-1 inhibitor z-YVAD-fmk altered IL-6 secretion by airway epithelial cells (Fig. 2.3C).

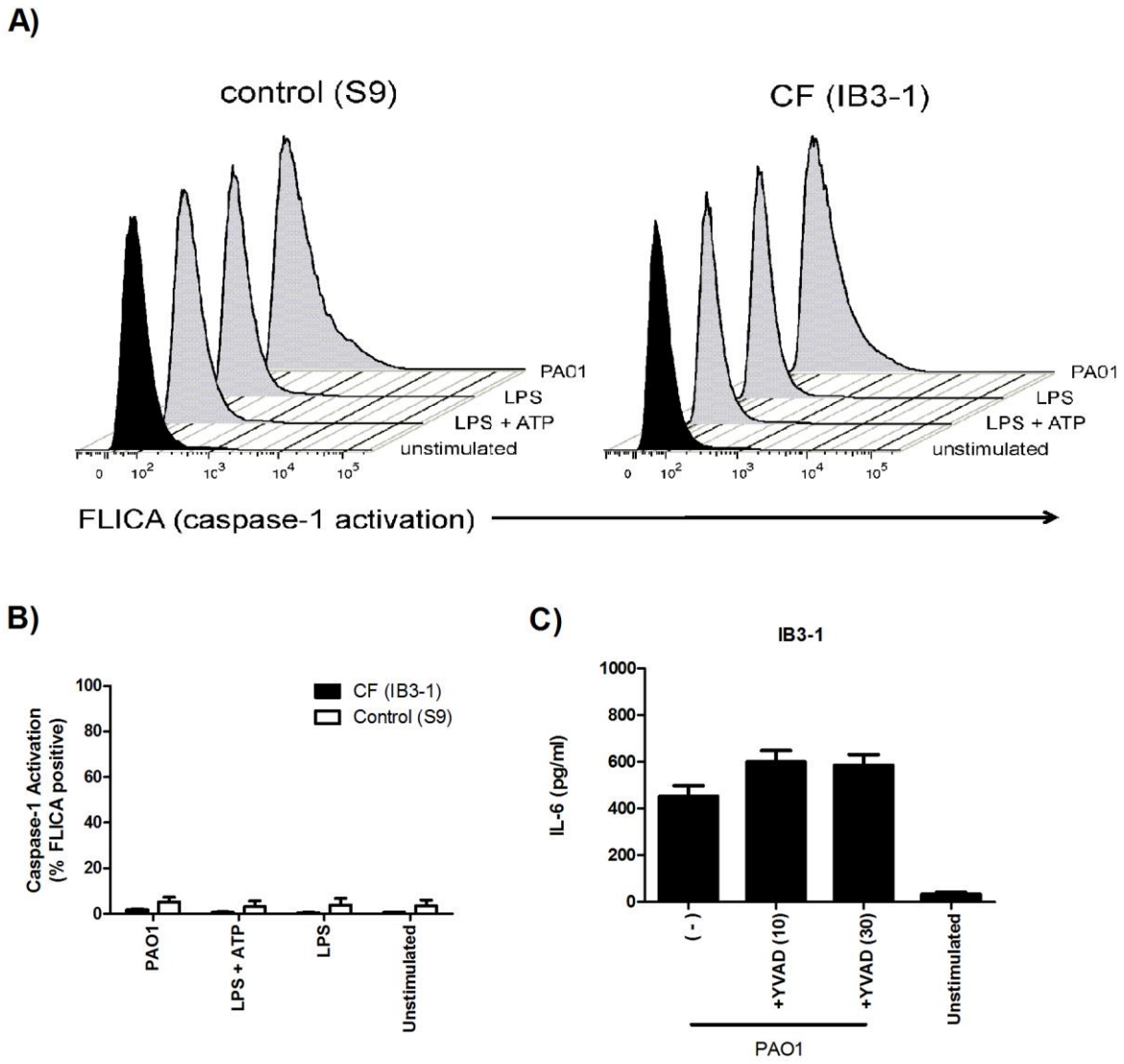


Figure 2.3: Airway cells do not strongly upregulate caspase-1 activity in response to inflammasome stimuli.

S9 and IB3-1 cells were examined for caspase-1 activation following inflammasome stimulation with *P. aeruginosa* strain PAO1 (MOI = 10) and ATP (5 mM). Cells were primed with LPS for 5 hours where appropriate. A representative histogram of % caspase-1-active cells is shown in (A) and the averaged values are shown in (B) (n = 3 independent experiments). (C) IB3-1 cells ($5x10^4$ cells/well) were pre-treated for 1 hour with either 10 or 30 μM z-YVAD-fmk prior to stimulation with *P. aeruginosa* (MOI = 50). Cell culture supernatants were collected after 6 hours and assayed for IL-6 by ELISA (n = 3).

CD14-positive PBMCs from CF patients and controls show similar increases in caspase-1 activity upon inflammasome stimulation

Monocytes were identified in PBMC populations using CD14 as a phenotypic marker. Unlike airway epithelial cells, CD14-positive PBMCs from both CF patients and healthy controls showed a strong increase in caspase-1 activation upon stimulation with LPS + ATP, PAO1, and LPS + Poly(dA:dT) (Fig. 2.4A), but this activation was not different between CF and control subjects (Fig. 2.4B). Inflammasome stimulation times prior to acquisition by flow cytometry were 3 hours for PAO1 and Poly(dA:dT) and 1 hour for ATP. Varying degrees of activation between stimuli may be representative of the speed at which each one acts, whereby Poly(dA:dT) seems to display delayed activation of caspase-1 whereas activation of caspase-1 by ATP is very strong and distinct signal even by 1 hour.

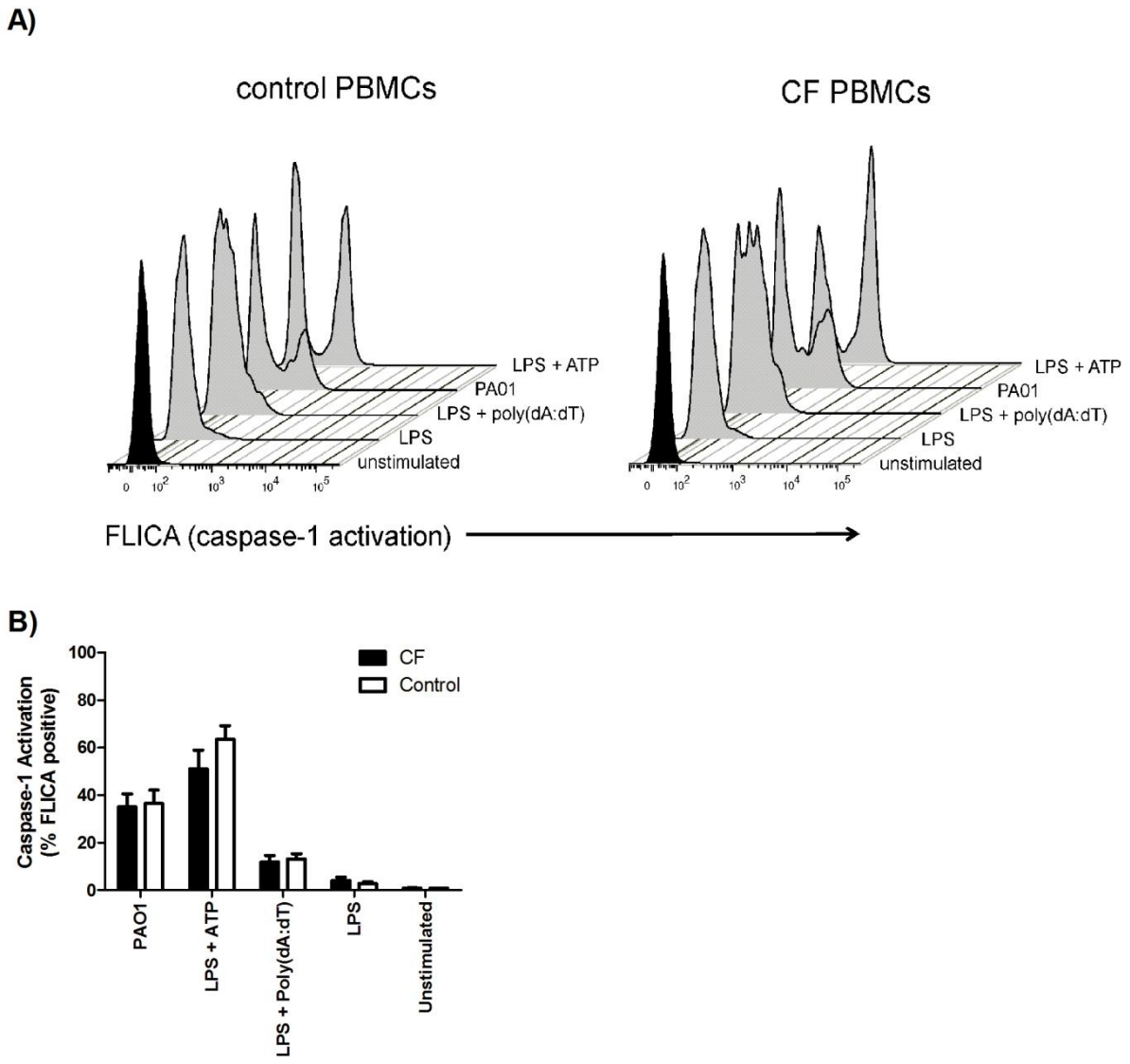


Figure 2.4: PBMCs from CF patients and controls show similar increases in caspase-1 activity upon inflammasome activation.

PBMCs from CF patients (n = 6) and healthy controls (n = 6) were primed with LPS (10 ng/ml) for 5 hours prior to stimulation with ATP (5 mM) for 1 hour or Poly(dA:dT) (1 μg/ml) for 3 hours. PBMCs were stimulated with PAO1 (MOI = 1) for 3 hours. A representative histogram of the % caspase-1-active $CD14^+$ PBMCs is shown in (A) with the averaged values shown in (B).

PBMCs from CF patients do not produce increased amounts of IL-1β upon inflammasome stimulation

Previous studies have found that the loss of CFTR results in increased NF-κB activity and pro-inflammatory cytokine secretion (193, 310, 311). To further examine this relationship, PBMCs from CF patients and healthy adult controls were stimulated with PAO1, LPS + ATP, and LPS + Poly(dA:dT), to activate the NLRC4, NLRP3, and AIM2 inflammasomes, respectively. By 24 hours of stimulation, CF PBMCs had not produced increased amounts of IL-1β (Fig. 2.5A) or IL-8 (Fig. 2.5B) when compared to healthy controls. However, we did notice a transient decrease ($P<0.001$) in the amount of IL-1β produced by CF cells in response to LPS + ATP at 6 hours (data not shown). Stimulation of PBMCs with *P. aeruginosa* that lacks *exsA* (PAO1Δ*exsA*), a key regulator of type III secretion, produced three-fold less IL-1β compared to the parental PAO1 strain by 24 hours (Fig. 2.5A). Inflammasome stimulation without priming did not result in any IL-1β production in either CF or control PBMCs. Contrary to our hypothesis, these results indicate that PBMCs from CF patients do not display increased production of IL-1β or IL-8 with inflammasome activation nor do they suggest any increased basal or induced NF-κB activity. These results are consistent with our observation that caspase-1 activity is not different between CF and control PBMCs (Fig. 2.4).

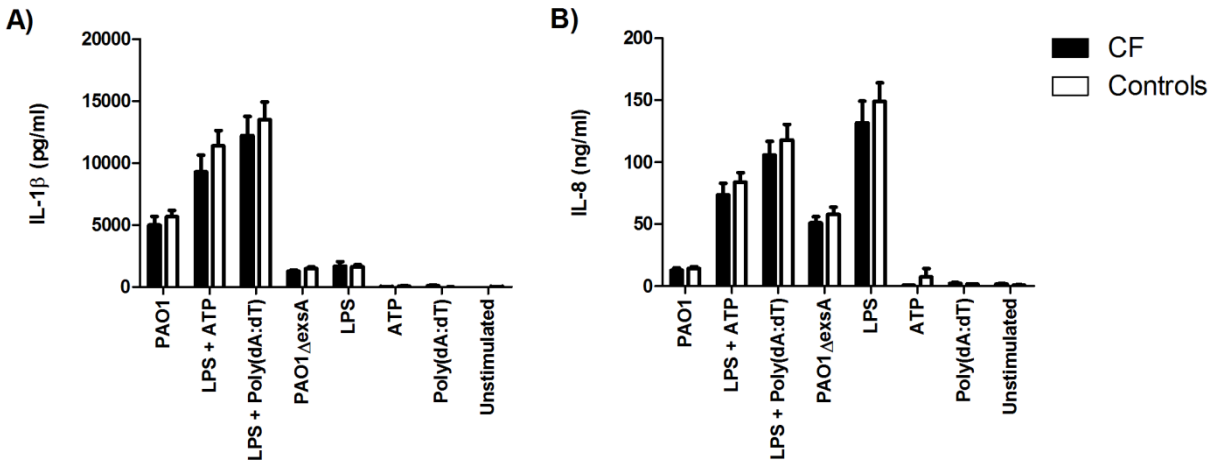


Figure 2.5: PBMCs from CF patients do not produce increased amounts of IL-1β.
PBMCs from CF patients (n = 17 - 20) and healthy controls (n = 15 - 19) were primed with LPS (10 ng/ml) overnight and stimulated with *P. aeruginosa* PAO1 (MOI = 1), *P. aeruginosa* PAO1 lacking *exsA* (MOI = 1),ATP (5 mM), or Poly(dA:dT) (1 μg/ml) for 24 hours. *P. aeruginosa* lacking *exsA* was used as a type III secretion control in comparison with wild-type *P. aeruginosa*. Supernatants were assayed for (A) IL-1β and (B) IL-8.

NF-κB activation is required for IL-1β and IL-8 responses to *P. aeruginosa*

An alternative explanation as to why CF airways contain increased IL-1β may be simply due to increased priming of NF-κB from chronic infections. Here, we confirmed the dependence of PAO1-induced IL-1β and IL-8 production on prior NF-κB activation using THP-1 X-Blue cells. We found that stimulation of primed THP-1 reporter cells with heat-killed PAO1 produced the highest levels of NF-κB/AP-1 activation (Fig. 2.6A) and this correlated with IL-8 secretion (Fig. 2.6B) but negligible amounts of IL-1β were secreted (Fig. 2.6C). On the other hand, stimulation of primed THP-1 X-Blue cells with live PAO1 did not significantly increase

NF-κB/AP-1 activity (Fig. 2.6A) or IL-8 secretion (Fig. 2.6B) over heat-killed PAO1 alone. However, IL-1β production was greatly induced by live PAO1 in unprimed cells, but also to a much higher degree in primed cells (Fig. 2.6C). This showed that NF-κB activation alone was not sufficient for maximal IL-1β secretion, but existing NF-κB activity could further augment IL-1β secretion in the presence of inflammasome activators. Dependency of these responses on NF-κB was confirmed by pharmacologic inhibition of NF-κB using the Bay11-7082 inhibitor of IκBα phosphorylation, which significantly reduced NF-κB/AP-1 activation ($P<0.001$) (Fig. 2.6D) and the subsequent production of IL-8 ($P<0.01$) (Fig. 2.6E) and IL-1β ($P<0.001$) (Fig. 2.6F) in response to both heat-killed and live PAO1. These results were also verified in CF and control PBMCs for each inflammasome examined ($P<0.001$) (Fig. 2.6G-H). Overall these results confirm that NF-κB is an important modulator of IL-1β production and that increased activation of NF-κB, by chronic infection for example, can augment inflammasome-mediated production of IL-1β.

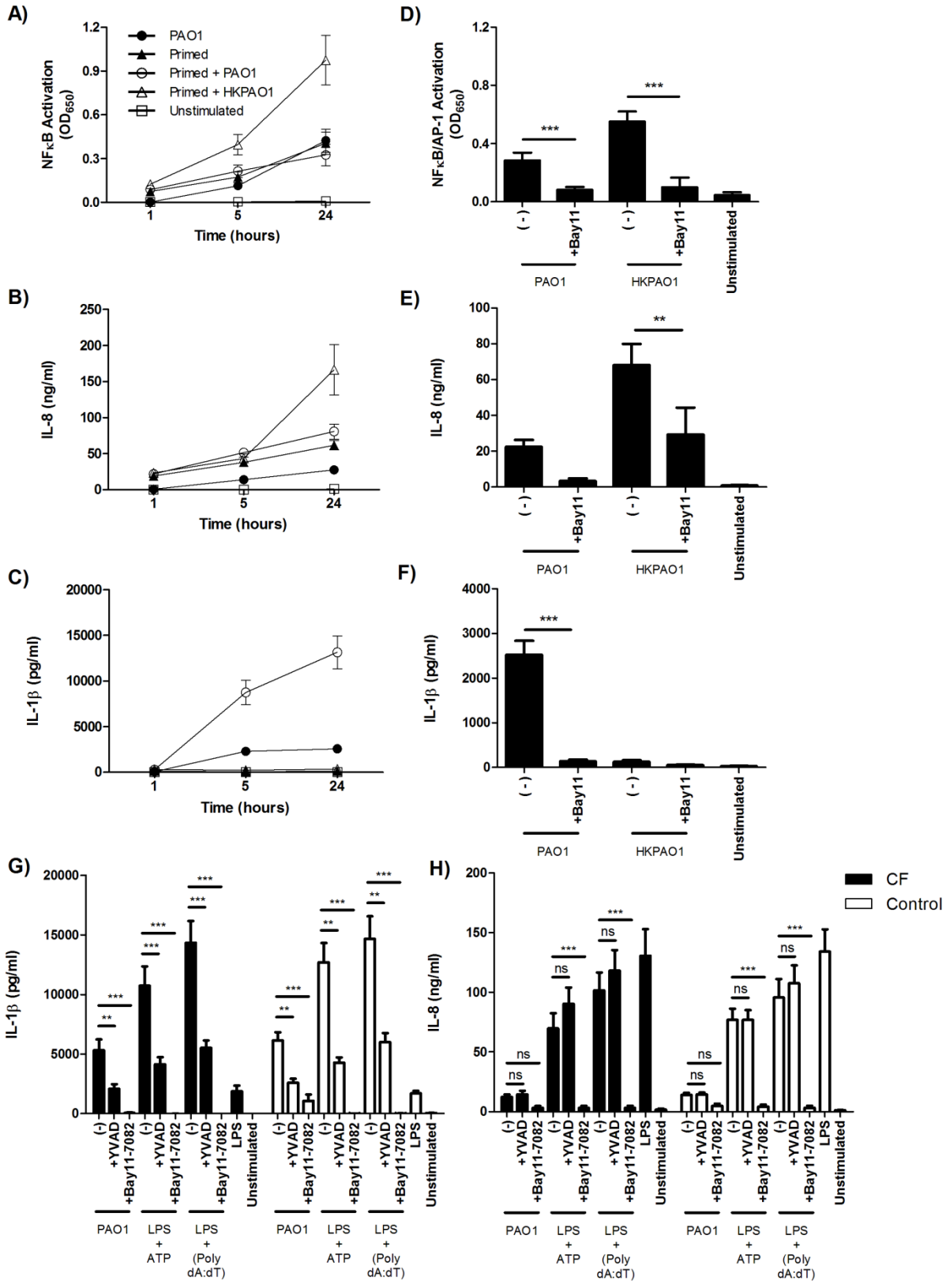

A)
NFκB Activation (OD650)
PAO1
Primed
Primed + PAO1
Primed + HKPAO1
Unstimulated
Time (hours)
B)
IL-8 (ng/ml)
Time (hours)
C)
IL-1β (pg/ml)
Time (hours)
D)
NFκB/AP-1 Activation (OD650)
+Bay11
PAO1
HKPAO1
Unstimulated
E)
IL-8 (ng/ml)
F)
IL-1β (pg/ml)
G)
IL-1β (pg/ml)
+YVAD
+Bay11-7082
LPS
LPS + ATP
LPS + (Poly dA:dT)
H)
IL-8 (ng/ml)
ns
CF
Control

Figure 2.6: NF-κB activation potentiates IL-1β production and secretion upon inflammasome activation.

THP-1 reporter cells were primed overnight with heat-killed *P. aeruginosa* and stimulated the next day with live *P. aeruginosa* or additional heat-killed *P. aeruginosa* for the times indicated. Cell culture supernatants were assayed for (A) NF-κB/AP-1 activity, (B) IL-8, and (C) IL-1β secretion (n = 3 - 6 experiments). Using the same stimulation method, THP-1 reporter cells were treated with Bay11-7082 (20 μM) for 1 hour prior to priming with heat-killed PAO1 or live PAO1. Supernatants were assayed at 24 hours for (D) NF-κB/AP-1 activity, (E) IL-8, and (F) IL-1β secretion (n = 3 - 5). PBMCs from CF patients (n = 11 - 15) and controls (n = 10 - 13) were treated with z-YVAD-fmk (20 μM) or Bay11-7082 (10 μM) and stimulated with live PAO1 (MOI = 1), ATP (5 mM), or Poly(dA:dT) (1 μg/ml) according to the schedule in Figure 2.1. (G) IL-1β and (H) IL-8 levels were measured at 24 hours. Statistical analysis was performed using two-way ANOVA with Bonferroni correction for multiple comparisons. *, **, and *** signify $P<0.05$, 0.01, and 0.001.

Bay11-7082 inhibits pro-IL-1β production in response to *P. aeruginosa*

CFTR inhibition has been reported to result in NF-κB activation (193), although somewhat controversially. In order to examine whether CFTR inhibition could have any priming effects in our system, we used the CFTR inhibitor $CFTR_{inh}172$ to inhibit CFTR activity in THP-1 cells (Fig. 2.7A). In addition to inhibition of NF-κB activity, Bay11-7082 can also directly inhibit the NLRP3 inflammasome (312). To validate its use in this study as an NF-κB inhibitor, western blots for its effects on pro-IL-1β were performed alongside $CFTR_{inh}172$ in response to PAO1 at 4 hours after stimulation (Fig. 2.7B). Our results indicate that Bay11-7082 prevents production of pro-IL-1β whereas $CFTR_{inh}172$ treatment did not seem to increase the levels of pro-IL-1β. This was further corroborated by the ability of Bay11-7082 to inhibit IκBα degradation at 0.5, 1, and 1.5 hours post PAO1 stimulation (Fig 2.7C).

