Interleukin 33, an inflammatory mediator in cystic fibrosis lung disease

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Abstract

Cystic Fibrosis (CF), is a systemic disease where chronic infection with *Pseudomonas aeruginosa* leads to a sustained increase in pro-inflammatory mediators. These mediators attract neutrophils to the lung with the objective of clearing the infection. However, this response is excessive and results in irreversible tissue damage. This tissue damage is further potentiated through the release of alarmins, also known as damage-associated molecular patterns (DAMPs). During physiological conditions, these mediators are inside the cell, where they play roles as regulators of different processes. However, following necrotic cell death, these mediators are released to the extracellular space where they act upon pattern recognition receptors (PRRs) on immune cells. IL-33, a member of the IL-1 family of cytokines, is a recently discovered DAMP. Intracellularly, IL-33 binds heterochromatin and acts as a transcriptional regulator. Extracellularly, IL-33 is a very potent neutrophil chemoattractant and a key amplifier of innate immunity. My central hypothesis is that decreasing IL-33 levels will reduce inflammation in CF.

My first aim is to study the expression profile of IL-33 in CF and non CF airway epithelial cells in the context both, chronic and acute infections with *P. aeruginosa*. Secondly, I will study the signaling pathways regulating IL-33 expression in CF airway epithelial cells following infection. Finally, I will characterize the role of IL-33 as a mediator of inflammation in our model.

My data show an increase of IL-33 mRNA in CF cells in an *in vitro* model of chronic infection as well as during an acute infection with *P. aeruginosa.* Signaling

through toll-like receptors (TLRs) regulates IL-33 expression since neutralization of both TLR2 and TLR5 prevented IL-33 mRNA upregulation in response to infection. Furthermore, experiments using inhibitors of specific kinases downstream TLRs show that TAK1, IKKβ, Tpl2, MEK1/2, ERK1/2 and p38 MAPK modulate IL-33 expression in response to *P. aeruginosa*. The increase in IL-33 mRNA is followed by an increase in intracellular protein, as assessed by immunoblotting. Interestingly, *P. aeruginosa* increases IL-33 in the cytoplasm of both, CF and non CF airway epithelial cells. However, IL-33 is not released in our model of acute infection. Finally, to assess the role of intracellular IL-33, airway epithelial cells stably expressing an NFκB luciferase reporter were transfected with wild-type, full-length IL-33 (FL IL-33) and with IL-33 R48, a construct with a mutation in the chromatin-binding motif (CBM). FL IL-33 is expressed in the nucleus and R48 IL-33 localizes in the cytoplasm of cells. Transfection with both, FL IL-33 and R48 IL-33 attenuated NFκB transactivation in response to TLR5 activation by flagellin. In line with the latter result, transfection of both IL-33 constructs significantly decreased IL-8 mRNA.

In conclusion, *P. aeruginosa* increases IL-33 expression in CF airway epithelial cells. The TAK1-IKKβ-Tpl2-MEK1/2 and TAK1-MKK3/6- p38 MAPK signaling pathways modulate IL-33 expression in response to bacterial infection. This is followed by an increase in intracytoplasmic IL-33. Intracellular IL-33 dampens NFκB transactivation in response to TLR5 signaling, resulting in a decreased expression of pro-inflammatory cytokine genes. Future experiments will aim to identify specific intracellular IL-33 binding partners and potential postranslational modifications occuring in airway epithelium.

Abrégé

La fibrose kystique (FK) est une maladie systémique ou les infections pulmonaires chroniques par *Pseudomonas aeruginosa* mènent à une réponse inflammatoire soutenue. Les médiateurs inflammatoires attirent des neutrophiles dans le poumon avec le but d'éliminer l'infection. Cependant, cette réponse est excessive et a comme conséquence des dommages tissulaires progressifs et irréversibles. Les dommages tissulaires peuvent potentialiser cette réponse inflammatoire à travers la libération d'alarmines, aussi connues comme DAMPs (Damage Associated Molecular Patterns). Durant des conditions physiologiques, ces médiateurs se retrouvent à l'intérieur des cellules ou ils jouent des rôles comme régulateurs de différents processus cellulaires. Cependant, suite à une mort cellulaire par nécrose, ces médiateurs sont libérés dans le milieu extracellulaire favorisant l'activation et le recrutement de cellules immunitaires. IL-33, une membre de la famille de cytokines IL-1, a récemment été découverte et classifiée comme alarmine. À l'intérieur de la cellule, IL-33 lie l'hétérochromatine et agit comme régulateur transcriptionnel. Dans le milieu extracellulaire, IL-33 est un puissant chemoattractant de neutrophiles et un amplificateur de la réponse immunitaire. Mon hypothèse est qu'une diminution des niveaux d'IL-33 reduira l'inflammation dans la FK.

Mon premier objectif est de caractériser l'expression d'IL-33 chez les cellules épithélilales FK et non FK lors d'une infection chronique, ainsi que durant une infection aigüe par *P. aeruginosa.* Ensuite, j'étudierai les voies de signalisation qui régulent l'expression d'IL-33 en réponse à cette infection. Finalement, je déterminerai le rôle d'IL-33 comme médiateur de l'inflammation dans notre modèle.

Mes résultats montrent une augmentation de l'ARNm d'IL-33 chez les cellules FK dans un modèle *in vitro* d'infection chronique, ainsi que durant une infection aigüe par *P. aeruginosa*. La signalisation à travers les récepteurs TLR (Toll-like receptors) régule l'expression d'IL-33 car l'inhibition de TLR2 et TLR5 empêche l'augmentation de l'ARNm d'IL-33 durant une infection. De plus, à l'aide de différents inhibiteurs de kinases en aval TLR, j'ai démontré que les kinases TAK1, IKKβ, Tpl2, MEK1/2, ERK1/2 et p38 modulent l'expression d'IL-33 dans le contexte d'une infection aigüe par *P. aeruginosa*. Cette régulation à la hausse d'IL-33 est suivie par une augmentation d'IL-33 dans le cytoplasme des cellules. Pourtant, IL-33 n'est pas relarguée dans notre modèle d'infection aigüe. Finalement, pour étudier le rôle d'IL-33 intracellulaire, des cellules épithéliales pulmonaires exprimant un rapporteur luciférase de NFκB ont été transfectées avec IL-33 sauvage (FL IL-33) ou avec IL-33 R48, un mutant ne liant pas la chromatine. FL IL-33 est exprimée dans le noyau tandis qu'IL-33 R48 est localisée dans le cytoplasme. La surexpression de FL IL-33 ainsi que d'IL-33 R48 diminue l'activité de NFκB en réponse à une activation de TLR5 par flagelline. En accord avec ce dernier résultat, la surexpression des deux constructions d'IL-33 diminue l'ARNm d'IL-8.

En conclusion, *P. aeruginosa* augmente l'expression d'IL-33 dans les cellules épithéliales pulmonaires. Les voies de signalisation TAK1-IKKβ-Tpl2-MEK1/2 et TAK1-MKK3/6-p38 régulent l'expression d'IL-33 lors d'une infection par bactérienne. Cette augmentation est suivie d'une augmentation d'IL-33 dans le cytoplasme. À l'intérieur de la cellule, IL-33 diminue la transactivation de gènes pro-inflammatoires par NFκB suite à une activation de TLR5. Des futures expériences viseront à identifier les

partenaires intracellulaires d'IL-33 ainsi que ses modifications post-traductionelles dans l'épithélium pulmonaire.

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Content

Statement of Contributions

Dr. Trevor Beaudoin and Julie Bérubé prepared *P. aeruginosa* diffusible material (PsaDM). Dr. Beaudoin also prepared *P. aeruginosa* biofilms.

Full length IL-33 and the chromatin binding mutant IL-33 R48 were cloned into pCDNA 3.1 by Dr. Lucie Roussel. These clones were re-amplified, cloned into pCMV4a and transfected into Beas-2B airway epithelial cells by Raquel Farias.

Beas-2B cells stably expressing a reporter for NFkB consensus binding sequences were made by Julie Bérubé and Dr. Guy Martel.

All experiments shown in this thesis were performed by Raquel Farias.

Dr. Simon Rousseau reviewed and edited the text in this manuscript.

Abbreviations

Aa	Amino acid
AP-1	Activator protein-1
Asn	Asparagine
Asp	Aspartic acid
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BALF	Bronchoalveolar lavage fluid
Bcf	*Pseudomonas* biofilm
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cAMP	Cyclic adenosisne monophosphate
CCR	Cys-Cys receptor
cDNA	Complementary Deoxyribonucleic Acid
CF	Cystic Fibrosis
CFLD	Cystic fibrosis lung disease
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
cm^2	Square centimeter
COPD	Chronic Obstructive Pulmonary Disease
CpG DNA	Cytosine triphosphate guanine triphosphate deoxynucleic acid
CXCL	Cys-Xaa-Cys chemokine ligand
CXCR	Cys-Xaa-Cys chemokine receptor
Cys	Cysteine

DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
FGF	Fibroblast growth factor
FL IL-33	Full-length IL-33 (wild type IL-33)
GAPDH	Glyceraldehyde 3 phosphate dehydrogenase
GTP	Guanosine triphosphate
h	hours
H_2SO_4	Sulfuric acid
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HMGB1	High-mobility group box 1
$HOCl^-$	Hypochlorite
HRP	Horse radish peroxidase
HSL	Homoserine lactone
ICAM-1	Intracellular adhesion molecule 1
IKKβ	I-kappa-B kinase beta
IRAK	IL-1 receptor-associated kinase
IRF3	Interferon regulatory factor 3
IκB	Inhibitor of kappa light polypeptide gene enhancer in B-cells
JNK	c-Jun NH_2-terminal kinase
K_D	Dissociation constant
kDa	Kilo dalton
L	Liter
LDH	Lactate dehydrogenase