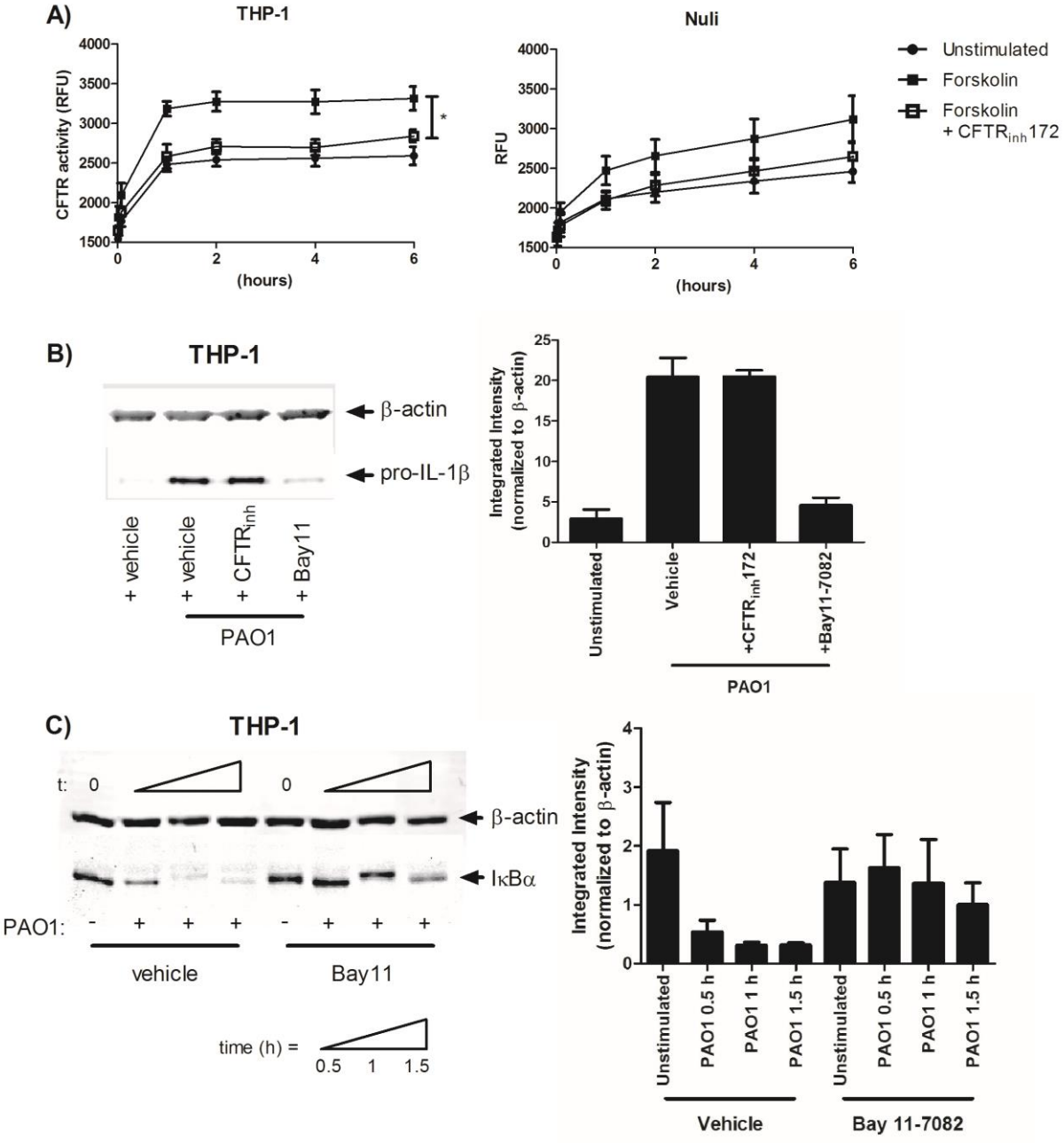

A)
THP-1
Nuli
CFTR activity (RFU)
RFU
(hours)
Unstimulated
Forskolin
Forskolin + CFTRinh172
B)
THP-1
β-actin
pro-IL-1β
+ vehicle
+ vehicle
+ CFTRinh
+ Bay11
PAO1
Integrated Intensity (normalized to β-actin)
Unstimulated
Vehicle
+CFTRinh172
+Bay11-7082
PAO1
C)
THP-1
t: 0
0
β-actin
IκBα
PAO1: - + + + - + + +
vehicle
Bay11
time (h) =
0.5 1 1.5
Integrated Intensity (normalized to β-actin)
Unstimulated
PAO1 0.5 h
PAO1 1 h
PAO1 1.5 h
Unstimulated
PAO1 0.5 h
PAO1 1 h
PAO1 1.5 h
Vehicle
Bay 11-7082

Figure 2.7: Bay11-7082 inhibits pro-IL-1β production in response to *P. aeruginosa*
(A) THP-1 and NuLi-1 cells were examined for CFTR activity with forskolin (10 μM) stimulation with or without $CFTR_{inh}172$ (10 μM) treatment. PMA-differentiated THP-1 cells were treated with $CFTR_{inh}172$ (10 μM) 18 hours or Bay11-7082 (20 μM) 1 hour prior to stimulation and harvested after (B) 4 hours (n = 3) or (C) 0.5, 1, and 1.5 hours (n = 3) stimulation with PAO1. One representative blot is shown with a graph of the averaged fluorescence intensity values over 3 experiments. Data were analyzed using one-way ANOVA and the Kruskal-Wallis test. * indicates $P<0.05$

Disruption of CFTR activity does not increase IL-1β production in PBMCs and THP-1 cells

A previous study has indicated a role for chloride ion concentration in suppression of NLRP3 inflammasome activation (313). To determine whether CFTR dysfunction alters IL-1β secretion through this mechanism, THP-1 cells and PBMCs from CF patients and healthy controls were treated with the CFTR inhibitor, $CFTR_{inh}172$, prior to simulation with live *P. aeruginosa*. Treatment with $CFTR_{inh}172$ did not alter IL-1β or IL-8 production in control subjects or CF patients (Fig. 2.8A-B). Similarly, IL-1β production was not different in monocyte-derived macrophages or THP-1 reporter cells treated with $CFTR_{inh}172$ (Fig. 2.8C-D). IL-8 (Fig. 2.8E) and NF-κB activity (Fig. 2.8F) were also unchanged in $CFTR_{inh}172$-treated THP-1 reporter cells.

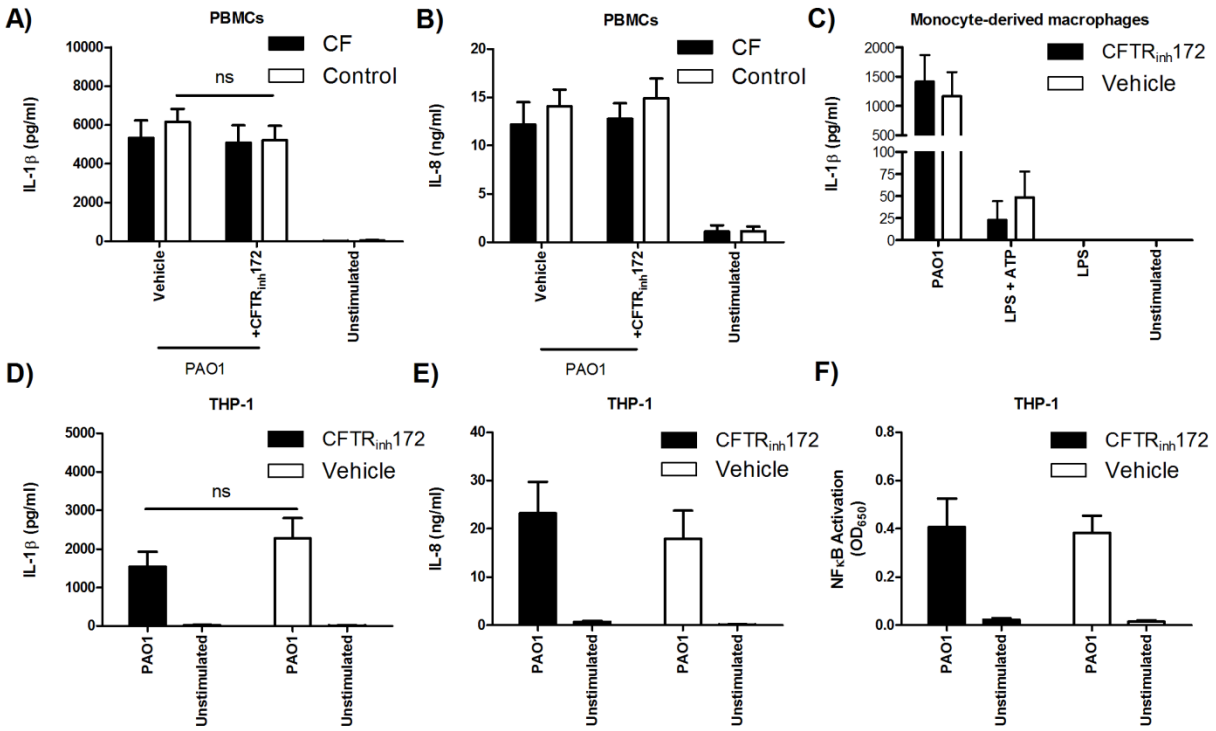


Figure 2.8: Disruption of CFTR activity does not increase IL-1β production in PBMCs or macrophages.

PBMCs from CF patients (n = 15) and controls (n = 13) were treated with $CFTR_{inh}172$ (10 μM) for 18 hours prior to stimulation with live PAO1 (MOI = 1). (A) IL-1β and (B) IL-8 production was measured at 24 hours. (C) Monocytes from controls (n = 3) were differentiated into macrophages. Macrophages were treated with $CFTR_{inh}172$, stimulated as per monocytes, and measured for IL-1β production at 24 hours. THP-1 reporter cells were treated with $CFTR_{inh}172$ 24 hours prior to stimulation with PAO1 and measured for (D) IL-1β secretion, (E) IL-8, and (F) NF-κB/AP-1 activity at 24 hours (n = 4).

2.5 Discussion:

Levels of IL-1β are increased in the BALF of CF patients but the cellular source of this cytokine and its production in the context of targeted inflammasome activation are still unclear.

We first studied airway epithelial cells due to their role in barrier function, proximity to infection, and ability to produce high levels of pro-inflammatory cytokines. However, we found that bronchial epithelial cells do not produce significant amounts of IL-1β and do not show a significant increase in caspase-1 activation in response to PAO1 and LPS + ATP, in comparison to hematopoeitic mononuclear cells. Other studies have since managed to detect IL-1β production in airway epithelia although usually in very small quantities (<20 pg/ml). Instead, hematopoeitically-derived cells, such as monocytes and macrophages, appear to be a principal source of IL-1β. CFTR is expressed in alveolar macrophages (310, 314) as well as in PBMCs at both the mRNA and protein level (315, 316), and its loss is frequently associated with an augmented inflammatory phenotype as noted above.

Despite our findings indicating that bronchial epithelial cells when grown *in vitro* are unlikely to be significantly involved in the direct production of IL-1β (Fig. 2.2A-D), others have shown their capacity to respond to alveolar macrophage-derived IL-1β and to amplify the inflammatory response through the induction of chemokines and recruitment of inflammatory effector cells (317). This interaction may constitute a critical component of effective host defense and diminishing the capacity of host cells to respond to IL-1β may leave the host susceptible to infections by pathogens such as *P. aeruginosa* (318). Conversely, overproduction of IL-1β can also play a key role in chronic inflammatory responses and cause damage to lung tissue (319, 320).

Although we hypothesized that CF cells would secrete increased amounts of IL-1β, we found that IL-1β production in CF PBMCs was not increased upon inflammasome stimulation as compared to controls (Fig. 2.5A-B). This was in contrast to a previous study from our group,

which showed increased IL-1β production by CF PBMCs in response to LPS (321), although this difference may be accounted for by technical issues in stimulation time and dose. Moreover, IL-1β production was not increased in CF PBMCs with inflammasome stimulation alone as would be anticipated if there were basal levels of NF-κB activation. Cells deficient in CFTR are thought to exhibit an increased basal level of NF-κB activity, which leads to increased pro-inflammatory cytokine production including an increased availability of pro-IL-1β for cleavage and secretion. Amplification of IL-1β secretion was shown by priming THP-1 monocytes and PBMCs with heat-killed *P. aeruginosa* or LPS prior to stimulation with live *P. aeruginosa*. This dramatically increased IL-1β secretion over stimulation with live *P. aeruginosa* without priming (Fig. 2.6C). Similarly, if CF PBMCs expressed increased basal NF-κB activity, there would be an increase in IL-1β secretion in the absence of LPS priming. However, no increase in IL-1β was observed under basal or primed conditions. Studies investigating the production of IL-1β have been somewhat inconsistent. A study by Reininger *et al.* (318) provided evidence that human bronchial epithelial cells possessing the ΔF508 CFTR mutation had a slightly reduced capacity to produce IL-1β and lacked the ability to induce an early NF-κB activation in response to *P. aeruginosa*. Conversely, a study by Kotrange *et al.* found that murine bone marrow-derived macrophages expressing ΔF508-CFTR produced increased amounts of IL-1β when compared to macrophages expressing normal CFTR in response to *Burkholderia cenocepacia* K56-2. The differentiation of monocytes into macrophages may partly account for the differences observed with our study (298). Inflammasome-mediated IL-1β production by monocytes and PBMCs does differ from macrophages and macrophages are also found to have higher expression of CFTR. However, as

monocytes and other PBMCs express CFTR and produce large amounts of IL-1β, they are adequate to model the effects of CFTR function on IL-1β production. Other hematopoeitic cells may also contribute to IL-1β production. For example, neutrophil counts can be significantly increased in the lungs of CF patients (322-324) and may produce mature IL-1β through caspase-1 independent mechanisms (325).

The role of NF-κB activation in inflammasome activation and IL-1β secretion is not straightforward. Studies have revealed an essential role for NF-κB activation in the production of pro-IL-1β and inflammasome components such as NLRP3 (303, 304). In contrast, deletion of IKKβ, a kinase essential in NF-κB activation, increases IL-1β secretion in murine macrophages (326, 327) and demonstrates a dual role for NF-κB in regulation of IL-1β. To address this variable, we also quantified IL-8, an important CF cytokine and marker of NF-κB activation, and found no differences between CF and control subjects. Similarly, levels of intracellular pro-IL-1β in THP-1 cells were dependent on NF-κB activity and did not increase with $CFTR_{inh}172$ treatment. Subsequent treatment of THP-1 cells and PBMCs with the NF-κB inhibitor Bay11-7082 significantly inhibited both IL-1β and IL-8 secretion (Fig. 2.6D-H). Therefore, the IL-1βand IL-8 responses observed were both dependent upon NF-κB activation. Priming with heat-killed *P. aeruginosa*, like LPS, is unable to induce a strong IL-1β response as compared to live *P. aeruginosa* (Fig. 2.6C), but generated greater NF-κB/AP-1 activation (Fig. 2.6A) and IL-8 secretion (Fig. 2.6B) than live bacteria despite stimulation at the equivalent MOIs. This may be indicative of the different degree and quality of the inflammatory response generated by live as opposed to dead bacteria (328).

Although the responses measured in peripheral blood cells may not completely reflect the responses occurring in the CF lung, PBMCs have a number of useful advantages: (8) PBMCs are not subject to alterations that may emerge from long-term cell culture, cloning and immortalization, and (ii) PBMCs express a large repertoire of innate immune receptors and secrete a broad array of cytokines and chemokines allowing comprehensive analysis of the modulation of inflammatory responses by CFTR. Consideration must also be given to the nature of *P. aeruginosa* infection and genotypic changes in *P. aeruginosa* as infection progresses. *P. aeruginosa* mediates inflammasome activation through its type III secretion system (T3SS) and the NLRC4 inflammasome (301, 302). However, clones of *P. aeruginosa* established during chronic infection may accumulate mutations in virulence factors such as *exsA* (140). By employing a deletion mutant in *exsA*, the key regulator in T3SS transcription, we confirmed that the T3SS is important for IL-1β secretion (Fig. 2.5A-B), and that depending on the adaptation in type III secretion, the host IL-1β response may be up or downregulated (140, 301, 329).

In conclusion, our data are consistent with a role for hematopoietic cells, not airway epithelial cells, as the major source of inflammasome-mediated IL-1β production in the lungs in response to ATP and *P. aeruginosa*. Furthermore, we find little evidence to support that increased IL-1β levels in CF is due to intrinsically increased NF-κB/Inflammasome activity in CF patients, but is more likely due to the additional activation of NF-κB through chronic infection. Further studies are warranted to determine if adaptations of *P. aeruginosa* during the course of chronic lung infection alters inflammasome activation and IL-1β production, and whether this can be correlated with disease severity in CF.

CHAPTER 3: ENDOPLASMIC RETICULUM STRESS REGULATES CHEMOKINE PRODUCTION IN CYSTIC FIBROSIS AIRWAY CELLS THROUGH STAT3

3.1 Rationale: In the previous chapter, we determined that extracellular factors, such as additional stimulation by PRR ligands during chronic infections, were likely responsible for the increased abundance of IL-1β observed in CF lungs. Informed by these data, we reasoned that the excessive inflammation observed in CF likely originated from cell extrinsic factors, and therefore decided to focus on other extracellular factors that could perhaps serve as good anti-inflammatory targets in CF. To this end, ER stress was an excellent candidate as a major influence on CF inflammatory response, since it has been reported to be caused by various infections (including the dominant CF pathogen, *P. aeruginosa*) in addition to oxidative stressors which are plentiful in the CF lung environment. To investigate the role of ER stress in driving inflammation, we employed a panel of chemical ER stressors (Table 3.1) in order to elicit strong activation of ER stress pathways and used them to model effects on inflammatory pathways. ER stress is a very attractive therapeutic target as it is considered to play an important role in a variety of inflammatory diseases including diabetes, inflammatory bowel disease, and cancer, in addition to CF.

Table 3.1: ER stressors employed in this study

ER STRESSOR	MECHANISM OF UPR INDUCTION
Tunicamycin (Tun)	A mixture of homologous nucleosides that inhibit N-glycosylation and cell cycle progression. Lack of proper glycosylation destabilizes native structure.
Thapsigargin (Thaps)	An inhibitor of the sarcoplasmic/endoplasmic reticulum calcium ATPase. It depletes sarcoplasmic reticulum/ER calcium stores which are required for proper protein folding while simultaneously raising cytosolic calcium levels.
MG-132 (MG)	An inhibitor of the β5 subunit of the proteasome. This prevents normal protein turnover and ER-associated degradation, resulting in the accumulation of the ubiquitinated proteins and increases burden on the ER.
Dithiothreitol (DTT)	A reducing agent that inhibits disulfide bond formation.
Brefeldin A (BFA)	A fungal metabolite that inhibits intracellular protein transport between the ER and the Golgi by preventing COPI-containing vesicle fusion to Golgi membranes. This results in an accumulation of proteins in the ER and activation of the UPR.

A panel of chemical ER stressors were used in this study to improve reliability of results.

3.2 Background:

ER stressors are stimuli that cause perturbations to cellular protein homeostasis, typically the result of an excess accumulation of unfolded or misfolded proteins in the cell. Under conditions of ER stress, cells activate the unfolded protein response (UPR) as a mechanism to help alleviate ER burden and restore proteostasis. The UPR functions through three primary ER-resident sensors which bind to unfolded proteins in the ER and proceed to activate corresponding downstream transcription factors: IRE1α-XBP1s, PERK-ATF4, and ATF6/cleaved ATF6 (Figure 3.1). These pathways employ complementary strategies to relieve

ER burden and restore normal cellular function, including upregulation of chaperone proteins (330), mRNA degradation (331), translational arrest (332), and facilitation of protein degradation (333, 334).

Interestingly, the UPR has also been documented to affect inflammatory responses (10). Hypomorphic polymorphisms in *XBP1* have been associated with inflammatory bowel disease in humans, validating the observation that mice carrying a targeted deletion of *xbp1* in intestinal epithelial cells develop enteritis (335). Furthermore, cells undergoing ER stress have been observed to elicit an inflammatory response through the NF-κB pathway in a stressor-specific manner (336). All three primary ERSR pathways have been described to activate inflammatory responses, albeit through distinct mechanisms. The IRE1α pathway is thought to follow classical NF-κB activation through IRE1α interaction with the TRAF2 adaptor protein and activation of the IKK complex, ultimately resulting in IκBα degradation and p65 nuclear translocation (337, 338). The PERK pathway has been suggested to activate NF-κB independently of IKK phosphorylation of IκBα, instead triggering an increased ratio of p65 to IκBα due to eIF2α-mediated translational interference (339, 340). The activation of the ATF6 pathway results in its translocation to and cleavage at the Golgi. The processed fragment (ATF6f) can then form a heterodimer with CREBH to mediate transcription of the acute phase proteins, serum amyloid P-component and C-reactive protein (341), while also activating the AKT pathway (342). In addition to these inflammatory mechanisms, there is evidence that the downstream transcription factors themselves, particularly ATF4 and XBP1s, can drive inflammatory responses through binding the promoter regions of IL-6 or both IL-6 and TNFα, respectively (Figure 3.1) (343, 344).

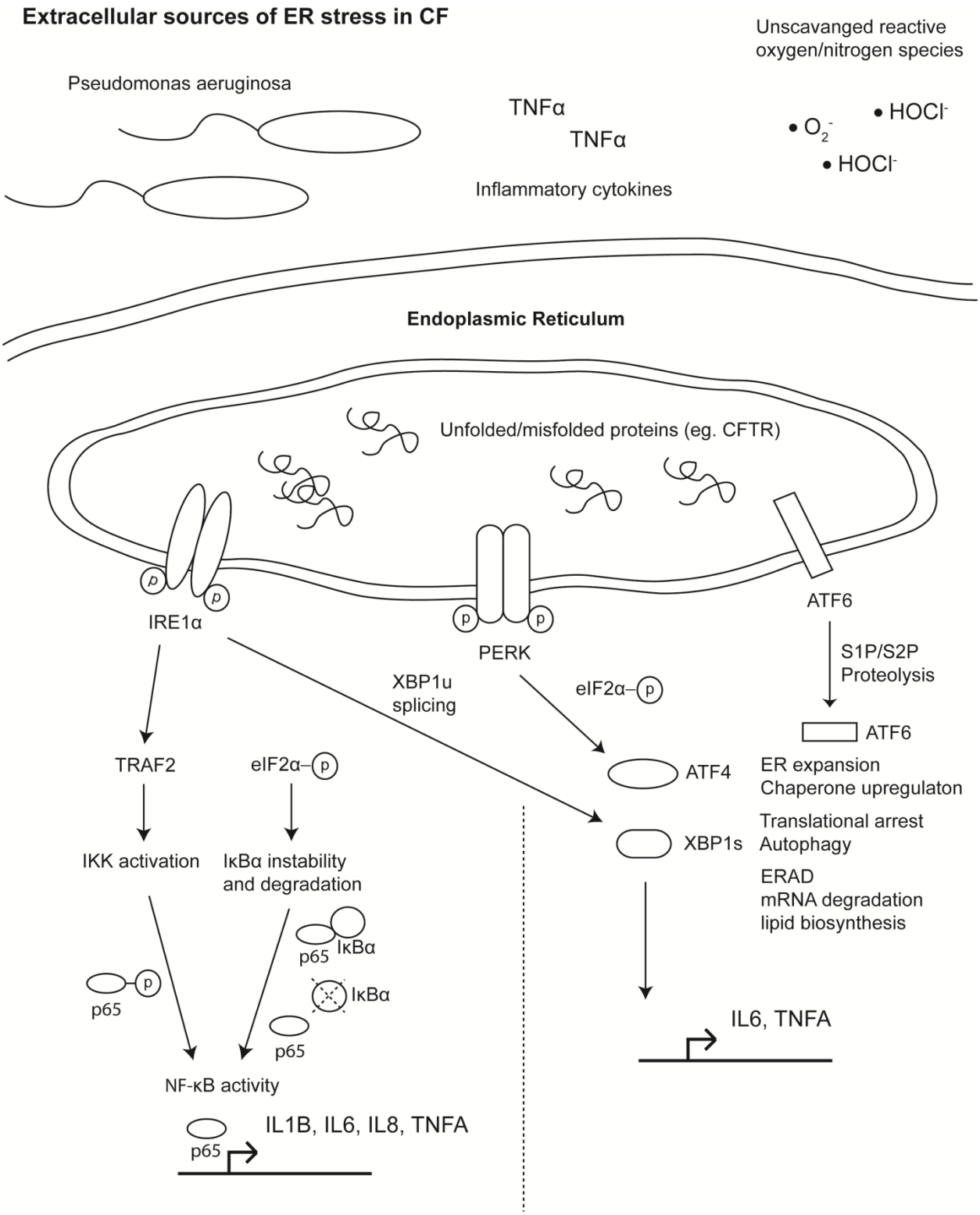

Extracellular sources of ER stress in CF
Unscavanged reactive oxygen/nitrogen species
Pseudomonas aeruginosa
TNFα
TNFα
Inflammatory cytokines
• O_2^-
• HOCl⁻
• HOCl⁻
Endoplasmic Reticulum
Unfolded/misfolded proteins (eg. CFTR)
IRE1α
PERK
ATF6
S1P/S2P Proteolysis
ATF6
XBP1u splicing
eIF2α–p
TRAF2
eIF2α–p
ATF4
ER expansion
Chaperone upregulaton
XBP1s
Translational arrest
Autophagy
ERAD
mRNA degradation
lipid biosynthesis
IKK activation
IκBα instability and degradation
p65
IκBα
p65
IκBα
p65
IL6, TNFA
NF-κB activity
IL1B, IL6, IL8, TNFA
p65

Figure 3.1: The UPR mediates proteostasis and inflammatory activation

Although the most common CFTR mutation in CF, F508del, can induce the UPR when expressed at high levels, extracellular sources of ER stress also exist including infection by pathogens such as *P. aeruginosa*, inflammatory cytokines such as TNFα, and also reactive oxygen species. The UPR mediates its proteostatic functions through the ER-resident proteins IRE1α, PERK, and ATF6, which induce expression of specific transcription factors including XBP1s and ATF4 which go on to mediate a variety of proteostatic effects including targeted mRNA degradation, translational arrest, endoplasmic reticulum associated degradation (ERAD) and chaperone upregulation, but also transcription of the inflammatory cytokines IL-6 and TNFα. ER stress can also induce activation of NF-κB-driven inflammatory responses. This occurs in an IRE1α-dependent fashion through TRAF2 recruitment and subsequent IKK-mediated phosphorylation of IκBα or through a PERK-dependent fashion through eIF2α-mediated translational arrest. This leads to IκBα instability and degradation, allowing for an increased ratio of free p65 to inhibitor IκBα.