LPS	Lipopolysaccharide
Lys	Lysine
m	Mili 10^{-3}
M	Molar
MAPK	Mitogen activated protein kinase
MAPKK or MKK	Mitogen activated protein kinase kinase
MAPKKK	Mitogen activated protein kinase kinase kinase
MEK	Mitogen activated protein ERK kinase
Mn^{2+}	Manganese
MOI	Multiplicity of infection
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation factor 88
n	Nano 10^{-9}
NADPH	Nicotinamide adenine dinucleotide phosphate
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor kappa B
PAGE	Polyacrylamide gel electrophoresis
PECAM-1	Platelet-endothelial cell adhesion molecule 1
PKA	Protein kinase A
PKC	Protein kinase C
PMA	Phorbol myristate acetate
Poly (I:C)	Polyinosine-polycytidylic acid
PRRs	Pattern recognition receptors
Psa	*Pseudomonas aeruginosa*

qRT-PCR	Quantitative real-time polymerase chain reaction
R48 IL-33	An arginine to alanine substitution at the 48^{th} residue of IL-33's sequence
Raf	Regulator of α-fetoprotein
RANTES	Regulated upon activation, normal, T-cell expressed and secreted
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
ST2	Suppression of tumorigenicity 2
TAK1	TGF-beta activated kinase 1
Thr	Threonine
TIR	Toll-IL-1 receptor
TIRAP	TIR-containing adaptor protein
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TPL2	Tumor progression locus 2
TRAF6	TNF receptor-associated factor 6
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adapter inducing interferon-β
Tyr	Tyrosine
v/v	Volume to volume
VEGF	Vascular endothelial growth factor
Zn^{2+}	Zinc
μ	Micro, 10^{-6}

Chapter 1: Introduction

1.1 Cystic Fibrosis

1.1.1From a mysterious syndrome to a genetic disease

Before the 20th century, cystic fibrosis (CF) was identified as a syndrome leading to severe malnutrition and death in infants. It was only in the early 1900's that case reports of this syndrome with histopathological analysis of different tissues were published in the medical literature. In 1923, a case of a severely malnourished infant whose state led to corneal perforation was reported. This female infant died at the age of 5 months, a few days after admission to the hospital. The autopsy reported inflammatory changes in lacrimal and salivary glands, as well as keratinization of the pancreatic epithelium accompanied by cystic cavities. Examination of the lungs showed peribronchitis, bronchiectasis and abscess formation. It was thought that the changes in the lung and in the pancreas were due to the effect of desquamated keratinized epithelium [1]. Several other reports described this syndrome of gastrointestinal disease, vitamin malabsorption, pneumonia and pancreatic dilatation and fibrosis, but it was only in 1938 that Dorothy Andersen described the condition as Cystic Fibrosis of the pancreas [2]. The disease was known to be severe and associated with high mortality early in life. A Canadian study analyzed ninety cases of CF and divided the patients into three groups. Group 1 included patients dying in the neonatal period, mostly due to stenosis of the intestinal tract. Group 2 included patients dying between the neonatal period and six months of age, these patients had failure to thrive and persistent respiratory infections. Group 3 included patients dying after six months of age and presented with similar manifestations to group 2, in addition of celiac syndrome [3].

It was later discovered that patients suffering from the disease had higher concentrations of chloride in sweat compared to healthy subjects and this was suspected to be mediated through abnormalities in the sweat glands [4]. This was the basis for the use of sweat chloride test as a diagnostic tool. Initially, patients suffering from the disease were wrapped around plastic bags to stimulate sweating in order to perform the measurement of chloride concentration. This inconvenience was circumvented by development of the pilocarpine iontophoresis sweat chloride test in 1959. This technique introduces the cholinergic agonist pilocarpine in the skin through a flow produced by two separate electrodes. The test is painless and efficient in inducing sweating that can then be collected to perform the measurement [5].

As life expectancy of CF patients increased, chronic lung disease became more prevalent. An important discovery was the abnormal electrolyte composition and low water content of airway secretions from CF patients [6]. In 1981 it was discovered that CF patients had increased nasal and lower airway potential differences as compared to healthy controls. This transepithelial potential difference could be corrected by a local infusion of amiloride, an inhibitor of sodium transport [7]. Two years later, the same group identified a defect in chloride permeability in the upper airways of CF patients [8].

Although throughout the years a greater insight into the pathophysiology of the disease had been gained, the exact genetic defect responsible for CF remained unknown. It was only in 1985 that the CF locus was identified and mapped to chromosome 7 [9, 10]. Four years later, through chromosome walking and jumping and cDNA hybridization, a 280 kb DNA segment in the CF locus was isolated. This discovery allowed for the cloning and characterization of CF gene. The structure of this gene was

predicted to code for a membrane-associated protein involved in ion transport, Cystic Fibrosis Transmembrane Conductance Regulator (CFTR). Additionally, a comparison of cDNA between CF patients and healthy individuals led to the discovery of a three base pair deletion that results in the loss of phenylalanine at position 508 (ΔF508) in CF individuals [11, 12]. Great improvements in the understanding of CF have been made since the discovery of the gene responsible for the disease. The following sections will give a brief overview of CFTR functions and the different CFTR mutations leading to disease. This will be followed by a review of the pathophysiology of cystic fibrosis lung disease (CFLD) with a focus on the pathways mediating inflammation in the context of infection.

1.1.2 CFTR structure and function

The gene coding for CFTR is found in the long arm of chromosome 7. CFTR mRNA codes for a 1480 aa protein (reference sequence: NP_000483.3, www.ncbi.nlm.nih.gov). The mature protein product corresponds to a transmembrane protein with two transmembrane domains (TMD), two nucleotide binding domains (NBDs) and one regulatory region I. CFTR belongs to the ATP binding cassette (ABC) C subfamily and functions as an anion channel at epithelial membranes [13]. Its activity as a channel depends on the conformational state it adopts which is in turn regulated by cAMP-dependent phosphorylation of the R domain by PKA and by the interaction of ATP with the NBDs [14]. Although CFTR is mainly known as a Cl^- channel, its anion selectivity is as follows: $I^- > Br^- > Cl^- > F^-$ [15]. In addition to acting as a channel for single anions, CFTR is also permeable to the polyatomic anions NO_3^-, HCO_3^-, formate and acetate [16].

Apart from its functions as an anion channel, CFTR acts as a regulator of diverse cellular processes. Through the release of ATP, CFTR indirectly regulates outwardly rectifying Cl^- channels (ORCCs), a distinct type of Cl^- channel [17]. CFTR is also known to regulate the amiloride-sensitive epithelial sodium channel (ENaC). In this context, induction of CFTR activity by cAMP agonists decreases ENaC activity, preventing excessive sodium absorption [18, 19]. Additionally, CFTR is a direct mediator of glutathione flux and therefore an important contributor of the extracellular redox state [20, 21].

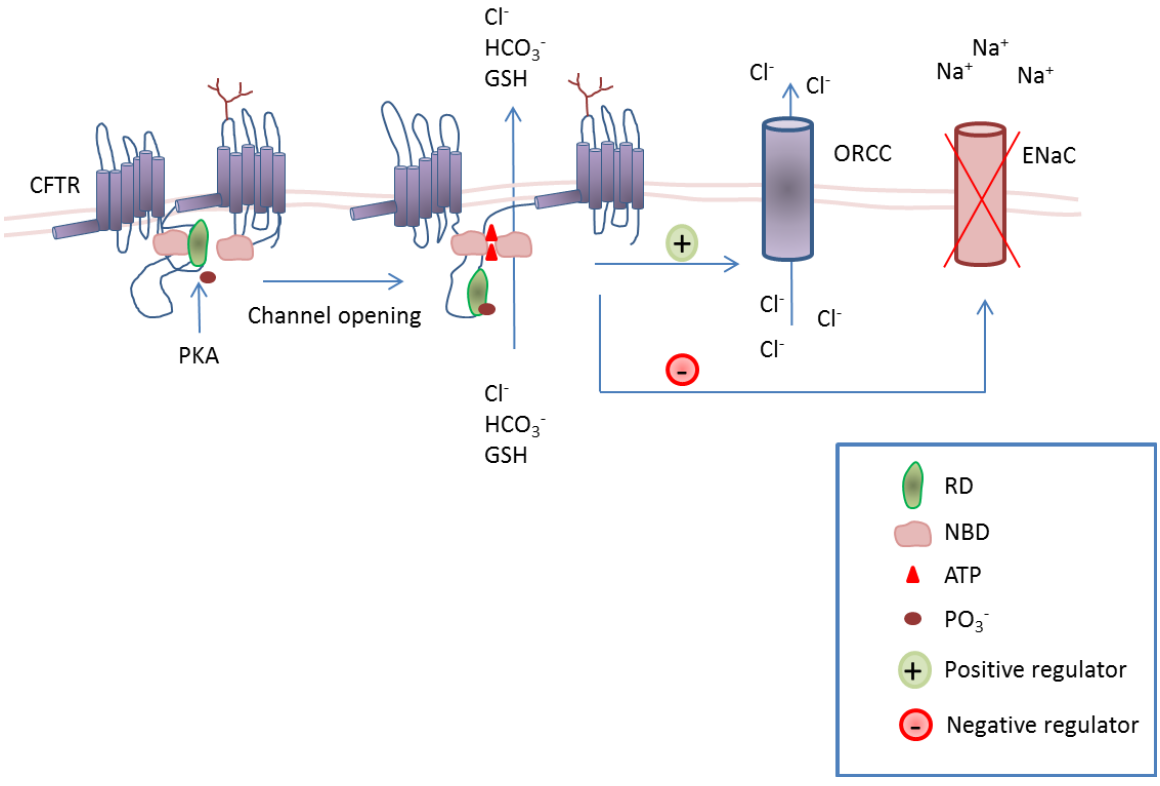


Figure 1.1: CFTR functions. From left to right: PKA induces cAMP-dependent phosphorylation of the RD in CFTR. This allows for the interaction of ATP with the NBDs and leads to channel opening. During the open conformation CFTR transports the anions Cl^- and HCO_3^-. CFTR also acts as a transporter of the antioxidant GSH. In addition to its functions as a channel, CFTR acts as a positive regulator of ORCCs and a negative regulator of the sodium channel ENaC. Abbreviations: cystic fibrosis

transmembrane conductance regulator, CFTR; protein kinase A, PKA; chloride, Cl^-; bicarbonate, HCO_3^-; reduced glutathione, GSH; outwardly rectifying chloride channel, ORCC; sodium, Na^+; epithelial sodium channel, ENaC; phosphate, PO_3^-; adenosine triphosphate, ATP; nucleotide binding domain, NBD; regulatory domain, RD.