ER stress is thought to contribute to inflammation in a variety of non-communicable diseases (10) and is a particularly appealing target in CF. Not only is the F508del CFTR mutation, which results in a misfolded protein product that may induce ER stress (195), present in close to 70% of all CF chromosomes worldwide (194), but sources of ER stress also occur externally in the CF lung environment. These include *P. aeruginosa* (345), one of the most common CF pathogens, viruses including respiratory syncytial virus (346), inflammatory cytokines (eg. TNFα) (347), and increased oxidative stress (196), which may be exacerbated by a CF-intrinsic oxidant:anti-oxidant imbalance (175, 176), and excessive neutrophil infiltration. Activation of the UPR has been previously reported to be increased in CF cells over controls and molecular inhibition of the UPR decreases IL-6 and IL-8 production (348, 349).

We hypothesize that ER stress and UPR activation will further exacerbate the inflammatory phenotype caused by chronic infection in the CF lungs and targeted inhibition of ER stress

pathways will help to alleviate excess inflammation. In order to emphasize reproducibility of our findings; we employed multiple cellular models including THP-1 monocytes and the CF airway epithelial cell lines IB3-1 and CuFi-1 to explore mechanism. We particularly focus on the chemokines IL-8 and CXCL1 because of their roles in neutrophil recruitment. Although CXCL1 is less commonly researched in CF, CXCL1 has been observed in increased amounts in CF airway washings (350), and has also shown to have an important role in neutrophil recruitment (351). Furthermore, we establish the importance of these chemokines and their relevance to disease by using genome-wide association study data combined from both North American and European cohorts (211).

Aim: To determine how the unfolded protein response modulates inflammation in CF cells and to identify potential anti-inflammatory pathways involving ER stress.

3.3 Materials and methods

Cell lines

IB3-1, CuFi-1, THP-1, and THP-1 X Blue cells were cultured as described in the previous chapter.

Cell culture and stimulations

IB3-1 cells were seeded overnight at a density of $2x10^4$ cells/well in a 96-well plate for stimulations or $1.5x10^4$ cells/well (80-90% confluency) for transfections followed by stimulation. THP-1 cells were differentiated using 50 ng/ml PMA (Sigma Aldrich) for 18 hours, washed with PBS and allowed to rest for an additional 48 hours prior to stimulation with ER stressors or TLR ligands. THP-1 cells were seeded at a density of $1x10^5$ cells/well of 96-well plate. The ER stressors used were tunicamycin (EMD Millipore), thapsigargin (EMD

Millipore), MG-132 (Cell Signaling Technologies), Brefeldin A (Life Technologies), and DL-Dithiothreitol (Sigma Aldrich). The TLR ligands and cytokines were LPS from *Escherichia coli* K12, (Invivogen), IL-1β (eBioscience), and TNFα (eBioscience).

Viability Assays:

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) was used according to the manufacturer's instructions (Promega). Briefly, MTS substrate was diluted 1in9 in RPMI1640 with supplements and incubated with cells for 2-2.5 hours at 37°C prior to reading at 490 nm on a Spectramax PLUS384.

qRT-PCR

RNA was isolated from cells using the RNeasy PLUS kit (Qiagen) and converted to cDNA using iScript reverse transcription supermix (Bio-Rad). qPCR was carried out using iTaq Universal SYBR green supermix (Bio-Rad). Reactions were carried out in 384-well plates (Axygen) in a ViiA-7 real-time PCR system (Applied Biosystems) and analyzed using the $2^{-\Delta\Delta CT}$ method. All primers were ordered from Integrated DNA Technologies (IDT) and confirmed for efficiency of > 90%. Primer sequences for ER stress genes have been previously published (348) and genes of interest were normalized to *ACTB* and *SDHA* using the geometric mean.

Polymorphonuclear cell isolation

PBMCs were separated from PMNs using Ficoll-Hypaque (GE Healthcare). The lower layer containing PMNs and red blood cells was subjected to dextran sedimentation (Sigma Aldrich) at a final concentration of 1.5%. Following sedimentation, the upper phase containing PMNs

was collected and residual RBCs were lysed using a hypotonic (0.2% NaCl) solution prior to resuspension in RPMI1640 as above.

Polymorphonuclear cell migration assay

PMNs were labeled with 10 μM CFSE (Life Technologies) for 30 minutes in serum-free RPMI1640. $1x10^6$ cells were seeded in the upper chamber of 3.0 μm transwell inserts. The lower chamber was filled with conditioned media from IB3-1 cells stimulated with IL-1β under conditions of ER stress (tunicamycin, thapsigargin, MG-132 treatment) and harvested at 24 hours. After 4 hours of incubation at 37°C, the degree of cell migration to the lower chamber was measured by fluorescence at 490 nm/520 nm ex./em. on a Tecan inifinite M200 plate reader.

Lysate preparation

Whole cell lysates were prepared by direct lysis in RIPA buffer supplemented with HALT protease inhibitor cocktail (PIERCE). RIPA buffer was prepared using1% Triton X-100, 0.5% Sodium Deoxycholate, 0.1% Sodium Dodecyl Sulfate, 140 mM NaCl, and 10 mM Tris-Cl (pH 8.0).

Nuclear enrichment

Cells were lifted using either trypsin (IB3-1) or EDTA (THP-1), pelleted at < 1,000 × *g*, and resuspended in a hypotonic buffer containing 10 mM HEPES, 10mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT and allowed to sit on ice for 10 minutes. Igepal (USB) was added to a final concentration of 0.5% and allowed to incubate on ice for an additional 5 minutes, over which time samples were vortexed twice for > 5 seconds each. Samples were pelleted at 18,000× *g* at 4°C. The resulting nuclei-enriched pellet was washed in PBS and then resuspended in RIPA

buffer. For samples in which more stringent fractionation of nucleus and cytosol were required (ie. the simultaneous blotting of nuclear and cytosolic fractions), the NE-PER nuclear protein extraction kit (PIERCE) was used according to the manufacturer's instructions.

SDS-PAGE and membrane transfer

All samples were sonicated, mixed with 4x Laemmli Buffer, and heated to 80°C for 10 minutes prior to running in 12% bis-acrylamide tris-glycine gels. Proteins were transferred in a trans blot system (Bio-Rad) in Towbin's Buffer containing 10% methanol onto Immobilon-FL membrane (Millipore) and blocked for 1 hour in TBS containing 5% BSA (Sigma) and 0.1% Tween-20 (Fisher).

Immunoblotting

Antibodies used for blotting: ATF4, p-p65, p-p38, β-actin, and IκBα (Cell Signaling Technologies); XBP-1, IL-1β, and Lamin B (Santa Cruz Biotechnology); p-STAT3 (Thermo Fisher); p-IRF3 (Abcam). All primary antibodies were diluted in 5% BSA in TBS-T overnight at 4°C. Secondary antibodies against donkey, mouse, and rabbit antibodies conjugated to 680 or 800 nm-emitting fluorophores were obtained from LICOR and were incubated on membranes for 1 hour prior to scanning in 5% BSA TBS-T. Nuclei-enriched samples were normalized to Lamin B and whole cell lysates were normalized to β-actin. Membranes were scanned on a LICOR Odyssey.

Enzyme-Linked Immunosorbent Assay

IL-1β, IL-6, and IL-8 ELISAs were purchased from eBioscience and used according to the manufacturer's instructions. Supernatants were centrifuged in 96-well U bottom plates to

eliminate any detached cells and stored at -20°C prior to analysis. Results were read at 450/570 nm on a Spectramax PLUS384 (Molecular Devices).

NF-κB activity assays

pGL4.32 (Genbank Accession Number EU581860) was purchased from Promega. It contains five copies of an NF-κB responsive element, upstream of the Luc2P reporter gene. Reporter plasmid was transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Samples were evaluated 24 hours after stimulation using the luciferase assay system with cell culture lysis reagent (Promega) and read on an Infinite M200 (Tecan). For THP-1 X-Blue cells, supernatants were collected 6 hours after ER stressor treatment and incubated at 37°C for 24 hours to allow colour development. Plates were subsequently read at 655 nm on a Spectramax PLUS384 plate reader.

RNA interference

XBP1 siRNA was purchased from Santa Cruz Biotechnology and ATF4 siRNA was modified from a sequence by Ryzmski*et al* (352) and ordered from IDT. 1 pmole of siRNA/well of a 96-well plate was transfected using RNAi max (Invitrogen) according to the manufacturer's instructions. Reagents were proportionally scaled for larger transfections.

Genetic association testing of CXCL1 and CXCL8 SNPs

The genome wide association study for lung disease severity in CF has been previously described (211). Briefly, samples were genotyped on Illumina platforms (CFN370, 610, 660W, and Omni5). Imputation was performed using MaCH/Minimac software, and Phase I Version 3 haplotype data from the 1000 Genomes Project was used as the reference population. Genome-wide association analysis was performed within each of several subgroups using linear

regression and linear mixed modeling with adjustment for sex and principal components reflecting genetic ancestry.

Lung disease severity was assessed using the previously described North American CF Modifier Consortium lung phenotype (353), in which age-specific CF percentile values of FEV1 were calculated using three years of data in CF patients 6 years of age or older. For the French subgroup, CF percentiles were calculated relative to other CF patients in France of the same age, sex and height.

For this analysis, genetic association results from 1642 individuals with CF from the Canadian CF Gene Modifier Study, 2963 CF individuals from the UNC/Case Western Reserve Gene Modifier Study, and 1222 individuals with CF from the French CF Gene Modifier Study were combined with a meta-analysis approach using PLINK. Both random-effects and fixed effects models were used to either account for effect size heterogeneity or maximize power, respectively. Gene based p-values were calculated for *CXCL1* and *CXCL8* using VEGAS2 (*v*ersatile *g*ene-*b*ased *a*ssociation *s*tudy 2) (354), a tool which computes gene based p-values by summarizing the full set of SNPs within a gene. This method uses simulation based on the LD structure of the 1000 Genomes population. In addition, a SNP-based analysis was performed including SNPs within the region 10 kb upstream of each gene's transcriptional start site, to 10 kb downstream of the transcriptional stop site. The critical value for significance for SNP-based P-values was determined using a previously described method (355, 356) to calculate the number of independent statistical tests required to correct for multiple comparisons.

Statistics

Statistics were performed in Graphpad Prism 5, using either two-way ANOVA with Bonferroni or one-way ANOVA with Dunnett's post-test. *, **, and *** signify $P< 0.05$, $P<0.01$, and $P< 0.001$, respectively. All data were expressed using the mean and the standard error of the mean, except for qPCR data, which were expressed by 95% confidence intervals.

3.4 Results

Limited pro-inflammatory cytokine secretion by ER stress stimulation

In order to examine the inflammatory effects of ER stress, we decided to look in a variety of model cell types (THP-1 monocytic cell line, and two CF bronchial epithelial cell lines) in order to ensure that our data was relatively consistent and could be replicated across models. To examine the functional consequences of ER stress induction, we quantified the ability of ER stress to drive the production of pro-inflammatory cytokines IL-1β, IL-6, and IL-8, which are all commonly found in increased amounts in the CF airways (185, 191, 357, 358). IL-6 and IL-8 transcripts were upregulated in IB3-1 cells ($P<0.05$) (Fig. 3.2A) while IL-1β transcript was upregulated in THP-1 cells upon treatment with several ER stressors (Fig. 3.2B). However, inconsistent with the literature linking ER stress to inflammation, production of inflammatory cytokines at the protein level was limited in response to a panel of ER stressors (Table 3.1) after 24 hours, with the only significant production seen in airway epithelial cells following exposure to thapsigargin ($P < 0.05$) (Fig. 3.2C). Similarly, THP-1 cells did not produce any secreted IL-6 or IL-1β after 24 hours of exposure (Fig. 3.2D).

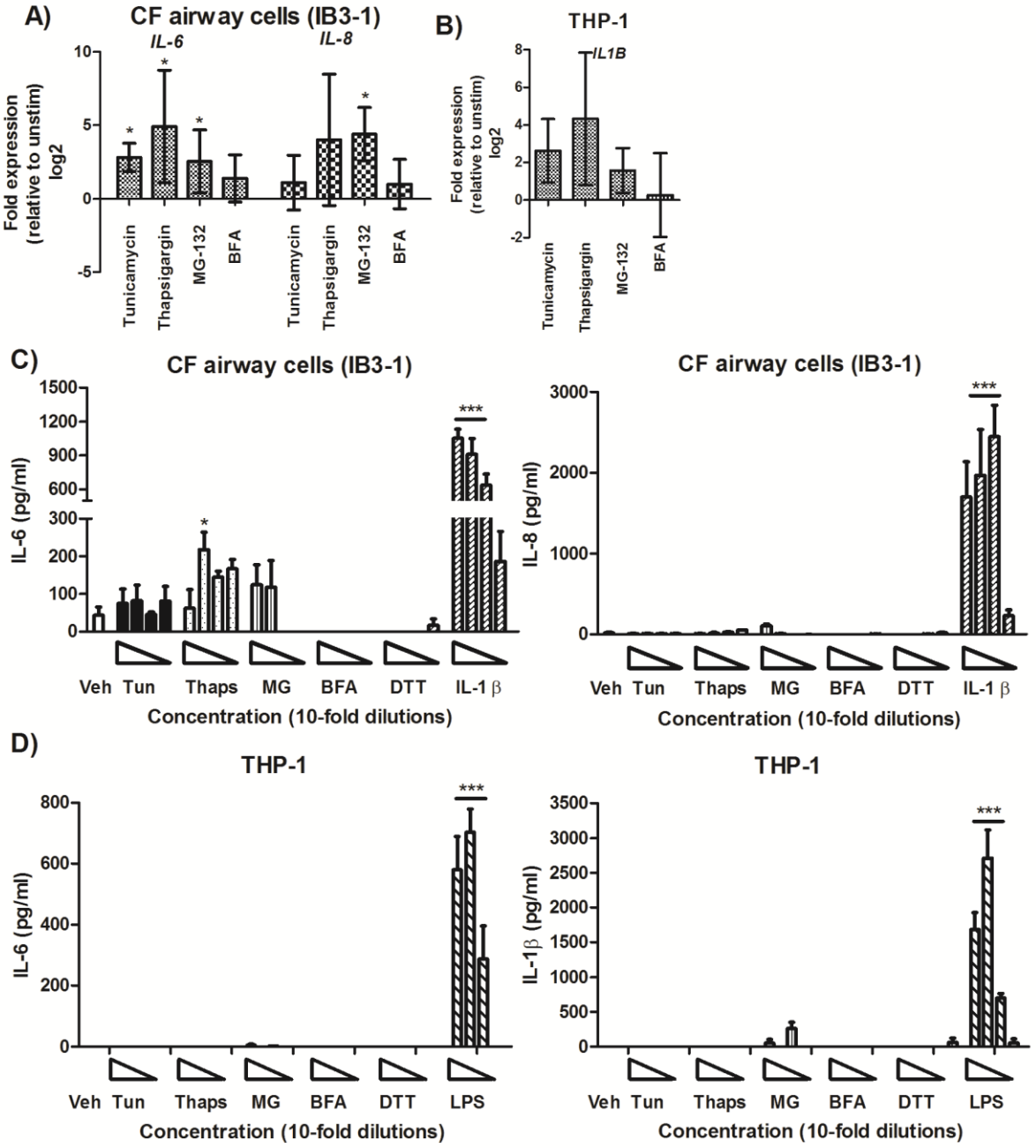

A)
CF airway cells (IB3-1)
IL-6
IL-8
Fold expression (relative to unstim) log2
Tunicamycin
Thapsigargin
MG-132
BFA
B)
THP-1
IL1B
C)
CF airway cells (IB3-1)
IL-6 (pg/ml)
IL-8 (pg/ml)
Veh Tun Thaps MG BFA DTT IL-1 β
Concentration (10-fold dilutions)
D)
THP-1
IL-6 (pg/ml)
IL-1β (pg/ml)
Veh Tun Thaps MG BFA DTT LPS
Concentration (10-fold dilutions)

Figure 3.2: Limited pro-inflammatory cytokine secretion by ER stress stimulation

qPCR measurement of inflammatory transcripts for (A) IB3-1 and (B) THP-1 cells after 5 hours of stimulation with Tunicamycin (10 μg/ml), Thapsigargin (10 μM), MG-132 (5 μM), and Brefeldin A (5 μg/ml). qPCR data are $\log_2$ transformed showing the mean with 95% confidence intervals. n = 3 for all experiments. (A) IB3-1 cells and (B) THP-1 cells were treated with a panel of ER stressors and examined for IL-6 and IL-8 (IB3-1) or IL-1β (THP-1) transcript generation over untreated cells. Four 10-fold dilutions were performed for each stimulus with the highest concentration being indicated: Tunicamycin (50 μg/ml), Thapsigargin (50 μM), MG-132 (50 μM), Brefeldin A (20 μg/ml), IL-1β (100 ng/ml), and LPS (10 ng/ml). Supernatants were collected after 24 hours and analyzed by ELISA. Data were analyzed by t-test or two-way ANOVA with Bonferroni post-test. * $P< 0.05$, *** $P< 0.005$.

NF-κB activation is inconsistent between ER stressors and cell types

Although ER stress has been reported to induce NF-κB activation (336, 337, 339), we were unable to consistently replicate these findings by either reporter assays or immunoblotting in either of the cell types employed: CF airway epithelial IB3-1 cells and THP-1 monocytic cells. Using a panel of ER stressors, IB3-1 cells transfected with an NF-κB reporter plasmid were exposed to a panel of typical ER stress-inducing chemicals over a range of concentrations and assayed at 24 hours, using IL-1β as a positive control (Fig. 3.3A). Similarly, THP-1 X-Blue cells expressing a secreted alkaline phosphatase (SEAP) reporter for NF-κB/AP-1, did not yield any appreciable or consistent activation after 6 hours of stimulation (24 hour development period), using LPS stimulation as a positive control (Fig 3.3C). These results were further validated by immunoblotting for p65 phosphorylation and IκBα degradation, both key events in NF-κB activation, in IB3-1 and THP-1 cells (Fig. 3.3B, D). There was no appreciable signal above baseline in most ER stressor-treated groups, nor was there consistent p65

phosphorylation or IκBα degradation, despite robust induction of the UPR as measured by ATF4 upregulation.

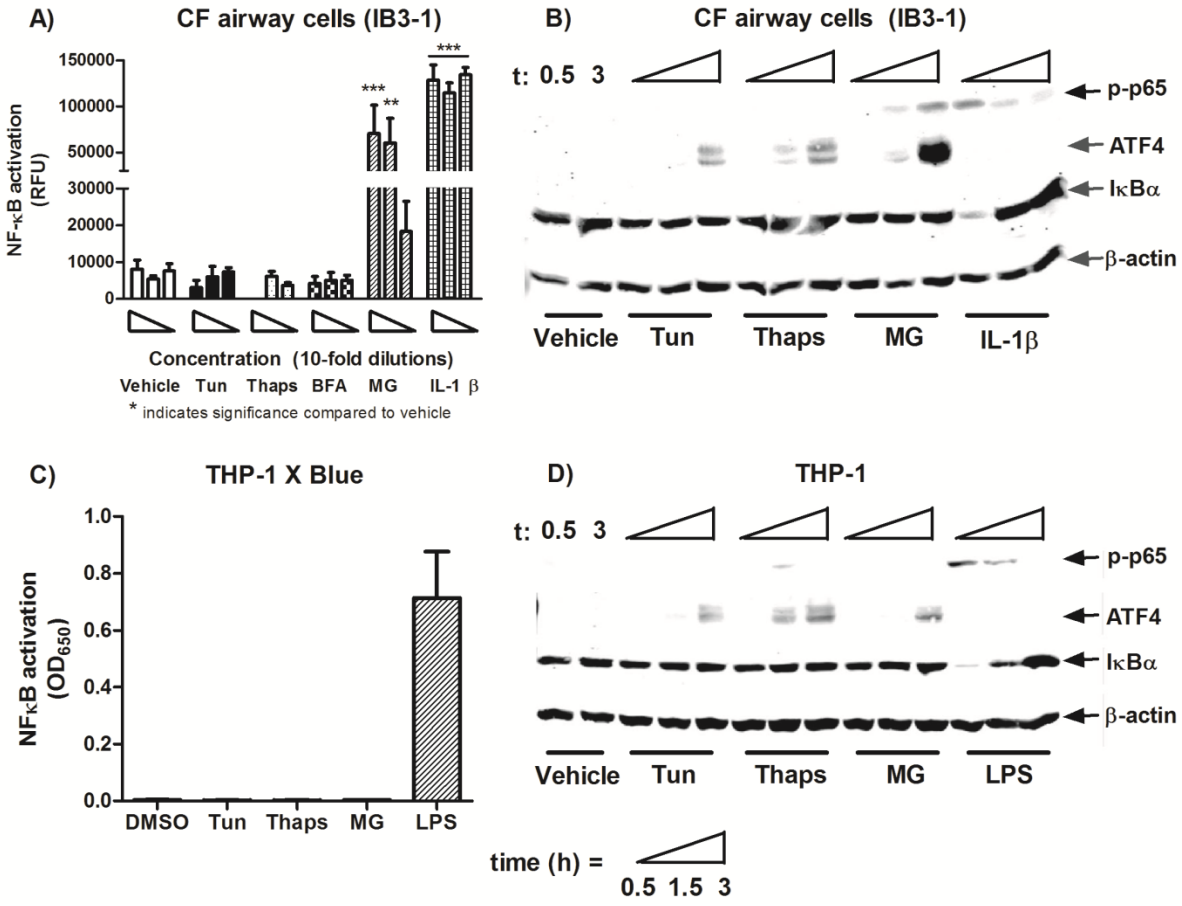


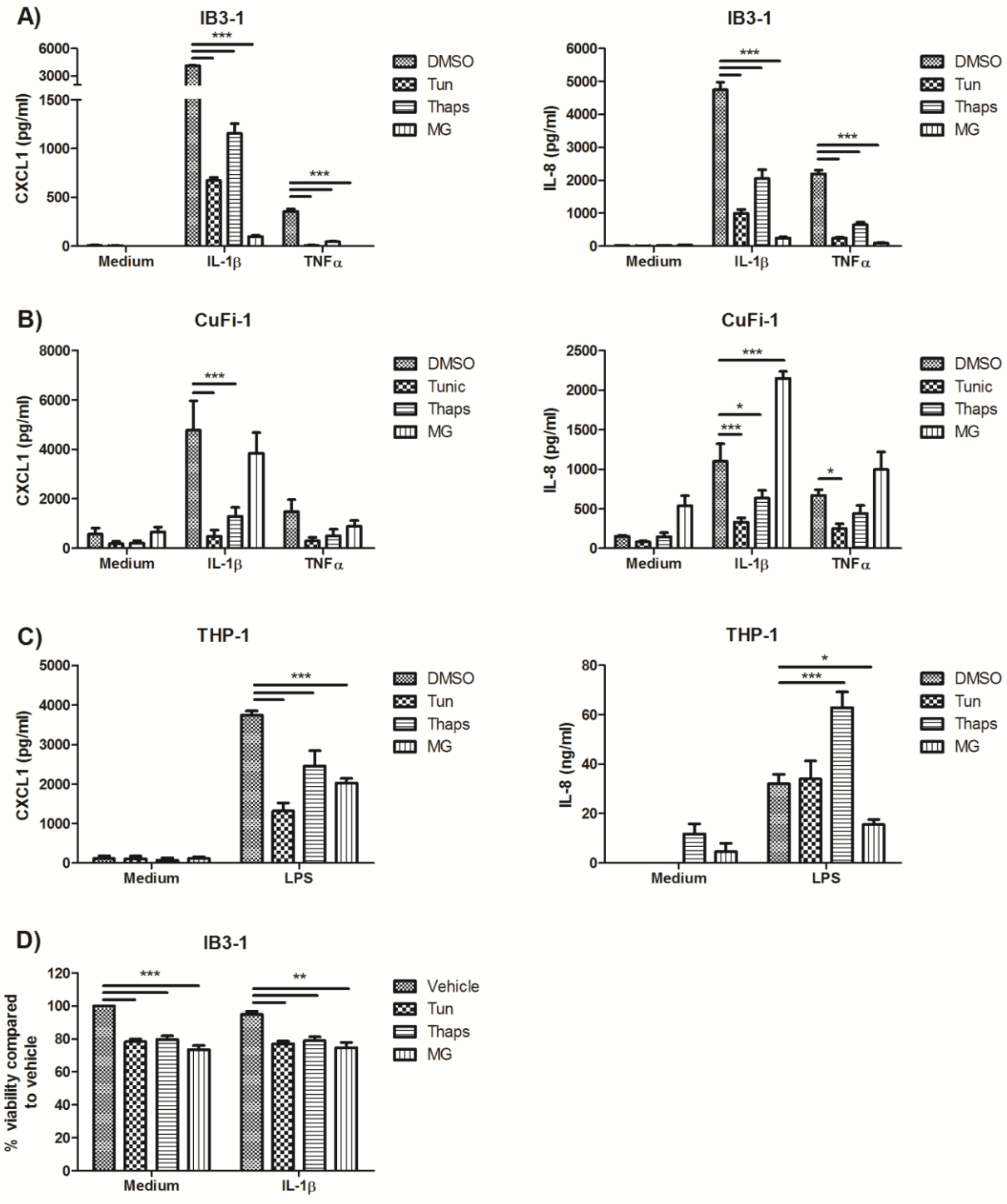
Figure 3.3: NF-κB activation is inconsistent between ER stressors and cell types