1.1.3 CFTR processing and trafficking

Following transcription, CFTR is translated in the cytosolic ribosomes and is then cotranslationally inserted into the ER. CFTR is then core-glycosylated at residues Asn894 and Asn900 (N-glycosylation), located in the fourth extracellular loop. This glycosylation is essential for glycoprotein folding, sorting and trafficking. N-glycosylation gives rise to an immature form of CFTR that can be observed as 'band B' in SDS-PAGE. CFTR then traffics through the Golgi complex, where it is further glycosylated, resulting in the fully mature protein that can be observed as 'band C' in SDS-PAGE [22]. CFTR folding is recognized by the ER quality control machinery. Firstly, CFTR folding conformation is monitored by the chaperone heat-shock proteins Hsp40/Hsc70 and Hsp90. The association of ΔF508 CFTR with Hsc70 is markedly increased, which allows for recognition by other chaperone proteins. ΔF508 CFTR is retained in the ER and is then degraded by the ubiquitin-proteasome pathway [23]. Upon successful passage through the first checkpoint, wild-type CFTR interacts with the ER chaperone calnexin, this interaction leads to further protein folding [24]. CFTR then exits the ER and enters the secretory pathway via the Golgi apparatus and is ultimately targeted to the cell membrane. CFTR retention and stability at the cell membrane is a complex process that partially depends on the activity of multiple small GTPases [25].

1.1.4 CFTR mutations

There are close to 2,000 documented mutations of the CFTR gene. An updated database of these mutations can be found at www.CFTR2.org. The disease is caused by mutations in both of the alleles coding for CFTR. Mutations are currently classified into six categories. Class 1 mutations are stop codons, for which no CFTR transcripts are produced by the cell, an example of this mutation is G542X. Class 2 mutations lead to defective protein maturation, which leads to the misfolded protein response and degradation of CFTR by the proteasome, examples of these mutations are ΔF508, N1303K, G85E and G91R. Class 3 mutations lead to defective CFTR regulation that result in reduced chloride channel function and are usually caused by defects in the NBD, an example is G551D. Class 4 mutations are caused by defective chloride conductance or channel gating, A455E is an example of this class. Class 5 mutations result in decreased CFTR transcripts leading to decreased membrane-bound CFTR and Class 6 mutations result in increased CFTR turnover or reduced CFTR stability at the cell membrane. Class II are the most common mutations occurring in CF patients, with the deletion of phenylalanine at position 508 (ΔF508) accounting for about two thirds of mutated alleles in Europe and North America [26, 27].

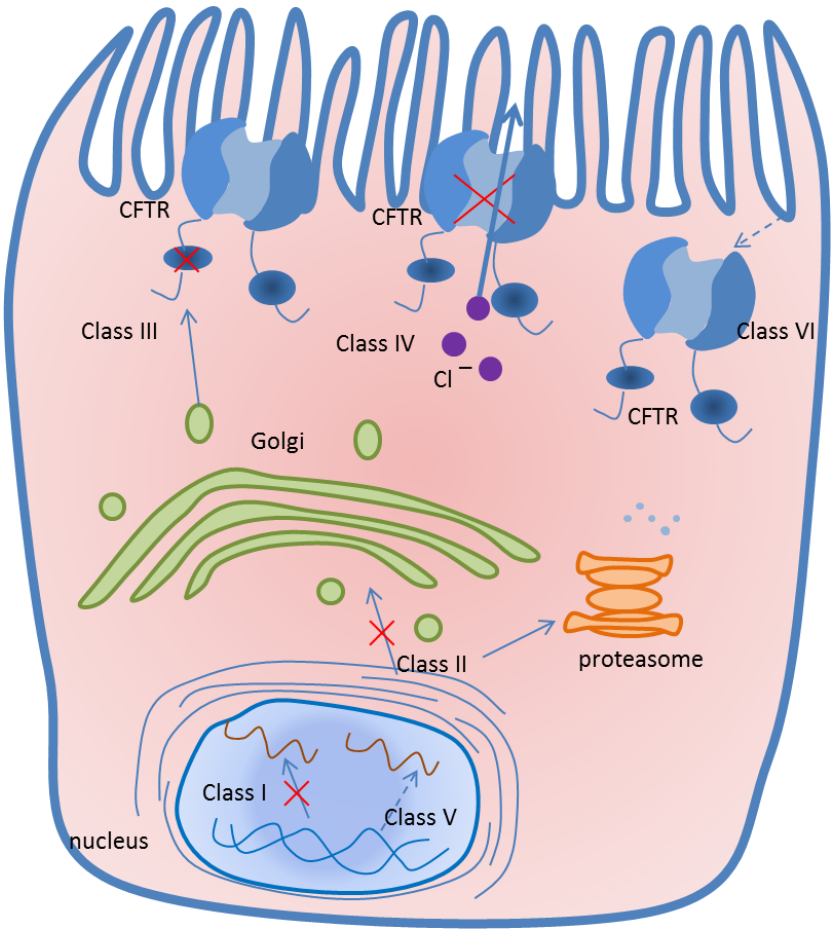


Figure 2.1: Classes of CFTR mutations. I) absence of synthesis; II) defective protein maturation and premature degradation (70%); III) disordered regulation; IV) defective chloride conductance or channel gating; V) reduced number of CFTR transcripts due to promoter or splicing abnormality; VI) accelerated turnover from the cell surface. Adapted from [27].

1.1.5 Pharmacologic strategies to target CFTR defects

An important strategy for the treatment of CF is the development of pharmacological compounds that target the basic CFTR defect. Small molecule CFTR modulators can be classified as follows: 1) molecules that prevent premature termination of protein synthesis; 2) compounds that revert processing and folding defects (correctors) and 3) compounds that increase channel gating and conductance (potentiators). Among these molecules, the potentiator VX-770 (Ivacaftor) has been approved for clinical use in

patients with at least one copy of the G551D mutation. Additionally, VX-770 improved CFTR channel activity of ΔF508 airway epithelilal cell cultures *in vitro*. However, VX-770 did not improve the lung function of ΔF508 homozygous patients in phase 2 clinical trials. The CFTR corrector VX-809 (lumacaftor) showed promise in restoring ΔF508-CFTR expression and function *in vitro*. However, this compound did not improve lung function in clinical trials. A combination of both the CFTR corrector VX-809 and potentiator VX-770 has also proven unsuccessful in correcting lung function of CF patients in clinical trials. This could be explained by the fact that VX-770 destabilizes CFTR at the ER and post-ER compartments, thus reducing ΔF508 cell surface CFTR function [14, 28].

1.2 Cystic Fibrosis Lung Disease

Defective CFTR function can lead to manifestations in diverse organs and systems, however, most of the complications arise from cystic fibrosis lung disease (CFLD). Chronic infections caused by different antibiotic-resistant pathogens and exacerbation episodes are associated with a progressive decrease in lung function [29, 30]. The following sections will address the pathophysiological changes occurring in the CF airway epithelium and the consequences of chronic bacterial colonization and sustained inflammation in the lung.

1.2.1 Innate Immunity and Inflammation CFLD

1.2.1.1 Airway epithelial defense mechanisms against pathogens

The airway epithelium is the first line of defense against microbial pathogens. In central airways, such as trachea and main bronchi, airway epithelial anatomy from the

base to the apex is as follows: submucosal glands; an epithelial layer with ciliated and non-ciliated, mucus-producing cells (goblet cells); a periciliary fluid layer and a mucus layer (figure 1.3 A). Peripheral airways such as terminal bronchioles lack the mucus layer, and the epithelium is composed of a single layer of cuboidal cells and the secretory Clara cells. [31].

Polarized airway epithelial cells secrete bactericidal factors into the airway surface liquid that allow for clearance of bacteria. Among these factors are β defensins and cathelcidins whose amphiphatic structure allows for disruption of microbial membranes [32]. Additionally, lactoferrin and lysozyme are important antimicrobial peptides present in airway secretions [33]. The pH of airway surface liquid is tightly regulated through the secretion of HCO_3^- by CFTR [34]. The adequate maintenance of airway surface liquid pH is essential for the maintenance of its antimicrobial properties. In CF, impaired CFTR function leads to decreased HCO_3^- secretion, which in turn acidifies airway surface liquid. The increased acidity of airway surface liquid decreases the antimicrobial activity of lysozyme and lactoferrin, allowing for bacteria to survive in the airways [35]. In addition to decreased pH, CF airway epithelium displays higher rates of airway surface liquid absorption, with the subsequent depletion of periciliary liquid. This results in a deficient mucus transport, which further allows for bacterial colonization [36]. Periciliary fluid has been measured in CF and non-CF human lower airway biopsies obtained by bronchoscopy. These measurements have failed to show a statistically significant difference in periciliary fluid height between non-CF and CF patients. However, periciliary fluid could not be quantified in half of the samples obtained and the technique for this measurement might require further optimization [37].

On top of the periciliary fluid layer is a mucus layer which provides the airways with a protective barrier. Mucus is composed of secreted mucins, mainly MUC5AC, MUC5B, MUC6 and MUC2. Structurally, mucins are O-linked glycoproteins produced by goblet cells and submucosal glands. Following secretion, mucins are polymerized and give mucus its characteristic viscosity and elasticity [38]. Glycan domains present in mucins bind bacterial components, protecting the epithelium from pathogens [39]. Additionally, α1,4-linked N acetylglucosamine present in mucins has direct antibacterial properties [40].

Mucociliary clearance is a key defense mechanism against pathogens. The ciliated epithelium acts in concert with the surface liquid and mucus layers to trap and transport pathogens out of the lung. Several evidences point towards an impairment in mucociliary transport in CFLD [41]. A recent *in vivo* study with CF piglets assessed mucociliary transport by tracking the movement of radioactive microdisks throughout the airway by x-ray computed tomography. They demonstrated that impaired mucociliary transport is due to thickened mucus that remains adhered to submucosal glands. This abnormality was replicated by inhibiting Cl^- secretion in non-CF airways. In this scenario, impaired Cl^- secretion due to abnormal CFTR function results in a thickened mucus layer that is unable to detach from submucosal glands, leading to a dysfunctional mucociliary transport [42]. Increased mucus viscosity and decreased mucociliary clearance allow for the establishment of chronic bacterial infection in CF. *Pseudomonas aeruginosa*, the most prevalent pathogen in adult CF lungs, takes advantage of mucociliary defects to form bacterial aggregates. These bacterial clusters undergo

phenotypic changes that lead to the formation of biofilms which allow for further pathogen survival in the airways [43].

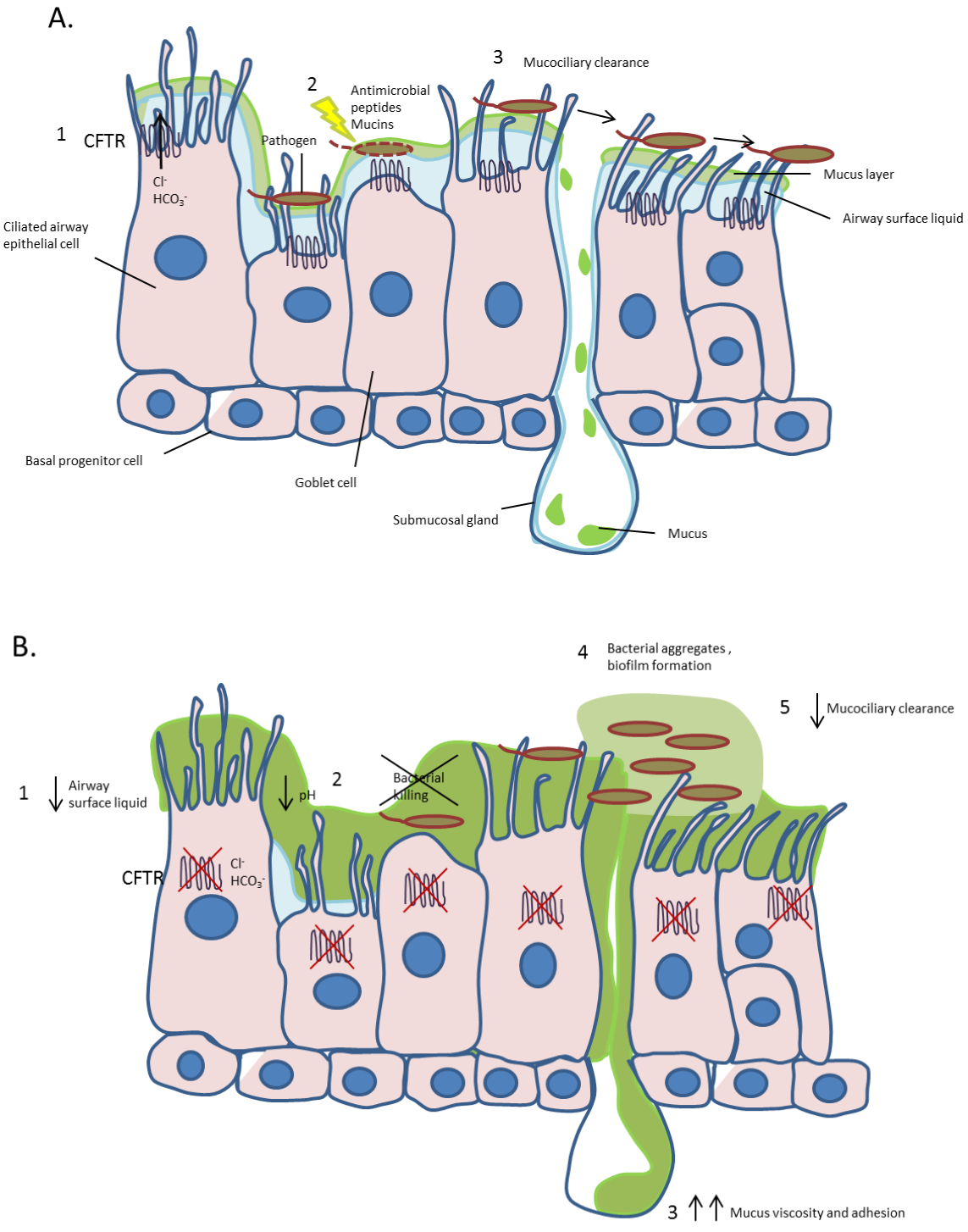


Figure 1.3: Airway epithelial defense mechanisms against pathogens in non-CF and CF lungs. A. 1) In physiological conditions, CFTR is expressed in the cell membrane, allowing for Cl^- and HCO_3^- secretion and adequate hydration of surface liquid. 2) When pathogens are introduced in the airway, antimicrobial peptides and mucins disrupt bacterial membranes. 3) Those pathogens who survive are propelled outside the airways through mucociliary clearance. Adapted from [31, 44]. B. 1) In CF airway epithelium, the absence of CFTR at the cell membrane prevents the secretion of anions ultimately leading to decreased amounts of airway surface liquid. 2) Changes in airway surface liquid composition and pH impair bacterial killing by antimicrobial peptides. 3) Mucus viscosity is markedly increased and mucus remains firmly adhered to submucosal glands. 4) Bacteria undergo phenotypical changes that allow them to grow in biofilm aggregates. 5) Thickened mucus and bacterial aggregate formation impair mucociliary clearance. Adapted from [36, 41-43]

1.2.1.2 Neutrophils

In addition to its intrinsic defense mechanisms, the airway epithelium produces several factors that attract innate immune cells to the lung. Upon contact with bacterial pathogens, airway epithelial cells produce diverse inflammatory mediators such as tumor necrosis factor α (TNFα), IL-1, IL-6, IL-8, granulocyte/monocyte colony stimulation factor (GM-CSF) and RANTES, among others [45, 46]. Neutrophils are guided to the site of infection by chemoattractant molecules that interact with receptors expressed in their cell surface. IL-8 (also known as CXCL8) is a very potent neutrophil chemoattractant that interacts with the receptors CXCR1 and CXCR2 in neutrophils. Other chemoattractants include IL-6, platelet activating factor, leukotriene B_4 and complement protein C_{5a}. Once neutrophils are recruited from the bloodstream, they are captured by adhesion molecules expressed in endothelial cells. Cytokines such as TNFα and IL-1 induce the expression of the adhesion molecule ICAM-1 on the surface of endothelial cells. ICAM-1 in turn interacts with the integrin CD18 expressed in neutrophils. Once neutrophils have adhered

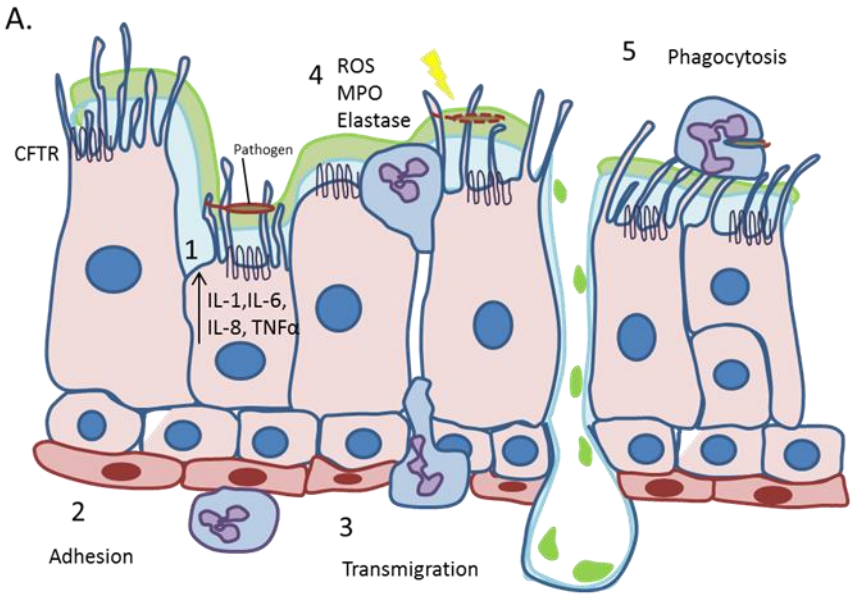
to the endothelium, they transmigrate through the endothelial cell layer and through the epithelium to reach the site of infection [47].