(A) IB3-1 cells were transfected with a plasmid containing an NF-κB responsive element upstream of a luciferase reporter gene (pGL4.32). Cells were stimulated for 24 hours and lysates were examined for luciferase activity. Three 10-fold dilutions were screened, starting with the concentrations indicated: tunicamycin (50 μg/ml), thapsigargin (50 μM), MG-132 (50 μg/ml), Brefeldin A (20 μg/ml), and IL-1β (100 ng/ml). NF-κB/AP-1 activation was also measured in THP-1 X-Blue cells using 10 μg/ml tunicamycin, 1 μM thapsigargin, 5 μM MG-132, and 10 ng/ml LPS in (C). Whole cell lysates for (B) IB3-1 and (D) THP-1 cells were blotted for NF-κB pathway activation by p65 phosphorylation and IκBα degradation as well as ER stress response activation by ATF4 expression at 0.5, 1.5, and 3 hours. n = 3 for all

experiments where one representative western blot is shown. β-actin was used as a loading control. Data were analyzed by two-way ANOVA with Bonferroni post-test. * P< 0.05, ** P< 0.01, *** P< 0.005.

ER stress modulates CXCL1 and IL-8 production in CF airway epithelial cells

Dissection of the interaction between ER stress and inflammatory signaling pathways has important implications for therapeutic developments in CF, where both UPR activation and inflammatory cytokine or TLR signaling occur simultaneously. In contrast to the modest cytokine responses triggered by treatment with ER stressors alone (Fig. 3.2C, D), we found that the CF airway epithelial cells IB3-1 and CuFi-1 displayed consistent reduction in the amount of IL-8 and CXCL1 produced ($P<0.001$) when stimulated with IL-1β or TNFα under ER stress conditions (Fig. 3.4A, B), although cell viability with ER stressor treatment was only 80% of the untreated control (Fig. 3.4D). ER stressors had differing effects on IL-8, but consistently reduced CXCL1 production by THP-1 cells in response to LPS (Fig. 3.4C), though it should be noted that the various ER stressors did not necessarily induce the UPR identically or to the same degree. Where tunicamycin and thapsigargin similarly induced XBP1s and ATF4 protein, MG-132 showed preference for ATF4 induction and Brefeldin A, used at 5 μg/ml, was a comparatively weak inducer of both when compared to the other ER stressors (Fig. 3.5A, B). Furthermore, quantification by qPCR did not necessarily reflect findings at the protein level (Fig. 3.5C).


A)
IB3-1
CXCL1 (pg/ml)
6000
3000
1500
1000
500
0
***
***
Medium
IL-1β
TNFα
DMSO
Tun
Thaps
MG
IB3-1
IL-8 (pg/ml)
6000
5000
4000
3000
2000
1000
0
***
***
Medium
IL-1β
TNFα
DMSO
Tun
Thaps
MG
B)
CuFi-1
CXCL1 (pg/ml)
8000
6000
4000
2000
0
***
Medium
IL-1β
TNFα
DMSO
Tunic
Thaps
MG
CuFi-1
IL-8 (pg/ml)
2500
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500
0
***
*
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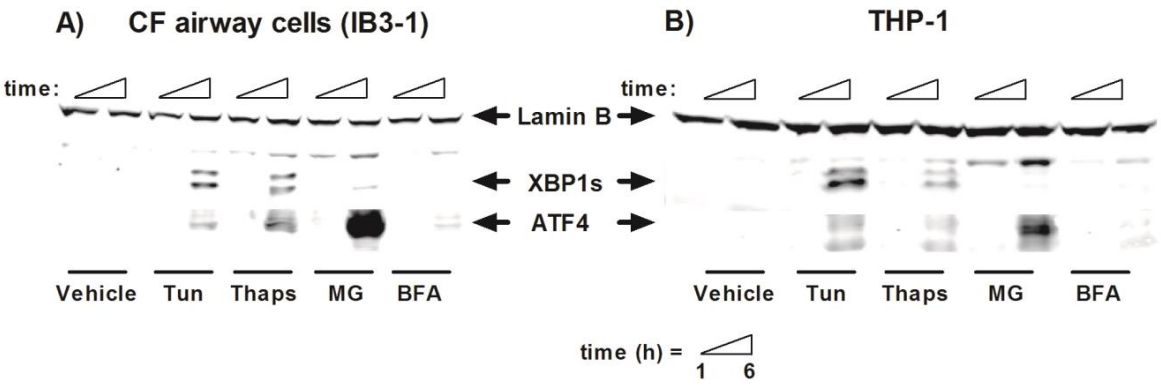
*
Medium
IL-1β
TNFα
DMSO
Tunic
Thaps
MG
C)
THP-1
CXCL1 (pg/ml)
5000
4000
3000
2000
1000
0
***
Medium
LPS
DMSO
Tun
Thaps
MG
THP-1
IL-8 (ng/ml)
80
60
40
20
0
*
***
Medium
LPS
DMSO
Tun
Thaps
MG
D)
IB3-1
% viability compared to vehicle
120
100
80
60
40
20
0
***
**
Medium
IL-1β
Vehicle
Tun
Thaps
MG

Figure 3.4: ER stress modulates CXCL1 and IL-8 production in CF airway epithelial cells

(A) IB3-1, (B) CuFi-1, and (C) THP-1 cells were pre-exposed to tunicamycin (10 μg/ml), thapsigargin (1 μM), and MG-132 (5 μM), and subsequently stimulated with either 2 ng/ml IL-1β (IB3-1, CuFi-1) or 10 ng/ml LPS (THP-1). After 24 hours, supernatants were subsequently examined for CXCL1 and IL-8 production by ELISA, and viability of IB3-1 cells were shown in (D). Data were analyzed by two-way ANOVA with Bonferroni post-test. * P< 0.05, ** P< 0.01,*** P< 0.005.



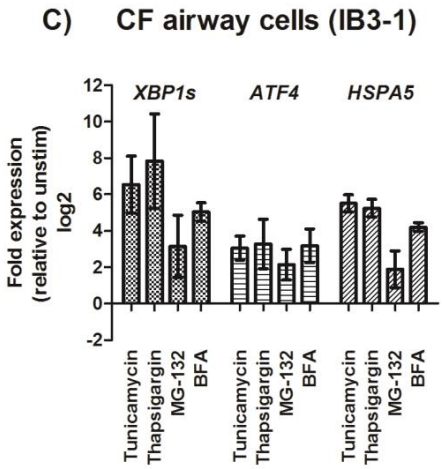


Figure 3.5: Differential induction of the ER stress response by chemical ER stressors

Nucleus-enriched (A) IB3-1 and (B) THP-1 lysates were blotted for the presence of XBP1s and ATF4 at 1 and 6 hours after ER stressor exposure: tunicamycin (10 μg/ml), thapsigargin (10 μM), MG-132 (5 μM), and BFA (5 μg/ml). Measurement of UPR transcription factors by immunoblot was compared with UPR activation as measured by qPCR. (C) IB3-1 cells were treated with various ER stressors for 5 hours and measured for increases in *XBP1s*, *ATF4*, and *HSPA5* transcript. qPCR data were expressed using a $\log_2$ transformation and show the mean with 95% confidence intervals. n = 3 independent experiments.

CXCL1 and IL8 are associated with lung disease severity in CF

CXCL1 and *CXCL8* (which encodes the IL8 protein) have both been shown to be elevated in CF (350), and may result in increased recruitment of neutrophils and progressive lung damage. In order to determine whether our findings could have any impact on disease in the general CF population, we decided to look at whether SNPs in these genes were associated with changes in lung function. To test whether genetic variation in *CXCL1* and *CXCL8* is associated with differences in CF lung function, we interrogated data from the recent international genome wide association study for gene modifiers in CF (211). Genetic variants in both *CXCL1* and *CXCL8* were significantly associated with lung disease severity in CF (P = 0.024 and 0.041, respectively) when examined using a gene based test statistic calculated using VEGAS2 (Table 3.2). Gene-based testing takes into account all SNPs in a gene (+/- 10kb of the gene of interest in this case) and is helpful for the detection of associations, especially when there are multiple causative variants which may only be weakly associated using individual SNP-based analysis (359). A subsequent SNP-based analysis identified 36 SNPs in *CXCL1* and 21 SNPs in *CXCL8* that were nominally associated with CF lung disease severity, however none of these SNPs were significant after Bonferroni correction for multiple comparisons (Table 3.3 and 3.4, Fig.

3.6). Together, these data indicate that variants in *CXCL1* and *CXCL8* significantly associate with lung disease severity in the CF population and targeting their gene products may therefore serve as a good therapeutic target.

Table 3.2: P-values for gene-based association tests for *CXCL1* and *CXCL8*

Gene	Number of SNPS	Number of Simulations	P-Value	Corrected P-Value	Meta-Analysis Model
CXCL1	247	1×10^4	0.024	0.016	Fixed Effects
		1×10^4	0.026	0.024	Random Effects
CXCL8	222	1×10^4	0.023	0.016	Fixed Effects
		1×10^4	0.044	0.041	Random Effects

Gene-based P-values displayed were calculated by VEGAS2. P-Values for both fixed and random effects are shown and the corrected P-value refers to correction for genome inflation.

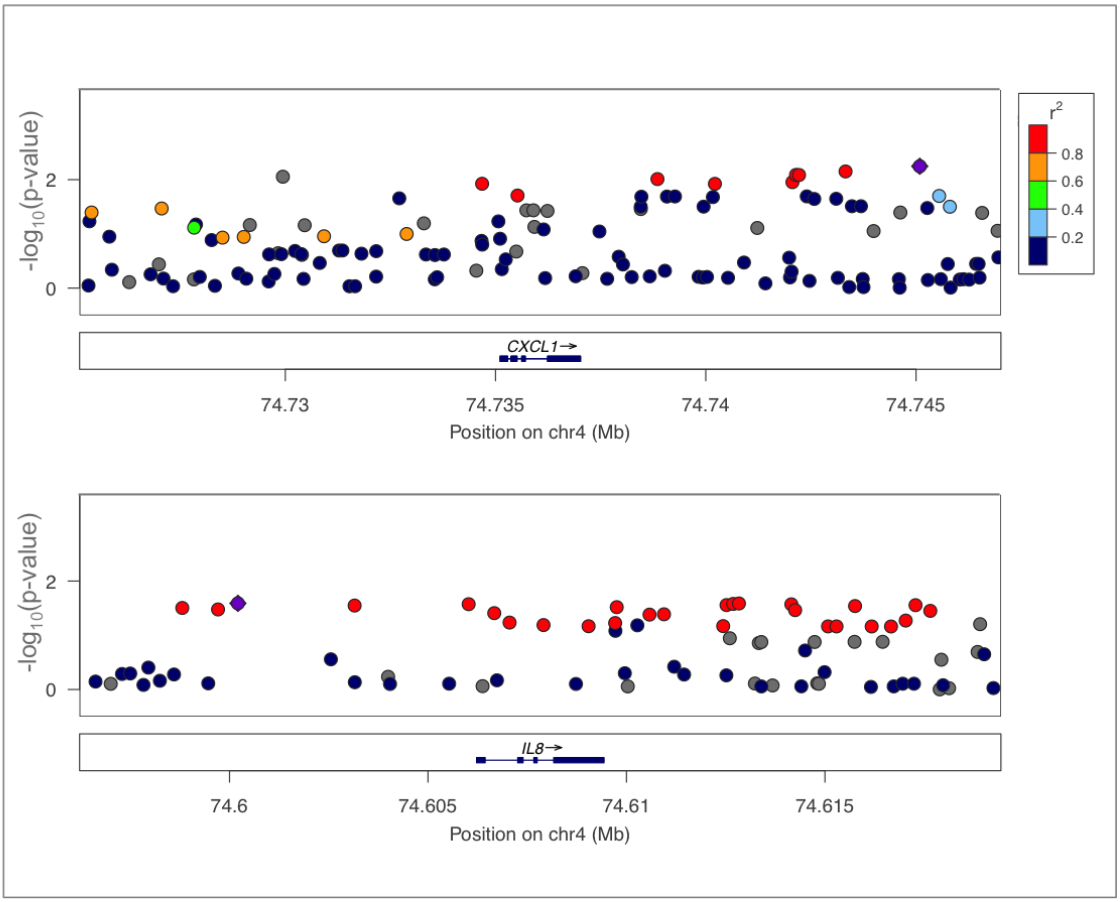


Figure 3.6: Locus Zoom plot for *CXCL1* and *CXCL8*

This plot shows the individual SNPs within 10 kb upstream of the transcriptional start or 10 kb downstream of the transcriptional stop site of *CXCL1* and *IL8*, along with their P-values and degree of linkage disequilibrium.

Table 3.3: P-values for genetic association of lung disease severity of *CXCL1* SNPs in CF

CHR	Position	SNP	Alleles	P-Value (Fixed Effects)	P-Value (Random Effects)	Location of SNP
4	74745089	rs183836557	T/C	0.006	0.006	intergenic
4	74745088	4:74745088:TC_T	deletion TC>T	0.006	0.006	intergenic
4	74745084	4:74745084:T_TC	insertion T>TC	0.006	0.006	intergenic
4	74743324	rs76212893	A/C	0.007	0.007	intergenic
4	74742146	rs58610473	C/A	0.008	0.008	intergenic
4	74742218	rs58324794	T/C	0.008	0.008	intergenic
4	74729943	rs2367290	C/A	0.009	0.047	intergenic
4	74738851	rs1951233	A/G	0.01	0.01	intergenic
4	74742061	rs61412577	T/G	0.011	0.011	intergenic
4	74734681	rs74544699	G/A	0.012	0.014	5’ region
4	74740217	rs1957069	C/T	0.012	0.012	intergenic
4	74735524	rs7656335	C/G	0.02	0.02	CXCL1 intron
4	74745550	rs10021230	A/T	0.02	0.022	intergenic
4	74742402	rs9991272	A/G	0.02	0.026	intergenic
4	74739267	rs2367293	C/T	0.02	0.026	intergenic
4	74739076	rs1366949	G/T	0.02	0.026	intergenic
4	74738469	rs1366946	C/T	0.021	0.026	intergenic
4	74740169	rs10007826	G/C	0.021	0.027	intergenic
4	74732711	rs3117601	A/T	0.022	0.032	intergenic
4	74732711	4:74732711:T_TA	insertion T>TA	0.022	0.022	intergenic
4	74743102	rs7674809	T/C	0.023	0.024	intergenic
4	74742580	rs10028419	C/T	0.023	0.025	intergenic
4	74743691	rs2115691	G/C	0.031	0.043	intergenic
4	74743475	rs3097409	C/T	0.031	0.043	intergenic
4	74739948	rs2968710	T/C	0.031	0.041	intergenic
4	74738454	rs3097411	G/C	0.032	0.041	intergenic
4	74745805	rs3111697	T/C	0.032	0.043	intergenic
4	74745268	rs28872183	T/C	0.033	0.169	intergenic
4	74727060	rs75019946	A/G	0.034	0.034	intergenic
4	74738456	rs1820888	G/T	0.035	0.1	intergenic
4	74735901	rs930113	A/C	0.037	0.092	CXCL1 intron
4	74735734	rs2018732	T/C	0.037	0.093	CXCL1 intron
4	74736235	rs1814092	T/C	0.038	0.093	CXCL1 intron
4	74725393	rs76797395	T/C	0.04	0.04	intergenic
4	74744628	rs1835263	T/C	0.04	0.089	intergenic
4	74746572	rs3097408	A/G	0.041	0.085	intergenic
4	74736144	rs4074	G/A	0.083	0.049	CXCL1 intron

CXCL1 SNPs nominally associated with lung disease severity in CF (fixed-effects P <0.05). The critical P -value for significance for *CXCL1* is 0.001 based on an effective SNP number in this region of 43.

Table 3.4: P-values for genetic association of lung disease severity of *CXCL8* SNPs in CF

CHR	Position	SNP	Alleles	P-Value (Fixed Effects)	P-Value (Random-Effects)	Location of SNP
4	74600211	rs11730667	G/A	0.026	0.066	intergenic
4	74612688	rs4694178	A/C	0.026	0.067	intergenic
4	74612834	rs4694637	G/A	0.026	0.067	intergenic
4	74614165	4:74614165:CTA_C	deletion CTA>C	0.026	0.066	intergenic
4	74606024	rs4073	T/A	0.027	0.068	5’ region
4	74614154	rs13109377	A/T	0.027	0.067	intergenic
4	74603151	rs2227521	C/G	0.028	0.071	intergenic
4	74612520	rs13106097	T/G	0.028	0.069	intergenic
4	74617282	rs1951242	T/C	0.028	0.069	intergenic
4	74615758	rs1951240	G/T	0.029	0.062	intergenic
4	74609755	rs13112910	A/G	0.03	0.066	3' region
4	74598809	rs4694636	T/G	0.031	0.071	intergenic
4	74599710	rs16849928	G/A	0.033	0.089	intergenic
4	74607514	4:74607514:T_TA	insertion T>TA	0.033	0.077	IL8 intron
4	74614249	rs74698542	A/T	0.034	0.071	intergenic
4	74617654	rs11730284	C/A	0.035	0.081	intergenic
4	74617680	4:74617680:G_GA	insertion G>GA	0.036	0.072	intergenic
4	74610150	4:74610150:C_CT	insertion C>CT	0.038	0.066	3' region
4	74606669	rs2227307	G/T	0.039	0.091	IL8 intron
4	74610945	rs12647924	A/C	0.041	0.073	intergenic
4	74610581	rs113976067	C/T	0.042	0.079	3' region

CXCL8 SNPs nominally associated with lung disease severity in CF (fixed-effects P<0.05). The critical P-value for significance for *CXCL8* is 0.002 based on an effective SNP number in this region of 29.

ER stress inhibits LPS and IL-1β-induced STAT3 signaling

Now that we had obtained both *in vitro* evidence derived from cellular models and also human evidence for the importance of CXCL1 and IL-8, we wanted to determine the specific mechanisms responsible for their modulation in response to ER stress. Thus, we examined various signalling pathways that have been previously shown to affect production of these cytokines. We decided to examine the STAT3 signaling pathway that has been previously implicated in both CXCL1 and IL-8 production as well as in ER stress (360, 361). Phosphorylation of STAT3 was diminished in IB3-1 cells following stimulation with IL-1β under ER stress conditions (Fig. 3.7A). This was confirmed in THP-1 cells where STAT3 phosphorylation was also decreased following stimulation with LPS under ER stress conditions (Fig. 3.7B) (indicated by arrowheads). Furthermore, we also examined the activation status of a number of classic inflammatory pathways including p65, IRF3, and p38 in THP-1 cells (Fig 3.7B). Of the pathways examined, only STAT3 phosphorylation was found to be inhibited by ER stress at 3 hours of stimulation with LPS and is therefore a compelling candidate pathway for the observed decrease in CXCL1 and IL-8 and production in our models.

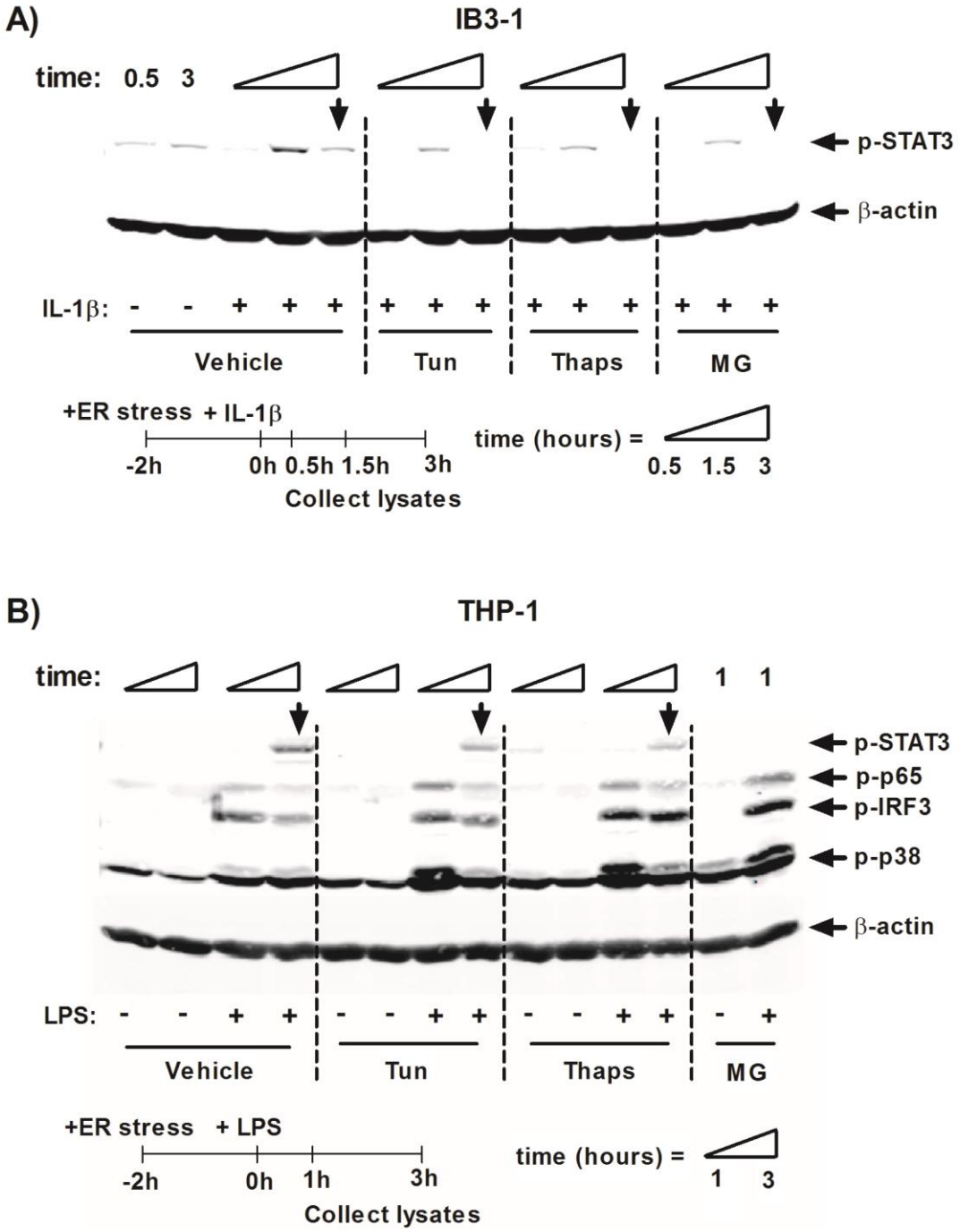


Figure 3.7: ER stress inhibits LPS and IL-1β-induced STAT3 signaling

(A) IB3-1 cells were pre-treated with ER stressors and subsequently exposed to IL-1β (2 ng/ml), with cells being harvested at 0.5, 1.5, and 3 hours. (B) THP-1 cells were pre-treated with ER stressors 2 hours prior to LPS stimulation (10 ng/ml) as in Figure 2 and cells were harvested at 1 and 3 hours post-stimulation.

STAT3, but not XBP1s or ATF4 modulate IL-8 and CXCL1 production in airway epithelial cells

Because the ER stress transcription factors XBP1s and ATF4 are well known to contribute to inflammatory cytokine production and affect inflammatory responses, we examined if they had any role in CXCL1 and IL-8 production in our system. Knockdown of ATF4 or XBP1 in IB3-1 cells (Fig. 3.8A) showed a dependency of IL-6 ($P< 0.01$) (Fig 3.8B), but not IL-8 or CXCL1 on the ER stress transcription factor ATF4 (Fig 3.8C, D). The ability of prior inflammatory stimulation to modulate ATF4 levels was also examined but did not yield any changes to ATF4 expression (Fig. 3.9A, B). Changes in XBP1 were observed (Fig. 3.9A, B), but XBP1 knockdown did not alter cytokine production (Fig. 3.8B-D). Conversely, inhibition of STAT3 using the inhibitor, Stattic, led to modulation of both IL-8 and CXCL1, although in somewhat differing manners. Stattic treatment inhibited CXCL1 production predictably in a dose-dependent manner (Fig. 3.8E), but its effects on IL-8 production were unusually varied: lower concentrations of Stattic increased IL-8 production, peaking at a concentration of 1.5 μM (Fig. 3.8E) prior to being inhibited at a concentration of 6 μM. This unusual pattern was also observed in the metabolic effects of Stattic on IB3-1 cells, where lower concentrations of Stattic increased metabolic activity as measured by MTS assay (Fig. 3.8F). Although this increase in activity could partially help explain the increased IL-8 production observed, it appears that taking into account the accumulated evidence in our cellular models, STAT3 inhibition has shown the most consistent outcomes on CXCL1, but not IL-8 production. Therefore, the decrease in IL-8 production in airway epithelial cells is likely mediated by other mechanisms affected by ER stress.

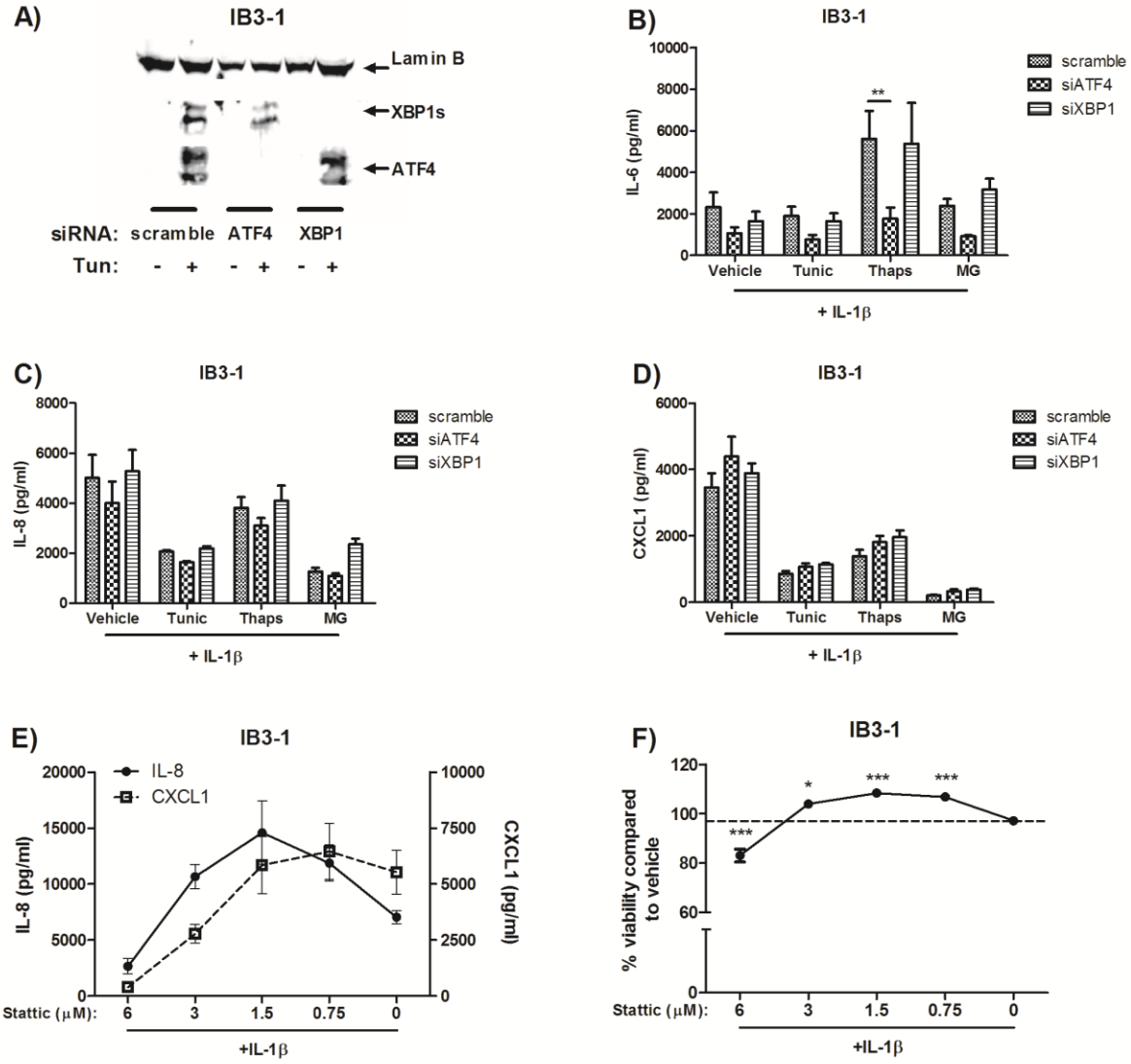

A)
IB3-1
Lamin B
XBP1s
ATF4
siRNA: scramble ATF4 XBP1
Tun: - + - + - +
B)
IB3-1
IL-6 (pg/ml)
scramble
siATF4
siXBP1
Vehicle
Tunic
Thaps
MG
+ IL-1β
C)
IB3-1
IL-8 (pg/ml)
scramble
siATF4
siXBP1
Vehicle
Tunic
Thaps
MG
+ IL-1β
D)
IB3-1
CXCL1 (pg/ml)
scramble
siATF4
siXBP1
Vehicle
Tunic
Thaps
MG
+ IL-1β
E)
IB3-1
IL-8
CXCL1
IL-8 (pg/ml)
CXCL1 (pg/ml)
Stattic (μM): 6 3 1.5 0.75 0
+IL-1β
F)
IB3-1
% viability compared to vehicle
Stattic (μM): 6 3 1.5 0.75 0
+IL-1β

Figure 3.8: STAT3, but not XBP1s or ATF4 modulates IL-8 and CXCL1 production in airway epithelial cells

(A) IB3-1 cells were transfected with siRNA against XBP1s or ATF4, stimulated for 4 hours with 10 μg/ml tunicamycin, and subsequently enriched for nuclei. siRNA-transfected IB3-1 cells were then exposed to ER stressors for 2 hours, and stimulated with 2ng/ml IL-1β for 24 hours, after which supernatants were examined for (B) IL-6, (C) IL-8, or (D) CXCL1 production. (E) IB3-1 cells were treated with varying concentrations of Stattic for 1 hour prior to stimulation with IL-1β. Supernatants were collected at 24 hours post stimulation and assessed for IL-8 and CXCL1 production, as well as (F) metabolic activity/viability by MTS assay. Data were analyzed by two-way ANOVA with Bonferroni correction for siRNA experiments and one-way ANOVA with Dunnett's post-test for Stattic experiments, where all concentrations were compared to the untreated condition. * $P< 0.05$, ** $P< 0.01$, *** $P< 0.005$.

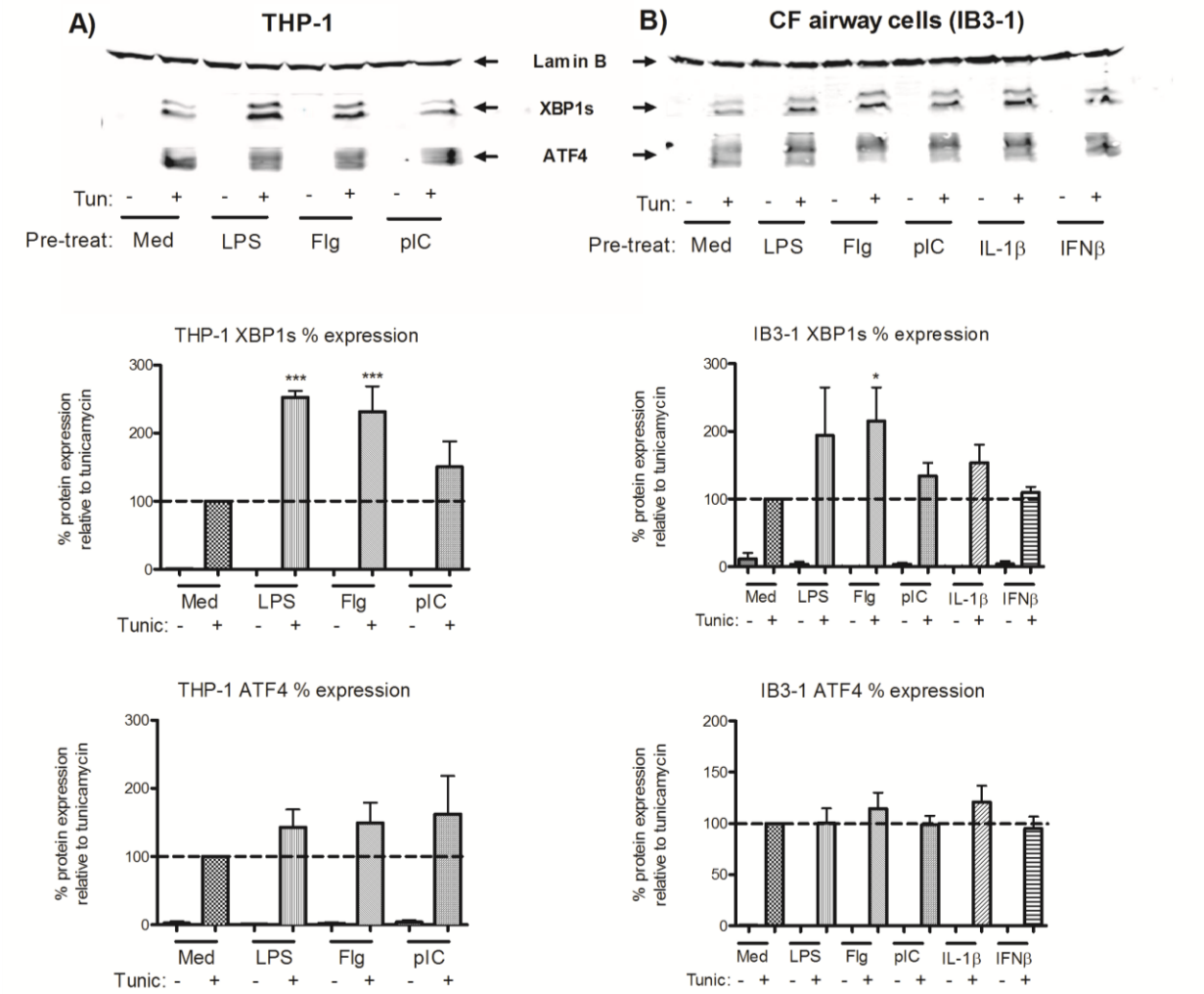


Figure 3.9: XBP1 but not ATF4 expression is modulated by inflammatory stimuli

(A) THP-1 (n = 3) or (B) IB3-1 (n = 4) cells were pre-treated with LPS (10 ng/ml for THP-1, 100 ng/ml for IB3-1), flagellin (500 ng/ml), poly(I:C) (20 μg/ml), IL-1β (10 ng/ml), or IFN-β (1000 U/ml) for 2 hours prior to exposure with tunicamycin (10 μg/ml) for 4 hours and then enriched for nuclei. Samples were normalized to tunicamycin treatment alone (taken as 100%). Pre-treatment with various TLR ligands and inflammatory ligands occurred 2 hours prior to tunicamycin exposure. XBP1s and ATF4 levels from THP-1 (A) and IB3-1 (B) were quantified. Data were analyzed by one-way ANOVA with Dunnett's post-test. * $P < 0.05$, *** $P < 0.001$.

ER stress decreases PMN migration towards epithelial-derived chemokines

As IL-8 and CXCL1 are primarily chemotactic for neutrophils, we sought to determine the effect that ER stress and STAT3 inhibition had on neutrophil migration to the bronchial epithelium as well as the impact that these cytokines could have on lung function in CF. Conditioned media from IB3-1 cells stimulated with IL-1β under ER stress conditions showed reduced chemotactic ability for PMNs isolated from healthy adult donors ($P < 0.05$) (Fig. 3.10A). Although conditioned media from Stattic-treated IB3-1 cells did not show a reduction in PMN migration, this is likely because a concentration was chosen where only CXCL1, but not IL-8, was decreased compared to control (3 μM from Fig. 3.8E) and further titration of inhibitor is necessary. Alternatively, this may be predictive of the inefficacy of monotherapy and the importance for the inhibition of both IL-8 and CXCL1 in order to effectively reduce neutrophil migration. It is important to note that as ER stressors were present in the conditioned media, induction of ER stress in PMNs may have also affected migration.

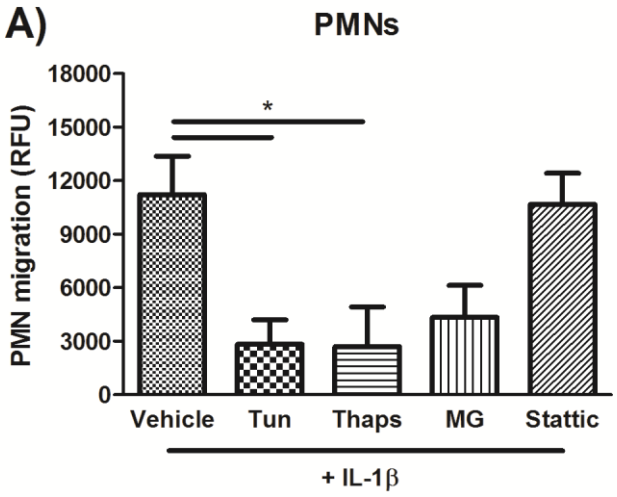


Figure 3.10: PMN migration is decreased towards supernatants from epithelial cells undergoing ER stress

(A) CFSE-labelled PMNs isolated from healthy adult donors were placed in the upper chambers of 3 μm inserts. The lower wells were filled with supernatants from IB3-1 cells stimulated with IL-1β (2 ng/ml) with or without ER stressors (same concentrations as in previous experiments) or Stattic (3 μM) pre-treatment. PMNs were allowed to migrate for 4 hours prior to reading. Data were analyzed using one way ANOVA with Dunnett's post-test. * indicates $P< 0.05$.

3.5 Discussion

ER stress-induced inflammation, or at least cross-talk between ER stress pathways and inflammatory pathways, has been suggested to be central to the pathogenesis of many human metabolic, intestinal, and airway diseases (10). Given the potential therapeutic value of targeting ER stress-induced inflammation, we sought to systematically define the relationship between ER stress and inflammation using a panel of commonly employed ER stressors in the context of CF lung disease. In our model we created an environment, such as in CF, where ER stress occurs concurrently with an abundance of inflammatory signals, such as TLR ligands and cytokines. Our main findings are that i) ER stress induction by itself did not consistently induce inflammatory cytokine secretion or NF-κB induction in either airway epithelial cells or monocytes. ii) The secretion of IL-8 and CXCL1 in response to IL-1β stimulation under conditions of ER stress is diminished in airway epithelial cells. iii) Polymorphisms in *CXCL8* and *CXCL1* are associated with lung disease severity in CF patients. iv) Decreases in IL-8 and CXCL1 are partially mediated by inhibition of STAT3 phosphorylation by ER stress.

Contrary to our predictions, we did not observe consistent NF-κB activation and inflammatory cytokine secretion alone in our cell types, despite strong induction of the UPR (Fig. 3.2 and Supplementary Figure 3.2). Instead, ER stress had a more significant role in affecting

inflammatory responses elicited from other sources including IL-1β and LPS. Instead of a strictly pro-inflammatory role, we found that the chemical ER stressors suppressed production of the neutrophil chemokines IL-8 and CXCL1 in CF airway epithelial cells. However, there were some differences depending on the cell type, where IL-8 production was not decreased in THP-1 cells stimulated with LPS after ER stressor treatment (Fig. 3.3C). ER stressors themselves may also affect the readouts of certain reporter assays. Brefeldin A, in particular is widely used to disrupt protein transport between the ER and Golgi and therefore multiple complementary methods have been used to validate these findings in multiple cell lines.

In addition to the well-documented role of ER stress in NF-κB induction, studies also point to an anti-inflammatory role for ER stress (362). This may occur through upregulation of the A20 deubiquitinase (363), inhibition of TRAF2 expression (338), and reduction of MCP-1 upon ER stress induction and subsequent stimulation with pro-inflammatory cytokines (364). Further work revealed the involvement of C/EBPβ in the inhibition of NF-κB activation by pro-inflammatory cytokines (365) and the inhibition of inflammatory cytokine mRNA after ER stressor treatment in conjunction with inflammatory cytokine stimulation. Pre-treatment with ER stress-inducing chemicals and XBP1s over-expression was also found to act antagonistically to NF-κB activation and cytokine secretion in retinal endothelial cells (366). Overall evidence indicates that ER stress can affect inflammatory processes through either pro- or anti-inflammatory mechanisms depending on the cell-type or stimulus involved.

In order to examine the relevance of IL-8 and CXCL1 to lung disease in the CF population, we looked for genetic association of SNPs in a combined data set of North American and French CF cohorts (n = 5,827) (211), which forms the largest CF modifier study to date. Although we did not identify any SNPs in either gene that significantly associated with lung disease severity

after correction for multiple comparisons, Locus zoom plots showed a considerable number of moderately-associated SNPs in low linkage disequilibrium (LD) for *CXCL1* that could be indicative of the presence of several causative variants. Therefore, we chose to employ gene-based analysis on our genes of interest using VEGAS2, which showed significant P values of $P < 0.024$and $P < 0.041$ for *CXCL1* and *CXCL8*, respectively. Several *CXCL8* SNPs identified to be of interest in our study have previously associated with lung disease severity in an association study performed on an American cohort of CF patients (209). These data showed a gender effect, where lung disease severity was only associated with *CXCL8* SNPs in male patients (albeit modestly). One variant of the associated SNPs, the T allele of rs4073, is located in the *CXCL8* promoter resulted in increased IL-8 levels compared to the A allele upon stimulation with IL-1β and TNFα (209). Interestingly, this IL8 promoter SNP (rs40873T/A) has also been implicated in several other chronic inflammatory airway diseases including asthma (367) and idiopathic pulmonary fibrosis (368), where the T allele was associated with disease. Complementary to our findings, a German cohort (n = 442) observed that specific haplotypes of *CXCR1* and *CXCR2* SNPs, receptors for IL-8 and CXCL1, were associated with lower lung function as measured by FEV1. Specifically, neutrophils carrying *CXCR1/2* haplotypes associated with decreased levels of CXCR1 but increased CXCR2 surface expression showed decreased bactericidal activity, indicating that differential responses to these cytokines may mediate alterations in bactericidal activity and neutrophilic inflammation (210). Although we did not find that single SNPs in either *CXCL1* or *CXCL8* strongly associated with lung disease severity, the complementary nature of these data warrants further pathway analysis which may help to identify gene-gene interactions that could significantly associate with CF lung function.

The STAT3 pathway has been noted to play critical roles in both neutrophil chemotaxis (66), and granulopoeisis (369). Interestingly, we found that although STAT3 inhibition by Stattic did decrease the amount of CXCL1 secreted, certain concentrations of the inhibitor unexpectedly increased the amount of IL-8 produced as well as the metabolic activity of the cells. Application of Stattic at a concentration where CXCL1 but not CXCL8 was decreased when compared to the untreated control, was not sufficient to inhibit overall PMN migration. This may suggest a requirement for the dual inhibition of these chemokines in order to achieve efficacy, but additional titration of the inhibitor is still necessary. Collectively, the data from the THP-1 cells, CF airway epithelial cells, and the Stattic inhibitor identify CXCL1 as being sensitive to STAT3 inhibition. On the other hand, IL-8 was inconsistently affected between cell types as well as between ER stressors and Stattic treatment, indicating that its reduction by ER stress in airway epithelial cells likely involves changes in other inflammatory pathways. This occurred independently of XBP1s and ATF4 as knockdown using siRNA did not show significant differences (Fig 3.4), although LPS stimulation did affect XBP1s levels upon tunicamycin treatment (Supplementary Figure 3.2). While there have been attempts at CXCR2 antagonism as a therapeutic in both CF and other diseases, their clinical successes have been limited (370). Supplementation of this strategy with STAT3 inhibition may help to increase the overall effect or may even stand alone as a therapeutic strategy if IL-8 levels can be decreased in combination with CXCL1.

In addition to cytokine production by epithelial cells, STAT3 also plays important roles in neutrophil chemotaxis by controlling expression of CXCR2 (66), one of the main receptors involved in neutrophil chemotaxis. Therefore, the use of STAT3 inhibitors may address the issue of inflammatory neutrophil recruitment in two ways: decreasing production of neutrophil

chemokines at the origin of inflammation and by dampening the subsequent migratory response to these chemokines. However, complete inhibition of STAT3 may also have other effects on neutrophil maturation and localization (369).