Once inside the airway epithelial lumen, neutrophils engulf and phagocytose bacterial or fungal pathogens. This results in the activation of NADPH oxidase with the subsequent production of reactive oxygen species such as O_2^- and H_2O_2 that are toxic to bacteria and fungus. To further potentiate bacterial killing, neutrophils release myeloperoxidase (MPO) from their cytoplasmic granules which in turn catalyzes the production of the highly toxic $HOCl^-$ [48].

In their azurophilic granules, neutrophils contain the serine proteases neutrophil elastase (NE), proteinase 3 (PR3) and cathepsin G (Cat G). These proteases act intracellularly to degrade microbial components and extracellularly by helping with the degradation of extracellular matrix components [49].

Due to chronic bacterial infection and to the subsequent production of inflammatory mediators, neutrophils are heavily increased in the lungs of CF patients [50]. In an effort to clear the infection, neutrophils secrete diverse bactericidal factors in the extracellular media. Bronchoalveolar lavage fluid (BALF) from CF patients contains elevated levels of active NE as compared to non-CF subjects, which causes proteolytic destruction of extracellular components [51]. In addition to being cytotoxic and damaging to basement membranes, a study demonstrated that NE can delay mucociliary clearance by decreasing ciliary beat frequency [52]. Furthermore, NE drives the production of IL-8 by airway epithelial cells, leading to a further recruitment of neutrophils to the airways [53]. Another mechanism used by neutrophils to clear infection is the formation of

extracelluar traps. These neutrophil extracellular traps (NETs) are composed of DNA, histones and granular proteins. NETs are highly cytotoxic to epithelial and endothelial cells [54]. Despite all the latter efforts, neutrophils fail to eradicate the infection in the CF lung. Evidence suggests that thickened mucus, such as observed in CF, decreases neutrophil motility. Furthermore, bacterial killing capacity is significantly decreased when neutrophils are trapped in concentrated mucus [55].



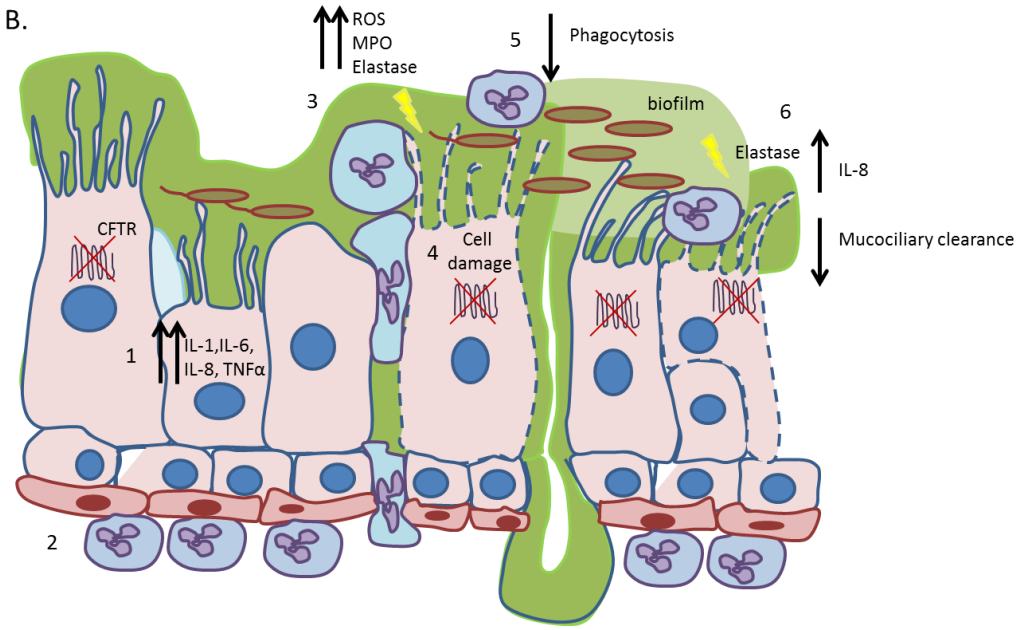


Figure 1.3: Neutrophil clearance of pathogens in the airways in physiological conditions (A) and in CF (B). A: 1) Upon contact with pathogens, airway epithelial cells release diverse cytokines and chemokines into the extracellular milieu. IL-8 is a potent chemoattractant that guides neutrophils to the site of infection. 2) Once recruited, neutrophils firmly adhere to endothelial cells through the actions of cytokines like TNFα and IL-1. 3) The latter process is followed by neutrophil transmigration through the endothelium and airway epithelium. 4) Neutrophils then release reactive oxygen species (ROS), myeloperoxidase (MPO) and proteases such as neutrophil elastase to kill bacteria. 5) Finally, neutrophils phagocytose bacteria to clear the infection. B: In CF, chronic infection leads to a sustained increase of pro-inflammatory mediators (1) which in turn recruit increased numbers of neutrophils to the lung (2). In an effort to clear the infection, neutrophils release MPO, ROS and elastase (3). However, these mediators fail to clear the infection and damage airway epithelial cells (4). Thickened mucus and biofilm formation by bacteria impair neutrophil motility and phagocytosis (5). Additionally, neutrophil elastase impairs ciliary clearance and induces IL-8 production (6), creating a vicious circle of inflammation and tissue destruction.

1.2.1.3 Monocytes and Macrophages

Monocytes and macrophages constitute another key line of defense of the innate immune system. There are two ways monocytes and macrophages fight infection in the respiratory tract. Firstly, the lung contains a population of resident alveolar macrophages

that is of a distinct origin from circulating monocytes [56]. Alveolar macrophages are in direct contact with external pathogens and respond immediately to signaling cues produced by epithelial cells to promote the host immune response [57]. Alveolar macrophages are primarily phagocytic cells and in the absence of infection, they phagocytose excessive surfactant protein to maintain alveolar homeostasis [58]. When faced with external pathogens, alveolar macrophages respond by phagocytosing and clearing the infectious agent [59].

Upon colonization with a bacterial pathogen, circulating monocytes are recruited from the bloodstream to peripheral tissues. Similarly to neutrophils, monocytes respond to cytokines and chemokines to express integrins and adhesion molecules that guide them towards the site of infection. Once recruited, monocytes can initiate an inflammatory response by secreting TNFα and chemokines. Additionally, monocytes can differentiate into alveolar macrophages or dendritic cells, which play key roles in phagocytosis and initiation of the adaptive immune response [60].

The presence of functional CFTR has been suggested to play a key role in phagolysosomal acidification and bacterial killing in alveolar macrophages. $CFTR^{-/-}$ alveolar macrophages display decreased intracellular bacterial killing when compared to WT macrophages [61]. This hypothesis was supported by another study that showed decreased intracellular bacterial killing capacity in monocyte-derived macrophages from CF patients [62]. CFTR is also expressed in peripheral monocytes and monocytes from CF patients have decreased phagocytosis and bacterial killing capacities. This is thought to be due to defective complement-mediated phagocytosis in CF monocytes [63].

1.2.2 Pathogens colonizing the airways in CFLD

The bacterial species most commonly found in cultures from the airways of CF patients are: *Pseudomonas aeruginosa, Staphylococcus aureus* and *Haemophilus influenza.* Other opportunistic pathogens commonly found during nosocomial infections include *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans.* Less prevalent pathogens include *Burkholderia cepacia, Burkholderia gladioli, Ralstonia* sp., *Cupriavidus* sp. and *Pandoraea* sp. [64]. The prevalence of pathogens changes with age, with *S. aureus* being the most prevalent pathogen in children while *P. aeruginosa* is more prevalent in cultures from adult CF patients in Canada. Overall, *P. aeruginosa* was found in 43% of sputum cultures of CF patients of all ages in Canada in 2013. Other than bacteria, the fungus *Aspergillus fumigatus* is highly prevalent in the airways of adult CF patients, with an overall 20% prevalence in patients of all ages in Canada [65].

1.2.2.1 *Pseudomonas aeruginosa*

P. aeruginosa is an opportunistic Gram-negative bacterium ubiquitously present in soil and aqueous environments. It is highly adaptable and antibiotic resistant, properties that allow it to survive in multiple settings. The great adaptability of *P. aeruginosa* is in part due to its high rate of mutations that cause phenotypic changes in response to specific environments. In addition to causing chronic infection in CF patients, *P. aeruginosa* causes serious nosocomial infections in mechanically ventilated and immunocompromised patients and is often found in the airways of patients suffering from chronic obstructive pulmonary disease (COPD) [66, 67].

1.2.2.2 *P. aeruginosa* virulence factors

Several strains of *P. aeruginosa* possess a wide array of virulence factors. The most common ones, usually present in clinical strains, are summarized in Table 1.1.

Flagella confer bacterial motility in aqueous environments as well as chemotaxis toward preferred substrates. In the lung, this allows for *P. aeruginosa* to spread throughout the respiratory tract. Furthermore, flagella are essential for the establishment of an initial infection through their binding to the asialyated glycolipid asialoGM1 in the host [30].

Pili are responsibe for twitching motility; the ability to move along solid and semisolid surfaces. Pili also allow for bacterial aggregation and biofilm formation. Additionally, pili mediate host cell adhesion and are a major virulence factor required for maintenance of infection [68].

The type 3 secretion system (T3SS) is a structure on the bacterial surface that transports proteins into host cells. Proteins exported by this system include exotoxin (Exo)Y, ExoS, ExoT and ExoU. The most virulent of these toxins are ExoS and ExoU, which induce changes in the host's cytoskeletal organization and plasma membrane, often leading to necrotic cell death [69]. Exotoxin A is another important virulence factor secreted by *P. aeruginosa* that inhibits the host protein synthesis by inhibiting elongation factor 2 (EF2). This results in a repression of the host immune response and cell death [66].