Although we found that ER stress in the presence of IL-1β stimulation decreased IL-8 and CXCL1 production, this is certainly not to say that ER stress is beneficial in CF, as the effects of ER stress on disease is quite variable. Rather, the use of chemical ER stress inducers in this study simply allowed for the identification of STAT3 as a pathway that may ultimately be helpful in reducing chemokine production and limiting inflammation in CF. Furthermore, it is important to emphasize the multi-factorial nature of neutrophilic inflammation, where many chemoattractants exist in the CF lung and must be targeted in concert to achieve good inhibition of neutrophil migration. This concept was well-illustrated by Mackerness et al (240) where inhibition of CF sputum samples with anti-IL-8 was insufficient to reduce the majority of neutrophil chemotaxis, and required additional inhibition of other chemotactic factors, including leukotriene B4, C5a, and platelet activating factor. Furthermore, phase I clinical trial results of a CXCR2 antagonist, while safe, yielded only modest effects (239). This ultimately shows that for inhibition of neutrophilic inflammation, a combined approach targeting several chemotactic factors or receptors may be necessary.

In conclusion, we report that ER stress decreases IL-8 and CXCL1 production by airway epithelial cells which, in turn, reduces neutrophil migration. Variation in the genes encoding both IL-8 and CXCL1 are associated with lung disease severity in CF, and modulation of ER stress signaling, at least partially through STAT3 inhibition, may help to mitigate production of these chemokines and improve health outcomes for people living with CF.

CHAPTER 4: DUAL PRO- AND ANTI-INFLAMMATORY FUNCTIONS OF PROTEASOME INHIBITORS: POTENTIAL OF TLR ADJUVANTS TO ELICIT IMMUNOGENIC CELL DEATH

4.1 Rationale: In the previous chapter we showed that ER stress and the UPR could be effectively manipulated to modify inflammatory pathways and subsequent leukocyte recruitment through ER stress modulation of STAT3. Because ER stress is also known to mediate inflammasome activation and IL-1β production (371, 372), we examined the effects of these inhibitors on IL-1β production in our system. Of the ER stressors tested, one in particular (MG-132) could strongly induce the production of IL-1β depending on when it was applied relative to an inflammatory TLR stimulus (Figure 4.3 A, B) Given the predicted detrimental role of IL-1β in cancer progression (253), it was interesting to find that this type of inhibitor is widely used as a first line therapeutic against multiple myeloma. Moreover, the combination of this therapeutic with TLR adjuvants has been recently investigated, with stimulation of TLR9 showing favourable effects by increasing tumour cell death (252, 373). Therefore, we sought to determine the effects that inclusion of a TLR adjuvant in conjunction with proteasome inhibitor treatment could have in the context of IL-1β processing and inflammatory cell death.

4.2 Background:

Proteasomes are very large protein complexes (~ 2.5 MDa) that degrade ubiquitinated substrates and are responsible for much of the normal protein turnover in the cell by degrading ubiquitinated substrates (374). Proteasomes also have important roles in cellular signaling, such as mediating degradation of IκBα in NF-κB signaling (375), as well as digesting protein antigens for presentation by MHC molecules during the immune response (376, 377).

Composed of a 20S catalytic core (378), proteasomes may be capped by one or two regulatory subunits (19S) which mediate recognition and entry of potential substrates (374). Catalytic core activity is characterized by three types of proteolytic functions: caspase-like activity mediating cleavage after acidic residues (β1 subunit), trypsin-like activity mediating cleavage after basic residues (β2 subunit), and chymotrypsin-like activity which mediates cleavage after hydrophobic residues (β5 subunit) (263).

The proteasome inhibitor bortezomib is employed quite successfully as a first-line treatment for multiple myeloma and shows efficacy in non-Hodgkin's lymphomas (eg. mantle cell lymphoma and Waldenström's macroglobulinemia) (379). However, its use is limited by its low effectiveness in other cancer types, particularly solid tumours (380), and bortezomib resistance can develop through mutations in *PSMB5* (381). Bortezomib primarily targets the β5 subunit (chymotrypsin-like activity) of the proteasome (382) and is thought to inhibit cancer growth through multiple mechanisms, including inhibition of NF-κB, regulation of the cell cycle, and induction of apoptosis (263). Although bortezomib alone is a good inducer of apoptosis, it also mediates immunosuppressive effects (383, 384). Instead, combination of bortezomib with TLR agonists may help to induce forms of immunogenic cell death that may more strongly stimulate subsequent development of anti-tumour immunity.

TLR agonists have been investigated as adjuvants in cancer therapy for many years now but have not seen much success as monotherapies (82). This may be due to the vast but confusing literature detailing both tumour-promoting and tumour-inhibiting effects of TLR stimulation. Currently, there are only two TLR agonists approved for use in cancer: the TLR7 agonist imiquimod (385) and the TLR2/4 agonist bacille de Calmette-Guerin (BCG) (386, 387). The TLR4 agonist MPL is approved by the FDA as an adjuvant (388) and is currently used in the

vaccine to human papillomavirus, Cervarix®. Here, we investigated the effects of TLR adjuvancy and bortezomib combination therapy on the production of IL-1β and cell death. We hypothesize that the addition of TLR adjuvants to proteasome inhibitors can result in immunogenic cell death through production of IL-1β.

Aim: To characterize the induction of IL-1β processing and cell death by proteasome inhibitor treatment in the presence of TLR stimulation.

4.3 Materials and methods:

Reagents:

MG-132 was from EMD Millipore and bortezomib and carfilzomib were from Selleck chemicals. z-YVAD-fmk and z-IETD-fmk were from R&D systems. VX-765 was from Invivogen. AEBSF-HCl was from Enzo Life Sciences. Nec1s was from Biovision, GSK'872 and V5 were from EMD Millipore.

Cell culture:

THP-1, U937, and primary blood cells were cultured in RPMI1640 supplemented with 10% FCS, 2mM sodium pyruvate, and 1 mM L-glutamine. CD14-positive cells were purified from PBMCs extracted from Ficoll using a magnetic bead-based enrichment kit (BD Biosciences). CD14-positive cells were differentiated into macrophages over 10 days using 50 ng/ml M-CSF (Peprotech) and stimulated the following day in the presence of 5 ng/ml M-CSF. Differentiation of both THP-1 and U937 cells were carried out using 50 ng/ml PMA for 24 hours. Cells were rested for 36-48 hours prior to stimulation. HEK293 null 1 (Invivogen) cells were grown in

high-glucose DMEM supplemented with 10% FCS, 2mM sodium pyruvate, 1 mM L-glutamine, with the addition of 100 μg/ml normocin every two passages.

NF-κB reporter assays:

HEK293 null 1 cells were seeded at 7.5 x 10^4 cells per well in a 96-well plate and allowed to grow overnight. They were then treated with conditioned media from THP-1 cells that had been previously exposed to LPS and MG-132 either in the absence or presence of IL-1Ra. After 24 hours, supernatants were collected and assayed for NF-κB/AP-1 activation by overnight incubation with Quanti-Blue. THP-1 X-Blue cells were used as previously described (see data Ch.1), with proteasome inhibition occurring before LPS treatment.

Immunoblotting:

Lysates were made in RIPA buffer (see Ch. 2). Antibodies were from Cell Signaling Technologies (IL-1β, Caspase-1, β-actin, IκBα), and R&D systems (Caspase-8). Samples were lysed in RIPA buffer, mixed with loading buffer and sonicated before and after heating to 90°C for 5 minutes. The procedure for nuclear enrichment has been detailed in Ch.3. 60-65 μg of protein per lane as determined by Bradford assay was run on 15% polyacrylamide gels. Blots were imaged on low autofluorescence 0.45 μm PVDF on a LICOR Odyssey scanner.

ELISAs

ELISAs for IL-1β (eBioscience) were carried out according to the manufacturer's instructions.

Viability Assays:

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) and lactate dehydrogenase (LDH) assays were from Promega and used

according to the manufacturer's instructions. Briefly, MTS substrate was diluted 1in9 in RPMI1640 with supplements and incubated with cells for 1.5 to 2 hours at 37°C prior to reading at 490 nm on a Spectramax PLUS384. LDH assays were performed on the same samples but using the collected supernatants (after centrifugation). Supernatants were diluted in PBS and added to LDH substrate to achieve a final composition of ~15% supernatant. This was allowed to develop at room temperature for at least half an hour prior to reading at 490 nm.

Cloning and transfections:

THP-1 cDNA was used to PCR amplify *IL1B*. This was inserted into pCMV6-entry vector (Origene) using AsiSI and MluI restriction enzymes. The product was transfected into THP-1 cells (0.5 μg per 2 $x10^6$ cells) using solution SG and the Amaxa 4D nucleofector. Immediately after transfection, cells were differentiated with 50 ng/ml PMA and seeded into 24-well plates. Cells were rested 36-48 hours prior to stimulation. The siRNA (400 nM each per 2 x 10^6 cells) employed has been previously described (see Chapter 2) and was transfected using the same methodology stated above.

Statistics

All experiments were performed at least 3 times unless otherwise indicated and analyzed as stated with *, **, and *** indicating P values of $P< 0.05$, $P< 0.01$, and $P< 0.001$, respectively.

4.4 Results:

Proteasome inhibitors induce IL-1β secretion

In order to determine whether or not proteasome inhibition could consistently induce IL-1β production, we employed three different proteasome inhibitors: MG-132, bortezomib, and carfilzomib (Table 4.1). We tested these inhibitors on two leukemic cell lines, THP-1 and U937, as well as primary monocytes that had been differentiated into macrophages (Fig. 4.1A-D). After priming with LPS, it was found that proteasome inhibition could induce significant IL-1β secretion in all of the above cells by 24 hours. The kinetics of IL-1β secretion where examined in THP-1 cells over 24 hours (Fig. 4.1A). This was observed over a range of concentrations corresponding to levels typically used in *in vitro* studies but also to maximum achievable plasma levels of bortezomib measured in patients ranging from 109ng/ml (283 nM) to 1300ng/ml (3.383 μM) (treatment dose of 1.3 mg/m^2) (389) (Fig. 4.1C, D). IL-1β production by proteasome inhibitors was also replicated in primary human cells (Fig. 4.1B) and was therefore not a unique effect of our cancer cell models.

Table 4.1: Proteasome inhibitors used in this study

	CLASS	**MECHANISM**	**CLINICAL USE**
MG-132	Aldehyde	Reversible inhibitor of β5 subunit primarily, but also β1 caspase-like activity at higher concentrations	No
BORTEZOMIB (Velcade®)	Boronate	Reversible inhibitor of β5 subunit	Front-line therapy against multiple myeloma, mantle cell lymphoma
CARFILZOMIB (Kyprolis®)	Epoxyketone	Irreversible inhibitor of β5 subunit	Used against refractory multiple myeloma

A description of the different proteasome inhibitors used in this study, of which two are currently used clinically.

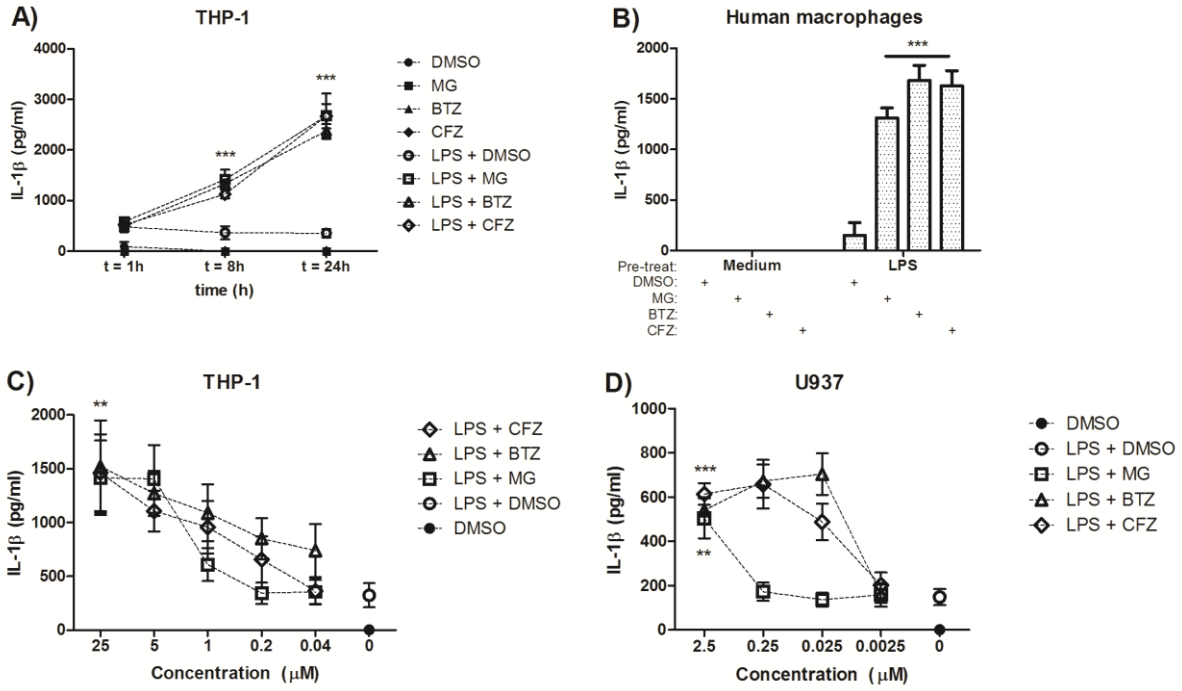


Figure 4.1: Proteasome inhibitors induce IL-1β secretion

(A) THP-1 cells were pre-treated with LPS (10 ng/ml) for 2 hours before exposure to 5 μM of proteasome inhibitors (MG-132, bortezomib, carfilzomib). Supernatants were collected 1, 8, and 24 hours after exposure and assayed for IL-1β secretion. (B) Peripheral blood-derived human macrophages were stimulated as in (A) and assayed for IL-1β secretion after 24 hours. (C) THP-1 and (D) U937 cells (n = 3 for all) were pre-treated with 10 ng/ml LPS for 2 hours prior to proteasome inhibitor treatment. Supernatants were collected and assayed for IL-1β levels after 24 hours. Data were analyzed using two-way ANOVA and the Bonferroni post-test. ** and *** indicate $P< 0.01$ and $P< 0.001$, respectively.

Proteasome inhibition induces processing of bioactive IL-1β

Proteasome inhibitors induce high levels of cell death which can result in pro-IL-1β release and quantification using ELISA may not adequately differentiate between pro and cleaved forms of IL-1β. Therefore, we examined the potential of secreted IL-1β by THP-1 cells to stimulate an NF-κB response in HEK293 null1 cells expressing an SEAP reporter for NF-κB/AP-1 activity. Conditioned media from THP-1 cells treated with LPS and MG-132, induced strong NF-κB/AP-1 activity which was abolished with IL-1 receptor antagonist (IL-1Ra) pre-treatment (Fig. 4.2A). Examining THP-1 supernatants by immunoblot showed that while pro-IL-1β was also present in the supernatant, cleaved IL-1β (p17) was present only in the LPS + MG-132 condition (Fig. 4.2B). Appearance of cleaved IL-1β by immunoblot (Fig. 4.2C) corresponded to the temporal increases in IL-1β observed by ELISA.

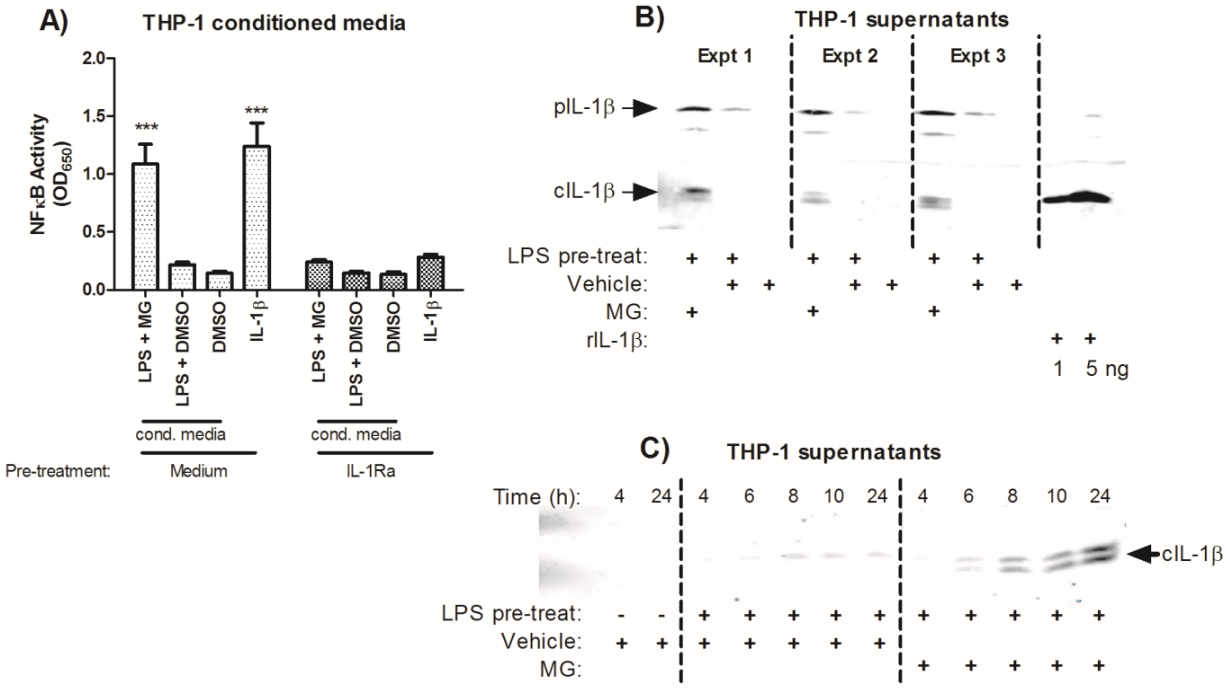


Figure 4.2: Proteasome inhibition induces processing of bioactive IL-1β

(A) Conditioned media from THP-1 cells stimulated with LPS (pre-treat 10 ng/ml) + MG-132 (5 μM), LPS only, or vehicle only, were used to stimulate HEK293 null 1 cells (n = 3). As a control, 2 ng/ml of recombinant IL-1β was used to stimulate these cells in parallel. Responses were measured in the presence of either media or IL-1Ra (200 ng/ml) pre-treatment. (B) Supernatants from THP-1 cells stimulated as previously indicated for 24 hours were blotted for IL-1β processing (n = 3). (C) THP-1 supernatants stimulated with LPS or LPS + MG were examined for the appearance of IL-1β over 24 hours (n = 2). Data were analyzed using two-way ANOVA and the Bonferroni post-test. *** indicates P< 0.001 compared with IL-1Ra-treated condition.

Il-1β secretion is dependent on the relative treatment of TLR vs proteasome inhibitor and correlates with NF-κB activation and pro-IL-1β synthesis

Proteasome inhibitors are generally thought to mediate anti-inflammatory activities (263) so it was unexpected when it also produced a pro-inflammatory response by mediating IL-1β maturation. To determine the role of the NF-κB-modulating effects of proteasome inhibitors, we tested MG-132 along with the other ER stressors tunicamycin and thapsigargin, by varying the timing of ER stressor treatment to occur either before or after LPS treatment. Interestingly, MG-132 treatment prior to LPS exposure inhibited IL-1β secretion (Fig 4.3A) whereas pre-treatment of LPS for 2 hours prior to MG-132 exposure resulted in an approximately 3-fold increase in secreted IL-1β over LPS alone (Fig 4.3B). This effect was also true for the other proteasome inhibitors bortezomib and carfilzomib, where pre-treatment with either inhibitor one or two hours prior to proteasome inhibitor exposure kept IL-1β production at baseline levels. Addition of proteasome inhibitor at the same time as LPS showed marginal increases in

IL-1β produced, whereas LPS treatment 1 hour prior to proteasome inhibitor exposure significantly augmented IL-1β production (Fig. 4.3C). We hypothesized that this difference was due to the limitation on pro-IL-1β production when proteasome inhibitors were applied prior to LPS treatment, which in turn was due to NF-κB inhibition. Pre-treatment of proteasome inhibitors prior to LPS exposure verified this, resulting in the inhibition of pro-IL-1β synthesis (Fig. 4.3D) and reduction of NF-κB activation by LPS (Fig. 4.3E). Therefore, the relative time of exposure of proteasome inhibitor to TLR agonist can dictate the level of NF-κB activation achieved and the amount of pro-IL-1β produced.

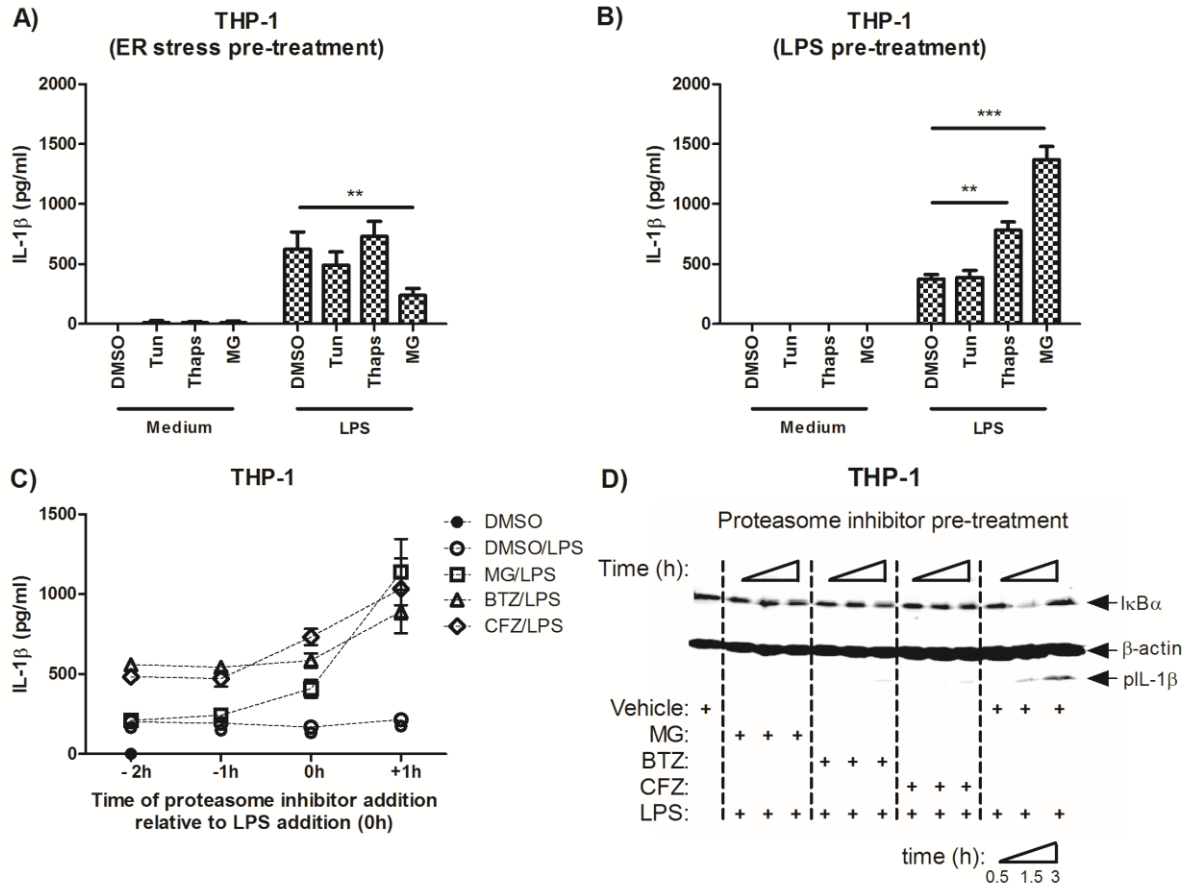

A)
THP-1
(ER stress pre-treatment)
IL-1β (pg/ml)
2000
1500
1000
500
0
**
DMSO
Tun
Thaps
MG
DMSO
Tun
Thaps
MG
Medium
LPS
B)
THP-1
(LPS pre-treatment)
IL-1β (pg/ml)
2000
1500
1000
500
0
***
**
DMSO
Tun
Thaps
MG
DMSO
Tun
Thaps
MG
Medium
LPS
C)
THP-1
IL-1β (pg/ml)
1500
1000
500
0
DMSO
DMSO/LPS
MG/LPS
BTZ/LPS
CFZ/LPS
- 2h
-1h
0h
+1h
Time of proteasome inhibitor addition
relative to LPS addition (0h)
D)
THP-1
Proteasome inhibitor pre-treatment
Time (h):
IκBα
β-actin
pIL-1β
Vehicle: +
MG: + + +
BTZ: + + +
CFZ: + + +
LPS: + + + + + + + + + + + +
time (h):
0.5 1.5 3
E)
THP-1 X-Blue
(proteasome inh pre-treatment)
NFκB Activity
(OD650)
1.2
0.9
0.6
0.3
0.0
***
DMSO
MG
BTZ
CFZ
Medium
LPS

Figure 4.3: IL-1β secretion is dependent on the relative treatment of TLR vs proteasome inhibitor and correlates with NF-κB activation and pro-IL-1β synthesis

THP-1 cells were exposed to the ER stressors tunicamycin (10 μg/ml), thapsigargin (10 μM), and MG-132 (5μM) either 2 hours (A) before or (B) after stimulation with LPS (10 ng/ml). (C) MG-132, bortezomib and carfilzomib (all 5 μM) were added to THP-1 cultures either before (2 hours, 1 hour), at the same time, or 1 hour after LPS stimulation (time = 0). Supernatants were harvested after 24 hours. (E) Proteasome inhibitors (5 μM) were applied 2 hours prior to LPS treatment (10 ng/ml). Cells were harvested at 0.5, 1.5, and 3 hours, and blotted for IκBα and pro-IL-1β. (F) THP-1 X Blue cells were treated as in (E), but supernatants were collected after 6 hours and assayed for NF-κB/AP-1 activity. n = 3 for all experiments. Data were analyzed using two-way ANOVA and the Bonferroni post-test. ** and *** indicate $P < 0.01$ and $P < 0.001$, respectively.

Priming occurs through various PRRs and correlates with cell death

Although up until this point we have strictly used LPS to stimulate TLR4, we found that priming can be successfully carried out with a number of TLR agonists, including flagellin, R848, and Pam3CSK4 (Fig. 4.4A) as long as cells showed responsiveness to that particular TLR ligand (Fig. 4.4B). In addition to IL-1β production, this responsiveness also correlated with cell death, where LPS, flagellin, and R848 all increased cell death over bortezomib treatment alone in a dose-dependent manner (Fig. 4.4C). However, THP-1 cells did not appear to be responsive to CpG stimulation, and subsequently did not result in IL-1β production (Fig. 4.4A) or increase in cell death (Fig. 4.4C). Although we had previously observed a strong dependency on TLR pre-treatment for production of mature IL-1β (Fig. 4.3), we found that regardless of whether LPS treatment occurred before or after bortezomib treatment, cell death was still increased over bortezomib alone (Fig 4.4D). We hypothesized that LPS stimulation prior to bortezomib treatment would protect against cell death due to pro-survival NF-κB functions but this was only observed at 10 nM of bortezomib ($P < 0.05$) and no differences were

observed at 100 nM of bortezomib. Finally, as we had shown in Figure 4.3 that pro-IL-1β production could be the limiting step at which IL-1β processing is inhibited, we transfected a pro-IL-1β expressing plasmid into THP-1 cells to see if IL-1β processing could still occur without TLR priming. Immunoblot of lysates and supernatants revealed that bortezomib stimulation alone could result in IL-1β maturation as long as pro-IL-1β was available (Fig. 4.4E). This therefore identifies the presence of pro-IL-1β as a key limiting factor in proteasome inhibitor-mediated IL-1β maturation.

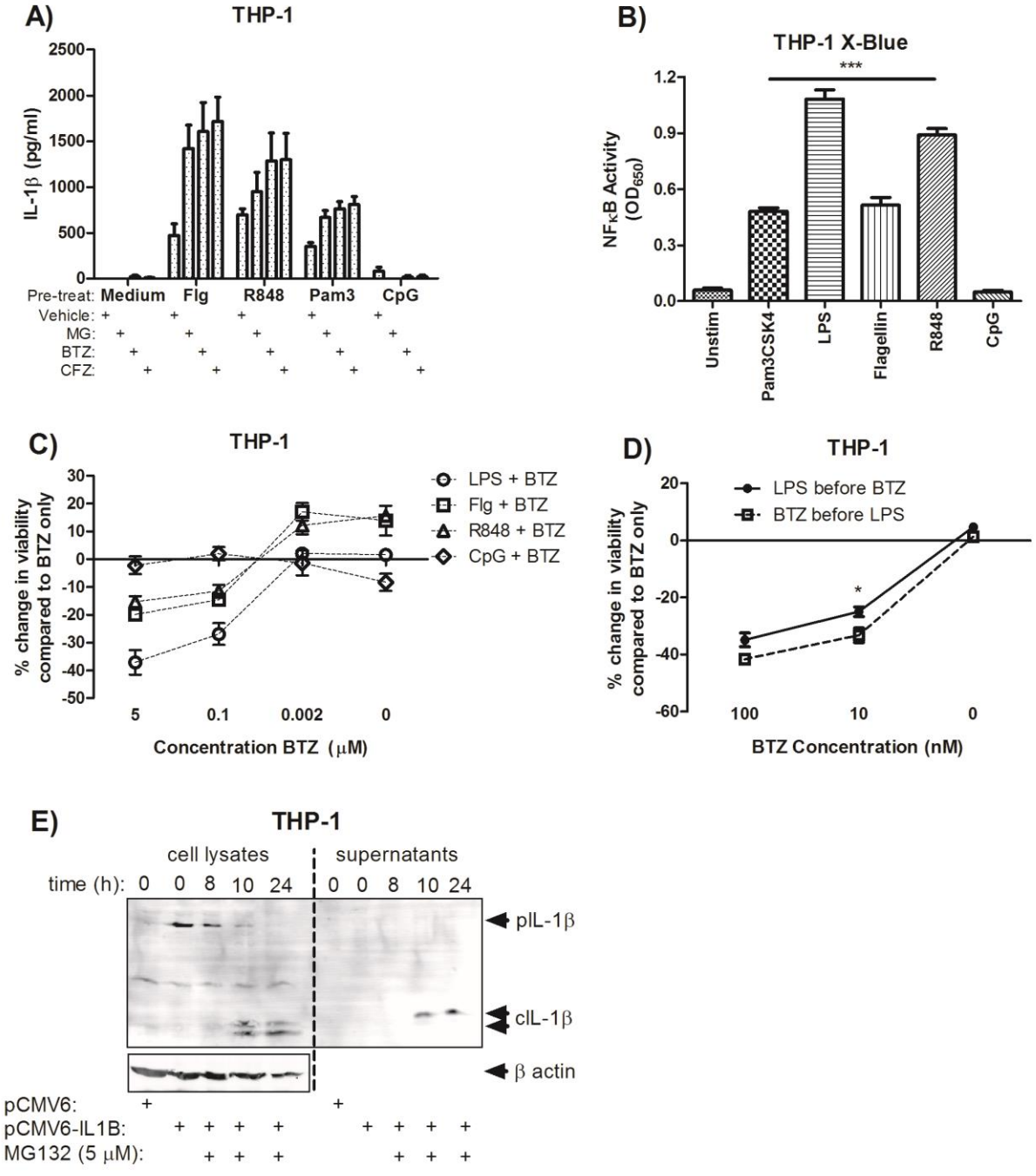

A)
THP-1
IL-1β (pg/ml)
Pre-treat: Medium Flg R848 Pam3 CpG
Vehicle:
MG:
BTZ:
CFZ:
B)
THP-1 X-Blue
***
NFκB Activity (OD650)
Unstim
Pam3CSK4
LPS
Flagellin
R848
CpG
C)
THP-1
LPS + BTZ
Flg + BTZ
R848 + BTZ
CpG + BTZ
% change in viability compared to BTZ only
Concentration BTZ (μM)
D)
THP-1
LPS before BTZ
BTZ before LPS
% change in viability compared to BTZ only
BTZ Concentration (nM)
E)
THP-1
cell lysates
supernatants
time (h): 0 0 8 10 24 0 0 8 10 24
pIL-1β
cIL-1β
β actin
pCMV6:
pCMV6-IL1B:
MG132 (5 μM):

Figure 4.4: Priming occurs through various PRRs and correlates with cell death

(A) THP-1 cells treated with 500 ng/ml flagellin, 100 ng/ml Pam3csk4, 2.5 ug/ml R848, or 5 μM CpG DNA two hours prior to proteasome inhibitor treatment for 24 hours (n = 3). (B) THP-1 X-Blue cells were examined for the ability of TLR ligands to activate NF-κB/AP-1 activity over 24 hours (n = 3 to 5). (C) LPS (10 ng/ml), flagellin, R848, and CpG-treated THP-1 cells were exposed to 5 μM, 100 nM, and 2 nM bortezomib and checked for viability after 24 hours (n = 3). (D) THP-1 cells were stimulated with LPS either before or after bortezomib (100 nM, 10 nM) exposure and examined for cell viability after 24 hours (n = 3). (E) THP-1 cells were transfected with pro-IL-1β-expressing plasmid (0.5 μg per $2x10^6$ cells) and treated with 5 μM MG-132 (n = 3). Lysates and supernatants were harvested at 8, 10, and 24 hours post-exposure and blotted for IL-1β processing (n = 3). Data were analyzed using one-way or two-way ANOVA with Kruskal-Wallis or Bonferroni post-tests. * and *** indicate $P< 0.05$ and $P< 0.001$, respectively.

IL-1β production can be dissociated from cell death by caspase inhibition

Because IL-1β processing typically results from inflammasome and caspase-mediated processing (particularly by caspase-1 and caspase-8), we examined the activation status and involvement of various caspases in our system. Similar to the cleavage of IL-1β in THP-1 supernatants (Fig. 4.2C), an increase in the appearance of active caspase-1 is also observed at 8 hours post-stimulation with MG-132 (Fig. 4.5A). Treatment of THP-1 cells with a variety of caspase inhibitors and controls including z-YVAD-fmk (Caspase-1, 4), z-IETD-fmk (Caspase-8), and VX-765 (Caspase-1, 4) but not AEBSF (serine proteases) decreased IL-1β secretion in a dose-dependent manner (Fig. 4.5B). Efficacy of these inhibitors in preventing activation of their target caspases was examined by immunoblot. Both z-YVAD-fmk and z-IETD-fmk inhibited activation of caspase-1 and caspase-8 to some degree, and ultimately inhibited processing of IL-

1β to its active form (Fig. 4.5C). Both of these inhibitors also reduced activation of the executioner caspase, caspase-3, which occurs downstream of caspase-8. Despite the profound effects of caspase inhibition on IL-1β maturation, caspase inhibition was incapable of rescuing these cells from death (Fig. 4.5D), implying the potential involvement of caspase-independent mechanisms in mediating cell death.

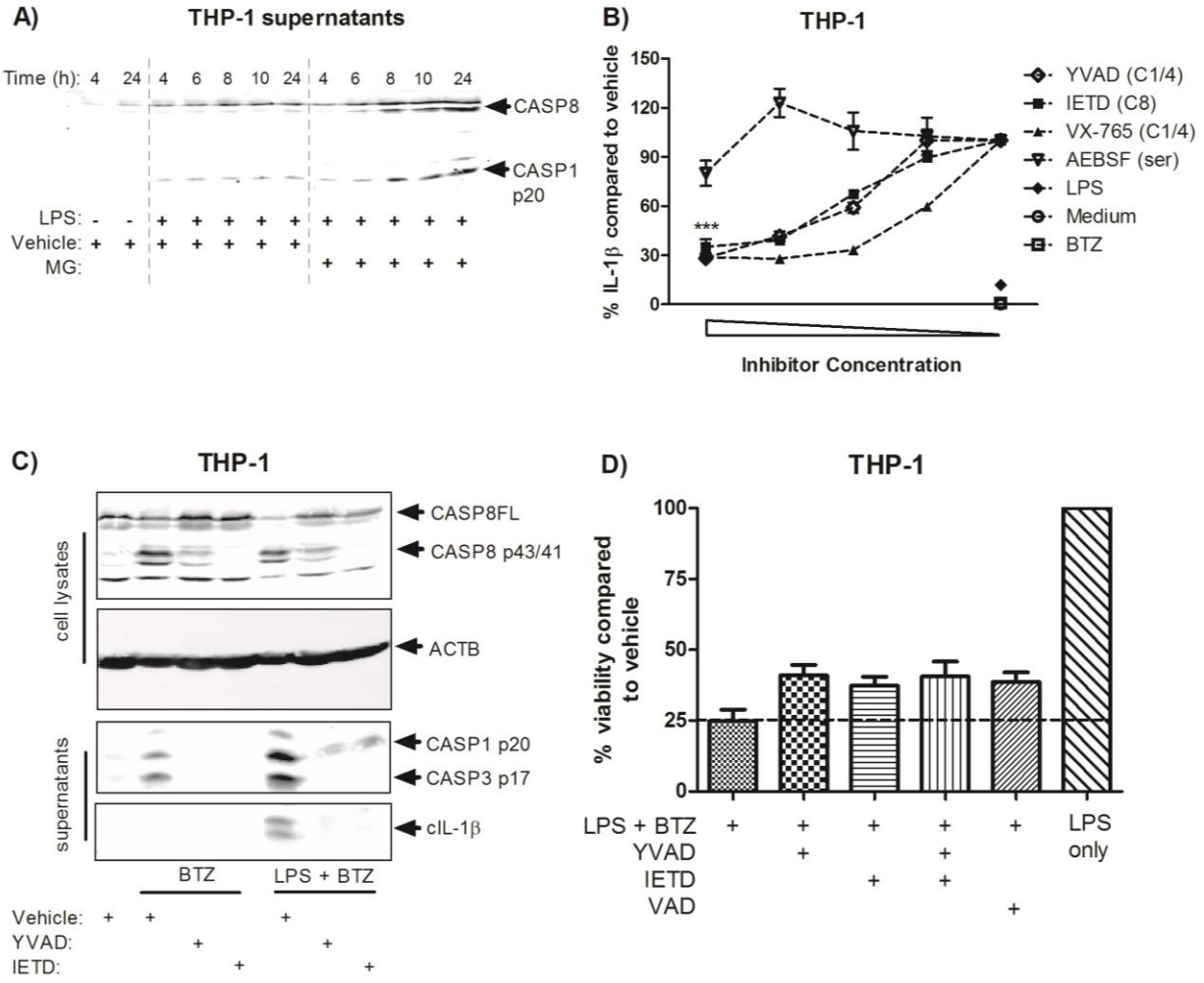


Figure 4.5: Dissociation of IL-1β production and cell death by caspase inhibition

(A) Supernatants from THP-1 cells stimulated with LPS (10 ng/ml) and MG-132 (5 μM) were blotted for the appearance of caspase-1 over 24 hours (n = 2). (B) THP-1 cells were titrated with various inhibitors in 10-fold dilutions, starting at the indicated concentrations: z-YVAD-fmk (20 μM), z-IETD-fmk (20 μM), VX-765 (20 μM), and AEBSF (300 μM) (n =3). (C) THP-1 cells treated with z-YVAD-fmk (20 μM) or z-IETD-fmk (20 μM) were blotted for the inhibition of caspase-8, caspase-1 and downstream caspase-3 and IL-1β processing in response to LPS (10 ng/ml) and bortezomib (100 nM) (n = 3). (D) Caspase inhibitors (all 30 μM) were examined for their ability to rescue cells from death after 20-24 hours of treatment (n = 3) and were stimulated as in (B) and (C). *** indicates $P < 0.001$ by testing with two-way ANOVA with the Bonferroni post-test.

Cell death mediated by LPS and bortezomib is not reversed by necroptosis inhibitors or ATF4 and XBP1 knockdown

Proteasome inhibitors have been identified to mediate cell death through numerous pathways including caspase-8 and caspase-9 mediated processing of caspase-3, as well as autophagic/ER stress dependent cell death mechanisms (390). Examination by simple light microscopy showed that by 24 hours, cells treated with LPS + bortezomib had very obviously undergone significant cell death compared to either LPS or bortezomib treatments alone. Cells treated with LPS and bortezomib had become smaller in size, fragmented, and very sparse when compared to other treatment conditions (Fig. 4.6A). Because caspase inhibition could not prevent cell death, we decided to look at necroptosis, a caspase-independent form of cell death, which may also be involved in inflammasome activation and IL-1β processing. While proteasome inhibition was capable of causing RIP1 and RIP3 cleavage (Fig. 4.6B), treatment with the RIP1 inhibitor Nec1s and the RIP3 inhibitor GSK'872 did not affect cell death as measured by MTS and LDH assays (Fig. 4.6C). Proteasome inhibitors are also well known to mediate autophagy and amino

acid homeostasis through ATF4 induction (Fig. 4.6D) and previous reports on bortezomib activity in multiple myeloma indicate resistance to cell death in XBP1s-negative myeloma cells. However, we did not find that siRNA against either ATF4 or XBP1 resulted in changes to cell viability when compared to control. Therefore, although the involvement of apoptotic caspases and ER stress pathways have been previously implicated in other models of proteasome inhibitor-mediated cell death (390-392), it appears that our system relies on a mechanism of cell death that cannot be suppressed by inhibition of these pathways individually.

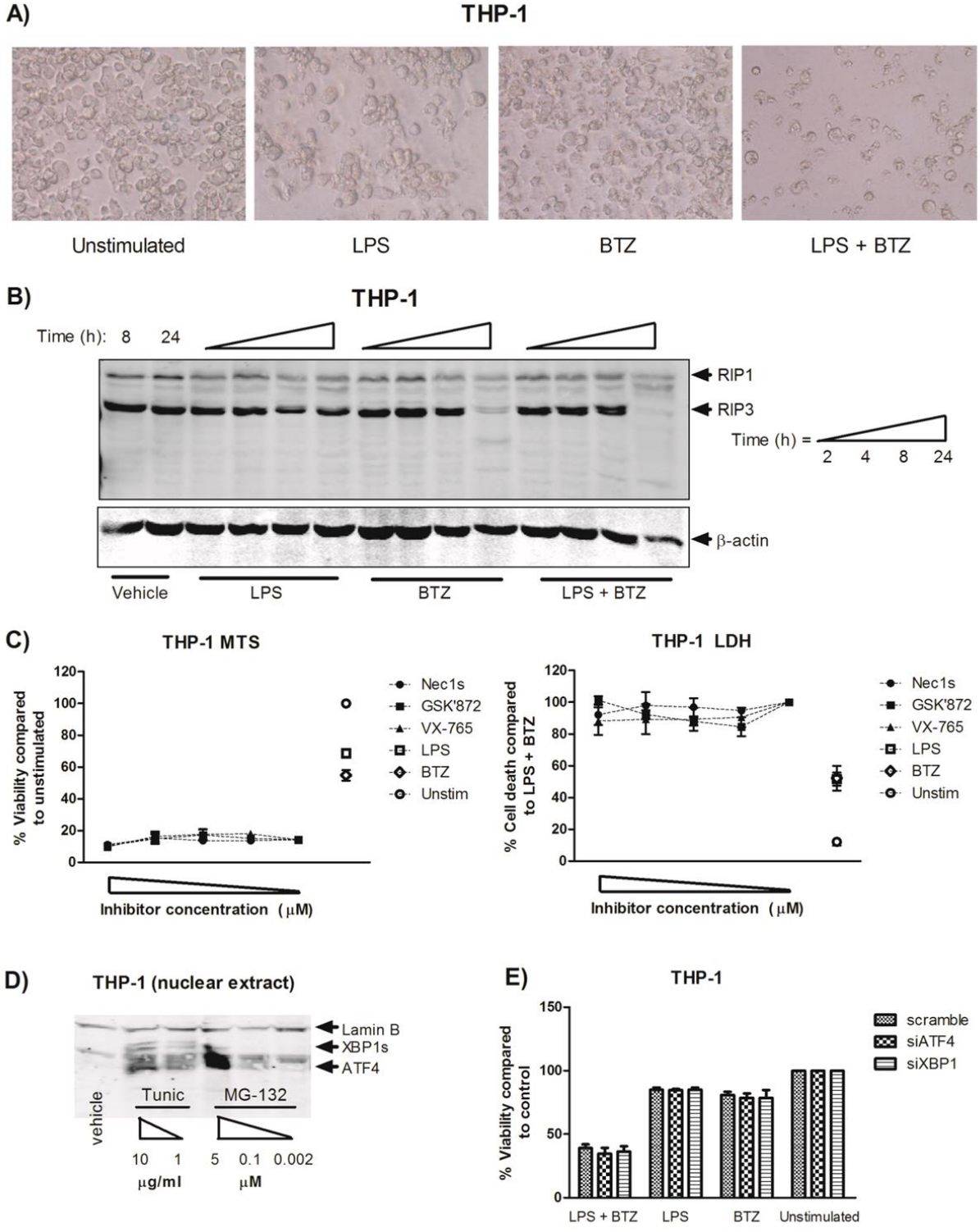

A)
THP-1
Unstimulated
LPS
BTZ
LPS + BTZ
B)
THP-1
Time (h): 8 24
RIP1
RIP3
Time (h) =
2 4 8 24
β-actin
Vehicle
LPS
BTZ
LPS + BTZ
C)
THP-1 MTS
% Viability compared to unstimulated
Inhibitor concentration (μM)
Nec1s
GSK'872
VX-765
LPS
BTZ
Unstim
THP-1 LDH
% Cell death compared to LPS + BTZ
Inhibitor concentration (μM)
D)
THP-1 (nuclear extract)
Lamin B
XBP1s
ATF4
vehicle
Tunic
MG-132
10 1 5 0.1 0.002
μg/ml
μM
E)
THP-1
% Viability compared to control
scramble
siATF4
siXBP1
LPS + BTZ
LPS
BTZ
Unstimulated

Figure 4.6: Cell death mediated by LPS and bortezomib is not reversed by necroptosis inhibitors or ATF4 and XBP1 knockdown

(A) Light microscopy images of THP-1 cells treated with LPS (1 ng/ml), bortezomib (100 nM), or both after 24 hours of stimulation. (B) THP-1 cells were treated with LPS (10 ng/ml), bortezomib (100 nM), or LPS + bortezomib. Lysates were harvested at the time points indicated and blotted for RIP1 and RIP3 (C) MTS and LDH viability assays on THP-1 cells treated with varying concentrations of the RIP1, RIP3 inhibitors Nec1s (5-fold dilutions starting at 50 μM) and GSK'872 (5-fold dilutions starting at 10 μM) as well as the Caspase-1/4 inhibitor VX-765 (5-fold dilutions starting at 40 μM). (D) Nuclear extracts of THP-1 cells stimulated with tunicamycin (10 or 1 μg/ml) or MG-132 (5, 0.1, and 0.002 μM) for 6 hours were blotted for XBP1s and ATF4 expression. (E) MTS viability assay of THP-1 cells knocked down for XBP1 and ATF4 prior to stimulation with LPS (10 ng/ml) and bortezomib (100 nM) (n = 3 for all experiments).