Quorum sensing is a bacterial cell to cell communication system through chemical cues. It works through two main systems: a signal generator (encoded by the gene *LasI*) and a signal receptor (encoded by *LasR*). Activation of *LasI* leads to the production of *N*-

(3-oxododecanoyl)-l-homoserine lactone (3O-C_{12}-HSL) which is sensed by the transcriptional regulator LasR. This is a positive feedback loop that increases with increased cell density. This allows for the activation of different autoinducer genes, ultimately resulting in the formation of biofilms that enable bacteria to survive in microcolonies and to establish a chronic infection [70].

P. aeruginosa produces several proteases that interfere with the host immune response. Alkaline protease is a fibrin and immunoglobulin lysing protease that has been implicated in the progression of acute lung injury by *P. aeruginosa*. Protease IV is a surfactant-degrading enzyme. The metalloprotease lasB disrupts the respiratory epithelium by damaging cell-to-cell adhesions. Phospholipase C degrades host membrane phospholipids and has also been implicated in acute lung injury [71].

Lipopolysaccharide (LPS) is a complex glycolipid and is the main structural component of the Gram-negative bacterial cell wall. It is composed by lipid A (anchored to the bacterial cell wall), a polysaccharide core region and variable O-specific polysaccharide (O-antigen). Lipid A alters the bacteria's sensitivity to antibiotics and antimicrobial peptides. Lipid A is recognized by the host immune system, through its interaction with MD2 and toll-like receptor 4 (TLR4). The O-antigen is recognized by the host immune system and induces an antibody response [72] .

Pyocyanin is a toxin that confers *P. aeruginosa* its color. It acts by inhibiting cell respiration, disrupting calcium homeostasis, affecting ciliary function and inactivating the α1-protease inhibitor, leading to lung damage [73].

In addition to all the above mentioned virulence factors *P. aeruginosa* has a variety of mechanisms that confer it resistance to antibiotics such as genetic adaptations for target enzymes and antibiotic efflux pumps [74].

Table 1.1: *P. aeruginosa* main virulence factors and their functions

Virulence factor	Functions
Flagellum	Motility in aqueous environment Establishment of infection
Type 4 pili	Motility on semisolid surfaces Bacterial aggregation, biofilm formation
Type 3 secretion system (T3SS)	Exotoxin (Exo)Y, v secretion
Exotoxin A	Mammalian protein synthesis inhibitor
Quorum sensing molecules	Cell survival, biofilm formation, virulence
Proteases	Lung surfactant degradation
Lipopolysaccharide (LPS)	Physical barrier against host defenses and antibiotics
Pyocyanin	Disruption of catalase, inhibition of host electron transport chain
Alginate (mucoid exopolysaccharide)	Decreased antibiotic uptake, impaired phagocytosis and killing by neutrophils
Source: [66]	

1.2.2.3 *P. aeruginosa* adaptations in CF

P. aeruginosa undergoes several genetic adaptations that allow for its chronic establishment in the airways of CF patients. In many occasions, the virulence factors required for an initial acute infection are lost during chronic infection. This allows for the bacteria to evade the host immune response and to establish a microenvironment favorable for their survival. Most of the mutations occurring during chronic infection affect protein function. For instance, a loss of function mutation of the gene *mexZ*, a negative regulator of a multidrug efflux pump, is commonly found in bacterial isolates from CF patients. This increases the activity of antibiotic efflux pumps and confers antibiotic resistance [75]. Additionally, *P. aeruginosa* strains from chronically infected patients show an attenuated phenotype for proteases, pyocyanin and T3SS [76].

It has been hypothesized that a single strain of *P. aeruginosa* initiates the colonization of the airways in CF patients. Over the course of time, this single strain can undergo extensive genetic and phenotypic adaptations. This leads to a significant diversification of *P. aeruginosa* in the lung. The main two phenotypes found during chronic infection in CF are mucoid cells and small colony variants (SCV). SCVs are slow-growing isolates that autoaggregate and show increased attachment to surfaces and are prone to biofilm formation [77, 78]. The biofilm matrix mainly consists of polysaccharides and chromosomal DNA. Exopolysaccharides are essential structural components of the matrix and extracellular DNA functions as a cell-to-cell interconnecting compound [79]. Biofilms confer physical and chemical barriers to bacterial communities thus decreasing the penetration of antibiotics and host defense molecules [66].

Analysis of CF lungs has revealed that colonies *P. aeruginosa* do not bind directly to the epithelial cells but they are rather found within mucus plugs in the airway lumen. *In vitro* studies demonstrate that *P. aeruginosa* firmly attaches to mucin in surfaces and then penetrates to hypoxic zones within mucus layers. Thereafter, bacteria undergo phenotypic adaptations to survive under hypoxic conditions [80, 81]. Another important adaptation of *P. aeruginosa* is the development of a mucoid phenotype that produces increased amounts of alginate, which in turn confers antibiotic resistance and impairs bacterial killing by neutrophils [82].

1.3 Pathogen recognition by the innate immune system: the toll-like receptors

Pathogens express diverse structural motifs that are recognized by the host innate immune system. Such motifs are called pathogen-associated molecular patterns (PAMPs) and are recognized by Pattern Recognition Receptors (PRRs) expressed in a variety of immune and structural cells. Toll-like receptors (TLRs) are the best characterized PRRs [83]. Toll receptors were initially discovered in the fly *Drosophila melanogaster* and were found to be homologous to the human IL-1 receptor. Toll receptors were first characterized for their role in antifungal defenses in the fly [84]. This was followed by the discovery of the highly conserved TLRs in mammals [85].

1.3.1 TLR structure and subcellular localization

TLRs are transmembrane proteins and are expressed either in the cell membrane or in the membrane of intracellular vesicles. Their ectodomain contains leucine-rich repeats that recognize PAMPs. This is followed by a transmembrane domain that connects the ectodomain with the cytosolic Toll-IL-1 receptor (TIR) domain, which in

turn activates downstream signaling pathways [86]. Currently, 10 and 13 TLRs have been identified in humans and mice, respectively [87].

TLR1, TLR2, TLR4, TLR5 and TLR6 are constitutively localized in the cell membrane and mainly recognize microbial proteins, lipoproteins and glycoproteins. Following recognition of their ligand, TLR2 and TLR4 are internalized and localized to the endosomes or phagosomes, where they mediate distinct signaling pathways. TLR3, TLR7, TLR8 and TLR9 are localized in intracellular vesicles of ER, endosomes, lysosomes or endolysosomes and recognize bacterial or viral nucleic acids [86, 88, 89].

1.3.2 TLR recognition of PAMPs

Upon recognition of bacterial, viral, fungal or parasitic PAMPs, TLRs can signal downstream as homo or heterodimers. TLR1 and TLR2 form a heterodimer that recognizes bacterial triacyl lipopeptides [90, 91]. TLR2 can also associate with TLR6 to recognize lipoprotein and diacyl lipopeptides such as lipoteichoic acid from gram positive bacteria [92]. TLR heterodimerization is necessary for discrimination and specific recognition of distinct PAMPs from different organisms. For instance, TLR2/TLR6 heterodimerization is necessary to sense the diacylated macrophage-activating lipopeptide 2 (MALP-2) from mycoplasma [93].

TLR3 recognizes viral double-stranded RNA (dsRNA), leading to the production of type 1 interferons [94]. The recognition of viral dsRNA by TLR3 in antigen-presenting cells is crucial for initiation of the adaptive immune response [95]. Additionally, TLR3 can recognize single-stranded RNA (ssRNA) from West Nile virus [96].

TLR4 is mainly known for its recognition of lipopolysaccharide (LPS), a cell wall component in gram-negative bacteria. When released from bacteria, LPS circulates in the bloodstream bound to lipopolysaccharide binding protein (LBP). MD-2 is essential for the recognition of LPS by TLR4. TLR4 then homodimerizes and triggers an initial signaling cascade that induces pro-inflammatory cytokine production. Subsequently, TLR4 is delivered to the endolysosome, where it activates a distinct signaling pathway leading to the production of type 1 interferons [92, 97, 98].

Flagella confer bacteria the ability to actively move from one place to another. TLR5 recognizes a structurally conserved domain of flagellin, the main component of bacterial flagella [99]. TLR5 mediates key inflammatory pathways in response to the presence of flagellin in airway epithelial cells. Polymorphisms in TLR5 binding domain increase the susceptibility to pneumonia in humans [100].

TLR7 was initially characterized for its recognition of imidazoquinoline antiviral compounds and the subsequent induction of TNFα and IFNα [101]. It was later discovered that both TLR7 and TLR8 recognize guanosine and uridine-rich single-stranded RNA (ssRNA) oligonucleotides from virus [102].

TLR9 recognizes unmethylated CpG dinucleotides from bacteria. Mammalian DNA is not recognized by TLR9 since the infrequent CpG dinucleotides are usually methylated [103]. Bacterial CpG DNA is endocytosed and transferred to the lysosomes, this in turn elicits the redistribution of TLR9 from the ER to the CpG DNA-containing lysosome. In the lysosome, CpG DNA binds directly to TLR9, inducing the recruitment

of adaptor molecules and downstream signaling [104]. Viral DNA also contains unmethylated CpG motifs that are recognized by TLR9 [105].

Table 1.2: Toll-like receptor ligands

TLR	Ligand	Reference
TLR1	Triacyl lipopeptides	[91]
	Pam3CSK4	[106]
TLR2	Mannuronic acid polymers	[107]
	Lipoprotein 1	[108]
	Slime-glycoprotein	[109]
	Lipoteichoic acid (diacyl lipopeptide)	[110]
	Peptidoglycan	[110, 111]
	MALP-2 (diacyl lipopeptide)	[93]
	Bacterial lipopeptide	[111]
	Pam3CSK4 (synthetic ligand)	[106]
TLR3	dsRNA	[94]
	Poly (I:C) (synthetic ligand)	[94]
	ssRNA	[96]
TLR4	LPS	[97]
	Lipoprotein 1	[108]
TLR5	Flagellin	[99]

TLR6	Lipoteichoic acid (diacyl lipopeptide)	[111]
	Peptidoglycan	[93]
	MALP-2 (diacyl lipopeptide)	[93]
TLR7	Imidazoquinoline antivirals	[101]
	ssRNA	[102]
TLR8	Imidazoquinolines	[112]
	ssRNA	[102]
TLR9	CpG DNA	[103, 105]

Abbreviations: Toll-like receptor, TLR; diacylated macrophage-activating lipopeptide 2, MALP-2; double-stranded ribonucleic acid, ds-RNA; single-stranded ribonucleic acid, ssRNA; cytosine triphosphate guanine triphosphate deoxynucleic acid, CpG DNA.

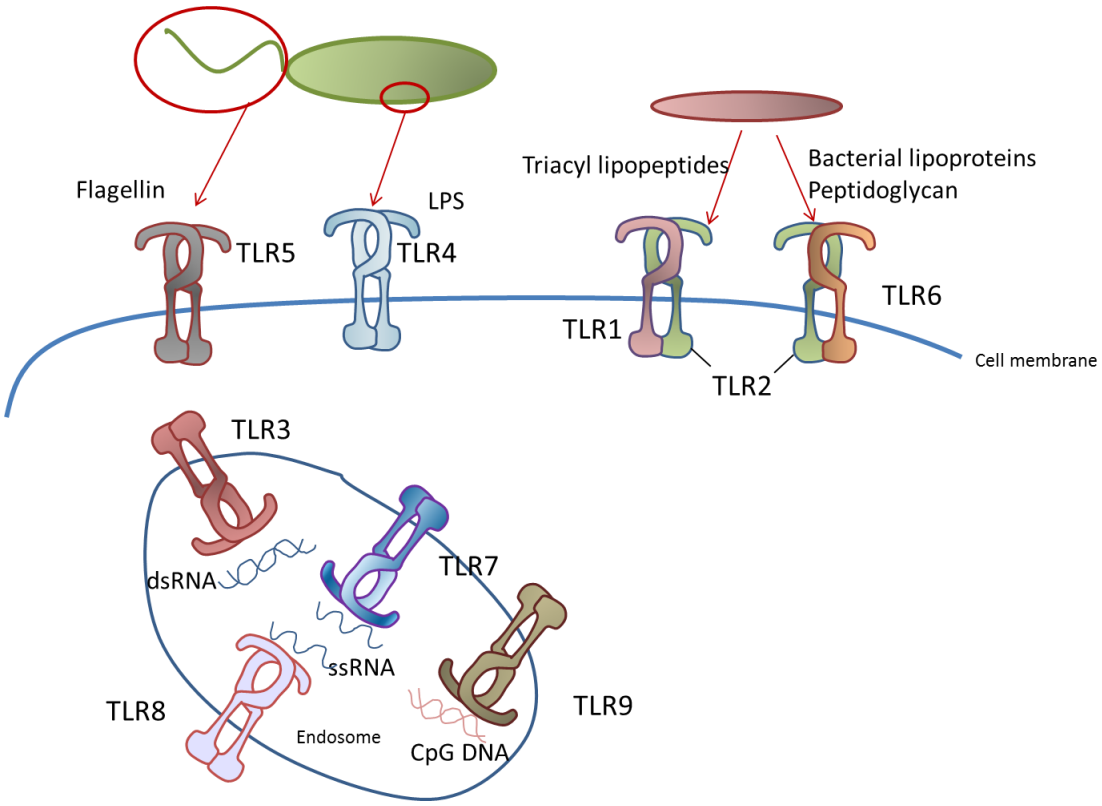


Figure 1.4: TLR localization and main ligands. TLR1, TLR2, TLR4, TLR5 and TLR6 are localized in the cytoplasmic cell membrane. TLR1 and TLR2 form a heterodimer that recognizes triacyl lipopeptides from bacteria. TLR2 can also associate with TLR6 to recognize bacterial lipoproteins and peptidoglycan. TLR4 recognizes lipopolysaccharide (LPS) from gram-negative bacteria and TLR5 senses flagellin from motile bacteria. TLR3, TLR7, TLR8 and TLR9 are mainly localized in the lysosomal membrane. TLR3 recognizes viral double-stranded (dsRNA) while TLR7 and TLR8 identify viral signle-stranded RNA (ssRNA). TLR9 recognizes unmethylated CpG DNA from bacteria and virus.

1.3.3 Toll-like receptor signaling pathways

Ligand recognition induces TLR dimerization with conformational changes that drive downstream signaling. The downstream signaling cascade triggered by each TLR depends on the recruitment of specific adaptor molecules by the TIR domain. The main adaptor molecules implicated in TLR signaling are: myeloid-differentiation primary response 88 (MyD88), TIR-containing adaptor protein (TIRAP), TIR domain-containing

adapter inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM). TIRAP serves as a bridge to signal through MyD88. TRAM in turn connects the TIR domain to TRIF. In general terms, TLR signaling can be divided into MyD88-dependent and TRIF-dependent downstream signaling. MyD88 signaling activates NFκB and mitogen-activated protein kinases (MAPKs), leading to the production of inflammatory cytokines. TRIF signaling leads to the activation of IRF3 and NFκB, which induce the production of type 1 interferons (IFNs) and pro-inflammatory cytokines [86, 113, 114].

1.3.3.1 MyD88-dependent pathways

With the exception of TLR3, MyD88 can be recruited either directly or indirectly by all TLRs. The interaction of MyD88 with the TIR domain or with the bridging molecule recruits members of the IL-1 receptor associated kinase (IRAK) family to the complex [115]. IRAK-1 and 4 have intrinsic kinase activity and their phosphorylation leads to a direct interaction with TRAF6 [116-119]. TRAF6 is a ubiquitin ligase and together with UBC13 and UEV1A catalyzes Lys-63 polyubiquitination of TRAF6 itself and of IRAK-1. Lys-63 polyubiquitin chains bind to TAB2 and TAB3, which activates the TAK1 complex [120]. TAK1 can in turn activate the IKK complex, formed by inhibitor of nuclear-factor κB kinase (IKK) α and IKKβ and NFκB essential modifier (NEMO). The aforementioned lys-63 polyubiquitin chains bind NEMO, keeping the IKK complex in close proximity to TAK1. TAK1 then catalyzes the phosphorylation of IKKβ [121]. IKKβ activation can lead to the phosphorylation, lys-48 ubiquitination and proteasomal degradation of IκB, with the subsequent translocation of the transcription factor NFκB to the nucleus [122]. IKKβ activation can also lead to the phosphorylation of the NFκB1 p105 subunit, resulting in the activation of the tumor progression locus-2

(TPL2) kinase, with the subsequent phorphorylation and activation of the mitogen activated protein kinase kinase (MKK) 1 and MKK2, followed by the activation of the mitogen activated protein kinases (MAPK) extracellular signal-regulated kinase (ERK) 1 and ERK2 [123]. TAK1 is also a mitogen activated protein kinase kinase kinase (MKKK or MAP3K) that can activate MKK3/MKK6 which in turn phosphorylate and activate p38 MAPK [124]. On the other hand, TAK1 can activate MKK4/MKK7 to induce jun-amino terminal kinase (JNK) signaling [125, 126].