4.5 Discussion:

In the previous chapter we showed that ER stress could mediate anti-inflammatory activity through suppression of STAT3 and inhibition of the chemokines CXCL1 and IL-8. However, in this study we show that ER stressors, and proteasome inhibitors in particular, can also mediate pro-inflammatory effects by activating IL-1β processing and cell death pathways. Because proteasome inhibitors are generally known to inhibit NF-κB activity, their effects on IL-1β maturation were dependent on when proteasome inhibition occurred relative to TLR stimulation. We reasoned that the success of proteasome inhibitors in multiple myeloma treatment could be further enhanced and applied to other haematological malignancies if we could somehow mimic an increase in intracellular protein load (391). To do this, we chose to apply TLR agonists in conjunction with proteasome inhibition because of their ability to stimulate immune responses, with the ultimate goal of eliciting an immunogenic anti-tumour response (393). Our main findings are that i) TLR priming prior to proteasome inhibition led to

IL-1β secretion and maturation in complementary monocyte/macrophage models whereas proteasome inhibition prior to TLR stimulation, strongly inhibited it. ii) Maturation of IL-1β by proteasome inhibition was primarily dependent on pro-IL-1β synthesis through prior TLR stimulation, and vector-driven expression of pro-IL-1β (in the absence of additional TLR stimulation) could be brought to maturation with proteasome inhibition alone. iii) Augmentation in proteasome inhibitor-mediated cell death occurred regardless of whether TLR stimulation occurred before or after proteasome inhibition, and iv) Cell death was found to proceed in both caspase-dependent and independent manners but IL-1β maturation was strictly dependent on caspase-1 and -8 inhibition. Whether or not these observations can be applied clinically will depend on the immunogenic effects of IL-1β and TLR stimulation in cancer.

The role of TLR stimulation in cancer progression is somewhat controversial. TLR stimulation can result in NF-κB-mediated survival and the expression of pro-inflammatory mediators (including IL-1β and TNFα) which may aid in tumour cell adherence, angiogenesis, and metastasis (82, 252, 394). However, there is also evidence that stimulation through particular TLRs, such as TLR3 and TLR5, can induce anti-tumour responses and lead to increased tumour cell death (82). Furthermore, two TLR agonist therapies, imiquimod and BCG, have currently met some clinical success as treatments for certain cancers (superficial basal cell carcinoma and bladder cancers, respectively). In our model, TLR stimulation may function through a mechanism analogous to that controlling multiple myeloma sensitivity to proteasome inhibition (391). This immunosuppression is thought to occur due to an increased protein load in cells already undergoing ER stress, as in many cancer cells. TLR stimulation may further push ER load past its capacity, and beyond the ability of the cell to successfully cope with the increased protein load, and driving cell death (391). Consequently, the use of specific TLR agonists could

also open the possibility for stimulation of specific cell populations based on their TLR or other PRR repertoires, thus allowing for more targeted effects on cell populations.

A potential concern in the use of proteasome inhibitors as a cancer therapeutic is their immunosuppressive effects. Bortezomib treatment has been shown to affect activation, migration, and immunostimulatory capacity in dendritic cells (383, 395) and may cause overall lymphopenia through reduction in T cell and B cell numbers (396-398). In fact, this property has made bortezomib an appealing therapeutic in preventing graft vs host disease (263). Because proteasomes mediate processing of protein antigens (ie. tumour antigens), their inhibition has been shown to affect MHC Class I surface expression (399) and may have the potential to decrease anti-tumour immunity mediated by cytotoxic T lymphocytes and limiting Th1 cytokines (400). This may manifest in an increased susceptibility to infections, particularly *Varicella zoster* reactivation in patients receiving bortezomib treatment (398, 401, 402). Despite this, bortezomib has been shown to also boost anti-tumour effects through the augmentation of tumour antigen expression through cell surface heat shock proteins 60 and 90 for dendritic cell uptake (403). Furthermore, bortezomib can sensitize tumour cells to exogenous cell death signals from activated dendritic cells and NK cells through the upregulation of tumour cell surface Fas and death receptor 5 (404) and the upregulation of activating NK cell NKG2D ligands (405). These factors may help to explain why in studies of graft rejection, bortezomib seems to be helpful in preventing graft vs host disease, while simultaneously maintaining a graft vs tumour effect (396, 406).

Because our data suggested that cell death occurred in both caspase-dependent and independent manners and we observed caspase-8 and IL-1β processing in our model, we decided to examine the involvement of necroptosis. Necroptosis is a form of caspase-independent cell death that is

mediated by RIP1 and RIP3 kinases (407). Similar to our observations, necroptosis is known to be tightly associated with caspase-8 as well as inflammasome activation and IL-1β processing (408, 409), making it a good candidate mechanism for our observations. Although we were able to identify a change in the amount of RIP1 and RIP3 proteins with bortezomib treatment (by 24 hours), potentially signifying involvement of this pathway, treatment with either RIP1 (Nec-1s) or RIP3 (GSK'872) inhibitors were ineffective at preventing cell death. Therefore, it is unlikely that necroptosis, at least through RIP1 and RIP3 activity, mediates cell death in our system. It is also possible that these proteins may possess scaffolding functions that are independent of their enzymatic activity, and may therefore have been unaffected by chemical inhibitors of their protease activity. New evidence also shows that caspase-2 may be important in mediating ER stress-mediating mitochondrial damage, leading to IL-1β production and cell death (410). Other groups have also reported amino acid starvation as a mechanism through which cell death occurs (279). However, knockdown of ATF4, which responds to low amino acids conditions, did not affect cell death in our system. Others have also described tumour cells that are XBP1s-negative as becoming bortezomib resistant (280). While we did describe LPS-mediated increases of nuclear XBP1s in response to the ER stressors tunicamycin and thapsigargin (Supplementary Figure 3.3), we did not find that proteasome inhibition induced observable amounts of XBP1s by immunoblot (Supplementary Figure 3.2) nor did siRNA against XBP1 improve cell viability (Figure 4.6D, E). This supports previous observations where proteasome inhibition is shown to suppress XBP1 splicing (411). Therefore, XBP1s does not seem to play an important role in mediating cell death/survival in our system. Whether or not IL-1β itself is beneficial or detrimental for tumour growth is somewhat debated. The effects of IL-1β on tumour growth can be determined by applying our model to an *in vivo* mouse model. An

additional IL-1β neutralization strategy (*Il1b*$^{-/-}$ mice, IL1Ra or anti-IL-1β administration) can be employed to neutralize IL-1β signalling and tumour growth/dissemination can be subsequently monitored by imaging for luciferase-expressing tumour cells.

Although IL-1β is frequently observed and associated with tumour progression (253), some studies show that it is important for anti-tumour immunity and its production by dendritic cells is important for generation of IFNγ and subsequent priming of cytotoxic T cells (393). Other studies have shown that IL-1R signaling is important for the function of the chemotherapeutic drugs doxorubicin and oxaliplatin in inducing IL-17A production by γδ T cells (412). On the other hand, IL-1β may also aid tumour spread by inducing angiogenesis and may also give rise to myeloid-derived suppressor cells that can inhibit anti-tumour NK and cytotoxic T cell functions. One of the benefits of our model system was that it allowed us to control IL-1β production simply by adjusting when bortezomib was administered relative to the TLR agonist, without compromising the increased tumour cell cytotoxicity. Interestingly, the importance of timing in bortezomib administration was recently observed in a model of graft rejection in mice. In this study, delayed bortezomib administration relative to engraftment resulted in increased IL-1β levels. Similar to our study, they found that varying the time of administration relative to TLR4 stimulation could inhibit IL-1β production and administration of the IL-1Ra Anakinra, decreased morbidity in mice (384).

Taking into account the potential cytotoxicity and immunomodulatory effects of proteasome inhibitors, addition of TLR adjuvants may be a useful way through which tumour cells can be activated to undergo increased cell death, using a lower dose or proteasome inhibitor. The effects of TLR stimulation in combination with bortezomib treatment should be further examined to determine whether combination therapy can help relieve some of the

immunosuppressive effects of bortezomib on immune cell activation, as well as their combined effects on the adaptive anti-tumour response.

CHAPTER 5: GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

Throughout these studies, our primary objective was to define the factors driving chronic inflammatory diseases and to subsequently identify therapeutic targets that may help mitigate them. We carried this out in a way that emphasized relevance to human disease and reproducibility by using multiple complementary model systems, including cell lines to interrogate mechanism but also primary patient samples and genetic data to validate our findings. Although we began with a focus solely on airway inflammation in CF, through experimentation, we made observations in basic inflammatory mechanisms that we soon realized also had good potential in improving treatment strategies of haematological malignancies.

5.1 Main contributions to the field of CF and inflammation research:

Because IL-1β is a major inducer of inflammatory responses and has been frequently observed in high amounts in the airways of CF patients (134, 185, 190, 191), we initially decided to evaluate its origins as well its potential as a therapeutic target. The body of research presented in this thesis, summarized in Figure 5.1, has shown that

1) Although airway epithelial cells are commonly considered to be an important source of inflammation in the CF airways, they do not appear to produce significant quantities of IL-1β and are therefore not likely to be an important source of IL-1β in the lungs (413). They are, however, highly responsive to IL-1β and produce substantial amounts of the neutrophil chemokines CXCL1 and IL-8, and may therefore play an important role in the propagation of the neutrophilic inflammation that is characteristic of CF lung disease.

2) Despite evidence in the literature showing that NF-κB activity is intrinsically dysregulated in CF cells due to loss of CFTR function (193, 311), we found no difference in the capacities of CF and non-CF PBMCs to activate caspase-1 and produce IL-1β. This was also replicated in THP-1 cells and non-CF PBMCs that had been treated with a CFTR inhibitor, indicating that CF cells do not have an increased propensity to produce bioactive IL-1β.

3) We found that environmental factors such as chronic infection and ER stress were more likely to contribute to the increased IL-1β observed in the airways of CF patients. Indeed, increased priming with bacterial products or the presence of ER stressors, significantly augmented IL-1β secretion. These responses could be inhibited by treatment with an NF-κB inhibitor, demonstrating the critical nature of the PRR priming step in determining the quantity of mature IL-1β produced.

4) Mutations in bacterial components, such as the type III secretion system can also affect IL-1β production. This may be important adaptation in chronic strains of *P. aeruginosa* which have been shown to acquire mutations in virulence factors as a method of immune evasion.

5) Although monocytic cells undergoing ER stress displayed increased IL-1β production, ER stress had the surprising effect of inhibiting IL-1β-induced CXCL1 and IL-8 production in CF airway epithelial cells. This inhibition was found to be partially dependent on suppression of STAT3 activity by ER stress and supernatants from IL-1β-stimulated cells undergoing ER stress had reduced chemoattractive properties. Therefore these pathways represent various levels at which therapeutic intervention may be successful at reducing

neutrophilic inflammation in CF: treatment of chronic infection with antibiotics, treatment of IL-1β with IL-1R antagonism, and the manipulation of ER stress and STAT3 to reduce production of neutrophil chemokines.

6) Genetic variation in *CXCL1* and *CXCL8* were found to significantly associate with lung disease severity in a meta-analysis of GWAS data from 5,827 CF patients from North America and France, confirming the potential benefit of therapeutically targeting CXCL1 and IL-8 in improving CF health outcomes.

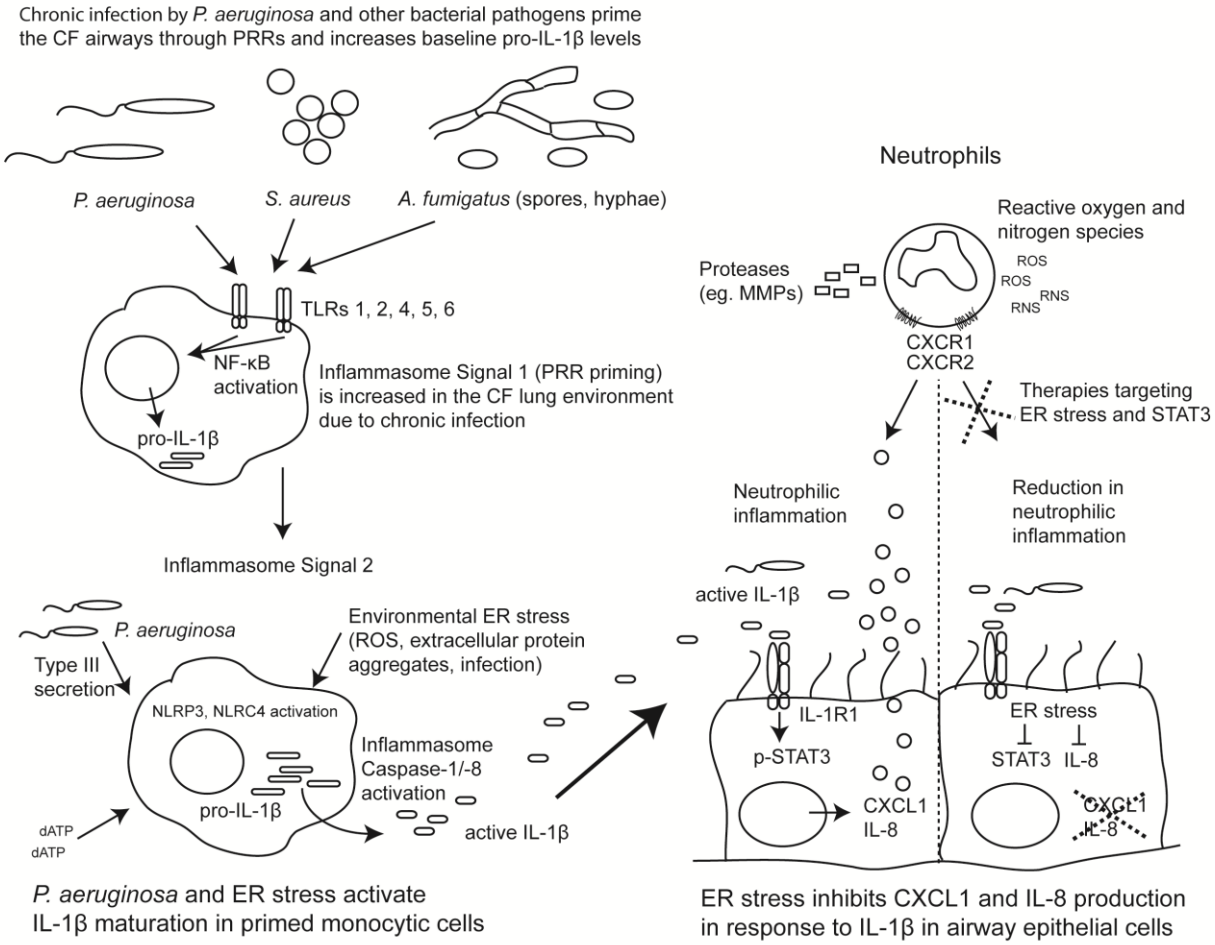
7) One of the chemical ER stressors we employed (the proteasome inhibitor, MG-132) in our CF models could both strongly activate or inhibit IL-1β production depending on when it was applied relative to TLR activation. We could replicate these observations in proteasome inhibitors currently used in the clinic (Bortezomib and Carfilzomib) and hypothesized that this differential response could have useful effects in cancer therapy.

8) Proteasome inhibitors have dual pro- and anti-inflammatory effects depending on when they are applied relative to an inflammatory stimulus. When applied after an inflammatory stimulus (ie. TLR ligand), they can function in a pro-inflammatory fashion by activating caspase-1 and -8 mediated production of mature IL-1β. When applied before an inflammatory stimulus, they can function in an anti-inflammatory by suppressing NF-κB activation and inhibiting production of pro-IL-1β production, thereby limiting maturation of IL-1β.

9) Co-stimulation of TLRs alongside proteasome inhibition also significantly augmented death of the THP-1 tumour cell line over proteasome inhibition alone. This increase in cell death occurred regardless of whether TLR stimulation occurred before or after

proteasome inhibition and was independent of IL-1β maturation and caspase activity. Therefore, addition of TLR adjuvants may serve as a useful method of augmenting tumour cell death, while simultaneously manipulating inflammation/tumour immunogenicity through IL-1β production.

Extracellular factors control degree of inflammasome activation and IL-1β secretion



Timing of proteasome inhibition relative to TLR stimulation alters IL-1β secretion but maintains increased effects on cell death

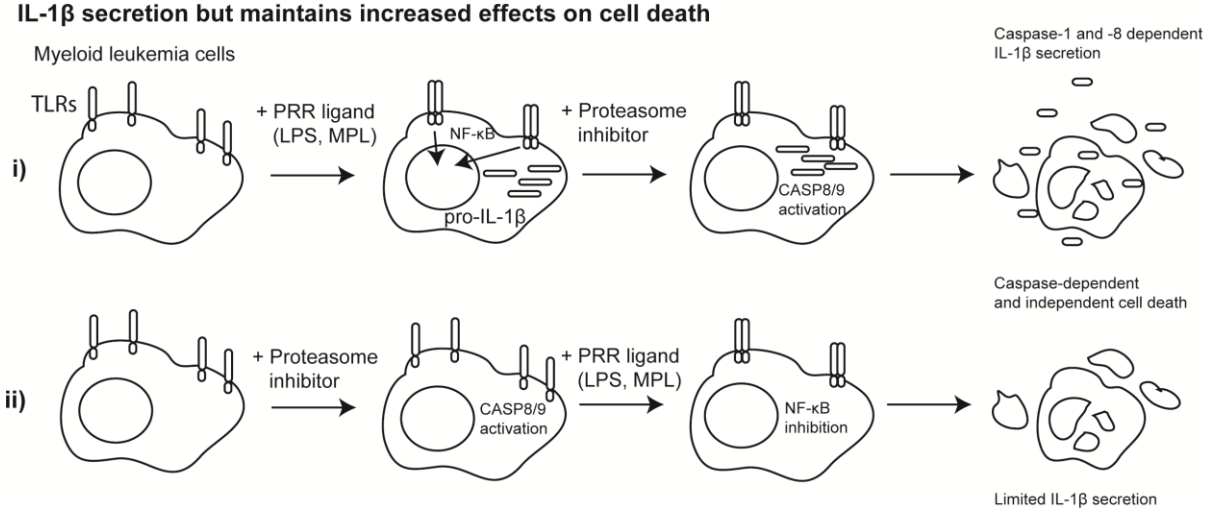


Figure 5.1: The roles of ER stress and inflammasomes in inflammatory disease

Chronic infection of the CF airways by opportunistic pathogens leads to increased priming of monocytic cells through the engagement TLRs (and other PRRs) leading to NF-κB activation and pro-IL-1β production (inflammasome signal 1). Increased priming results in more mature IL-1β being secreted when inflammasome activation by pathogens (*P. aeruginosa*) or ER stress occurs (inflammasome signal 2). Mature IL-1β can proceed to stimulate airway epithelial cells to produce the neutrophil chemokines CXCL1 and IL-8, recruiting and activating neutrophils which can contribute to airway damage through secretion of proteases and reactive oxygen/nitrogen species. ER stress inhibits secretion of both CXCL1 and IL-8 in airway epithelial cells, with CXCL1 suppression occurring through inhibition of STAT3 signaling.

In contrast to its anti-inflammatory effects through inhibition of CXCL1 and IL-8 secretion in airway epithelia, ER stress (via proteasome inhibition) is also capable of pro-inflammatory effects by mediating IL-1β maturation in monocytic cells. This may be useful in certain haematological malignancies due to the induction of immunogenic cell death. Production of IL-1β is dependent on the treatment order of TLR stimulus and proteasome inhibitor, where proteasome inhibitor treatment prior to TLR stimulation inhibits NF-κB activation and pro-IL-1β synthesis but maintains effects on cell death.

5.2 Future directions:

While my findings advance knowledge in a number of areas outlined above, the research has raised a number of unanswered questions:

Can key findings be validated in complementary model systems?

We have identified several potential therapeutic targets in CF and haematological malignancies and the logical next step would be to validate these findings in complementary systems. One option would be to use *in vivo* models. Given that mouse models of CF do not produce a similar lung disease phenotype to human disease, these studies are better carried out in pig and ferret models (414), which require more specialized skills, facilities, and time allowances than locally

available. Another more feasible alternative would be to replicate our results with *ex vivo* samples from CF patients (using sputum and polarized primary airway epithelia in conjunction with clinical phenotype data). Although *ex vivo* human models may only represent certain "snap shots" of disease and must be controlled for certain confounding factors (eg. age, gender, differences in infection profile, and differences in treatment regimens between individuals), they are the best and most relevant representation of disease. Furthermore, advances in culture methodology including the differentiation of primary airway cells into ciliated epithelia, development of pulmonary organoid models, and the use of induced pluripotent stem cell models have greatly increased our options to more accurately and easily model human CF disease (415).This would most likely need to be carried out in collaboration with a CF center where a large number of these samples may be more readily obtained and the results more easily linked back to their clinical characteristics. Furthermore, because we only demonstrated partial effectiveness of STAT3 inhibition in reducing CXCL1 (and to a different extent IL-8) production when compared to ER stressors, our findings could be further refined to identify either additional targets affected by ER stress or to identify a target that may better limit production of both CXCL1 and IL-8. In this case, a high throughput siRNA screen of UPR components using CXCL1 and IL-8 production as a primary readout would be appropriate for determining a more effective molecular target.

Will co-treatment of proteasome inhibitors and TLR ligands be effective in an in vivo cancer model?

Although we have obtained very promising results *in vitro*, translation to an *in vivo* model could potentially prove challenging due to differences in *in vivo* drug/adjuvant kinetics and

bioavailability as well as variable effects of the drug on various cell types. Fortunately, many studies have previously examined bortezomib administration *in vivo* and administration of THP-1 cells into NOD Scid gamma (NSG) mice is a fairly common tumour model. In terms of our proteasome inhibitor study, we have already begun development of a mouse model of acute myeloid leukemia (using THP-1 cells) in which we will progressively track tumour burden using luciferase-expressing tumour cells. So far, THP-1 cells have been transduced with lentivirus expressing a dual luciferase and GFP reporter and are being selected for highly fluorescent populations. This model is being performed in NSG mice, which lack functional T cells, B cells, and NK cells (416), resulting in a lack of a functional adaptive immune system, and will therefore be observed over a relatively short time period (< 2 weeks). Effectiveness of the treatment regimen will be evaluated by multiple readouts, including weight, mortality, and most importantly, by serial measurements of *in vivo* fluorescence of tumour cells. This will allow us to progressively track treatment effects on tumour burden and localization over time in a non-invasive manner (417). As a first step, our observations will be based on the cytotoxic and inflammatory effects of proteasome inhibition in conjunction with TLR ligands, while ignoring the possible immunogenic effects of IL-1β on the adaptive immune response. Development of T, B, and NK cell responses will eventually also be examined but using a different mouse model that would require the development of an isogenic tumour cell line for fluorescent tracking. Effects on resistance upon re-challenge of tumour cells as well as the differentiation of T helper cells into specific subsets (Th1, Th2, Th17) as well as its effects on cytotoxic T cell function would serve as especially interesting experimental outcomes.

What is the mechanism of cell death induced by co-treatment with proteasome inhibitors and TLR agonists?

Additionally, although we will be proceeding with the *in vivo* model, we are also concurrently attempting to define the precise mechanism through which cell death occurs. We have already explored several primary cell death pathways including caspase 3-mediated apoptosis as well as necroptosis. Other possible pathways including autophagic cell death, pyroptosis, and ER-stress mediated mechanisms of cell death will be subsequently examined. So far, it does not appear that cell death is wholly-dependent on caspase or RIP kinase activity, as assayed with chemical inhibitors. Although chemical inhibitors serve as an excellent screening tool, because of their non-specific effects (418) we will ultimately need to confirm our results using specific molecular inhibition/over-expression of our molecules of interest. Interacting proteins and potential pathways will be identified using co-IP and mass spectrometry. After identifying a specific molecular pathway/target, we will use multiple strategies to validate this mechanism including generation of a stable knockdown in our THP-1 model and show resistance to cell death *in vivo,* as well as using specific knock-out mouse cell lines.

Overall, we have identified several key stages in the propagation of inflammatory signalling between monocytes and airway epithelial cells that can be targeted to disrupt neutrophilic inflammation in CF: NF-κB priming of IL-1β production by inflammasomes, modulation of IL-1β signalling by ER stress pathways and STAT3, as well as suppression of CXCL1 and IL-8 production. We predict that disruption of signalling or any of these molecules will produce some alleviation of neutrophilic inflammation in CF. Additionally, our findings on the differential effects of proteasome inhibition on IL-1β maturation in the presence of TLR

signalling could develop into a useful approach through which tumour cell immunogenicity can be augmented through MPL stimulation or IL-1β release while simultaneously increasing tumour cell cytotoxicity.

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