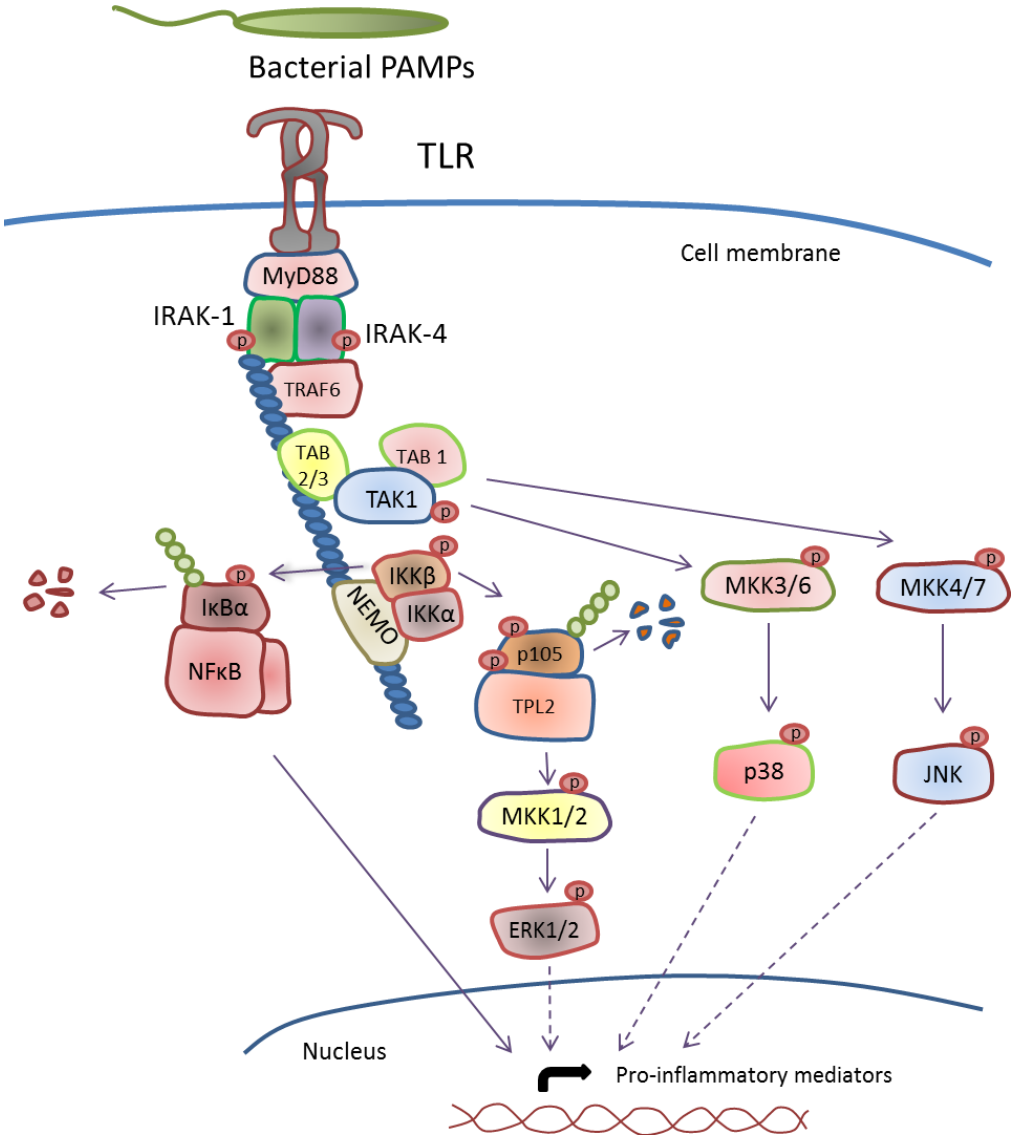


Figure 1.5: MyD88-dependent TLR signaling. Binding of ligand to TLRs induces conformational changes that lead to TLR dimerization and the recruitment of the adaptor MyD88. MyD88 recruits and activates IRAK1 and IRAK4, whose phosphorylation recruits and activates TRAF6. This, in turn leads to the formation of lys-63 polyubiquitin chains that bind TAB2 and TAB3, leading to the activation of TAK1. Lys-63 polyubiquitin chains also bind NEMO in the IKK complex, allowing for TAK1 phosphorylation of IKKβ. IKKβ can phosphorylate IκBα, leading to its ubiquitination and proteasomal degradation. IKKβ can also phosphorylate NFκB p105 subunit, allowing for the activation of the TPL2-MKK1/2-ERK1/2 signaling pathway. Finally, TAK1 is a MAP3K that can phosphorylate and activate MKK3/6, which in turn activate p38 MAPK. TAK1 can also phosphorylate MKK4/7 leading to JNK activation.

1.3.3.2 TRIF-dependent pathways

Similar to MyD88, TRIF can be recruited to TLRs directly or indirectly through the interaction with the adaptor TRAM. The N-terminal portion of TRIF can interact with TRAF3 and TRAF6. TRAF3 is an E3 ubiquitin ligase that, upon interaction with TRIF, undergoes lys-63 self-ubiquitination, leading to the activation of TRAF family member-associated NFκB activator (TANK)-binding-kinase 1 (TBK1). TRAF3 can also activate inducible IKK (IKK-i, also known as IKK-ε). TBK1 or IKK-i phosphorylation leads to the dimerization and nuclear translocation of interferon regulatory factor (IRF) 3 and IRF7. This is followed by the production of type 1 IFNs (IFNβ or IFNα). On the other hand, TRAF6 activation ultimately leads to the translocation of NFκB to the nucleus with the subsequent transcription of NFκB target genes [127, 128].

Table 1.3: Toll-like receptor adaptor molecules and main signaling pathways

TLR	Adaptor molecules	Signaling pathways
TLR1	TIRAP, MyD88	NFκB, MAPKs
TLR2	TIRAP, MyD88	NFκB, MAPKs

	TIRAP, MyD88 (endosome) TRAM, TRIF	IRF3, IRF7 IRF3
TLR3	TRIF	NFκB, IRF3
TLR4	TIRAP,MyD88 TRAM, TRIF	NFκB, MAPKs NFκB, IRF3
TLR5	MyD88 TRIF	NFκB, MAPKs NFκB, MAPKs
TLR6	TIRAP, MyD88	NFκB, MAPKs
TLR7	MyD88	NFκB, MAPKs IRF7
TLR8	MyD88	NFκB, MAPKs IRF5
TLR9	MyD88	NFκB, MAPKs IRF7
References: **[86, 114, 127, 129, 130]**		

1.3.4 TLR downstream effectors

1.3.4.1 NFκB

The five NFκB proteins expressed by mammals are NFκB1 p50, NFκB2 p52, RelA (p65), RelB and c-Rel. NFκB1 and NFκB2 are synthesized as precursors of 100 and 105 kDa (p100 and p105, respectively). These precursors are proteolysed to produce the mature NFκB1 p50 and NFκB2 p52 subunits. NFκB1 p50 and NFκB2 p52 subunits are unable to promote transcription and have to be heterodimerized with Rel subunits, which

contain a transactivation domain. NFκB dimers are negatively regulated by members of the IκB family. In the absence of stimuli, NFκB forms homo or heterodimers that are sequestered in the cytoplasm by IκBs. Upon stimulation, IκBs are phosphorylated by upstream kinases, this leads to degradation by the ubiquitin-proteasomal pathway. The major kinases that phosphorylate IκBs are IKKs, protein kinase A (PKA), protein kinase C (PKC), protein kinase R (PKR) and glycogen synthase kinase 3 β (GSK3β). NFκB can regulate a wide variety of genes implicated in inflammation, survival, proliferation, oncogenic transformation, among many other functions. In the context of innate immunity, NFκB activation leads to the transcription of TNFα, IL-1, IL-6, CXCL8, RANTES, E-selectin and VCAM-1, among many others [131, 132].

1.3.4.2 MAPKs

As described on the previous section, MAPKs are important components of pro-inflammatory signaling pathways and they can be activated through a variety of upstream mediators. MAPKs are a group of ubiquitously expressed, proline-directed, serine/threonine kinases that mediate cellular responses such as growth, differentiation, inflammation and apoptosis by integrating and processing extracellular signals. Extracellular signal-regulated kinases 1 and 2 (ERK 1/2), p38 MAPK and c-Jun N-terminal kinase 1 (JNK1) are the three most common MAPKs studied in mammalian cells. Each subfamily consists of several isoforms with distinct functions. The MAPK signaling cascade has three main components, the MAPKKKs, the MKKs and the MAPKs, which mediate a series of phosphorylation cascades that lead to the activation of the downstream effectors. MAPKs are activated upon dual phosphorylation on tyrosine and threonine residues by the MKKs and this is reversed by the action of MAPK

phosphatases. MKKs are in turn activated upon phosphorylation on serine and threonine residues by the action of MAPKKKs. Induction of MAPK signaling pathways results in activation of transcription factors, other protein kinases, cytoskeletal proteins, translational machinery components and metabolic enzymes, ultimately resulting in cellular differentiation, migration, inflammation, proliferation, cytoskeletal rearrangement and oncogenic transformation [133, 134].

1.3.4.2.1 p38 MAPKs

The p38 MAPK family consists of the four isoforms α, β, γ and δ. Among the four members, p38α is the best characterized and is known to mediate diverse cellular signaling pathways in response to stress and inflammation. p38 MAPKs are activated upon dual phosphorylation at the Tyr and Thr residues within a Thr-Gly-Tyr motif by MKK3 and MKK6, which causes conformational changes that enhance access to the substrate, therefore increasing their kinase activity [135, 136]. MKK3 and MKK6 are in turn activated upon phosphorylation by a MAPKKK, which is cell type and stimulus specific. The MAPKKKs ASK1, TAK1 and some members of the MEKK family activate p38 MAPK signaling through their activating phosphorylation of MKK3/6 [137, 138]. Upstream of the MAPKKKs are low molecular GTP-binding proteins from the Rho subfamily, heterotrimeric G-protein coupled receptors and TLRs [139]. Upon activation, p38 MAPK can modulate different downstream responses. For instance, it can regulate the activity of transcription factors such as ATF-2 and CHOP-1, implicated in cell growth and differentiation. Other transcription factors activated by p38 MAPK include c-Fos, p53, p73, Stat 1/4, Elk1, Pax6, Mac, c-Myc and c-Jun [140]. p38 MAPK can also activate other protein kinases such as MAPK-activated protein kinase-2 (MAPKAP-K2), which in

turn mediates the inflammatory response [141]. A crucial role of p38 γ and δ MAPKs is the stabilization of the MAPKKK TPL2 in the context of TLR4 activation. *In vitro* studies show that p38γ and p38δ are required for ERK1/2 MAPK activation in macrophages in response to LPS and TNFα. The mechanism through which p38γ and p38δ maintain steady-state levels of TPL2 has to be further elucidated but potentially involves posttranslational modifications of the TPL2/ABIN2 complex by p38γ and p38δ MAPKs [142].

1.3.4.2.2 ERK1 and ERK2 MAPKs

The extracellular signal-regulated protein kinases (ERK) 1 and 2 are serine/threonine kinases that regulate diverse cellular processes such as cell growth, differentiation, survival and apoptosis. ERK1 and ERK2 share 84% homology and have identical substrate specific activity *in vitro* [143, 144]. ERK1/2 are activated upon phosphorylation of tyr and thr residues by the MAPKKs MAP/ERK kinase (MEK)1 and MEK2 [145]. The main MAPKKKs known to activate MEK1/2 are regulator of α-fetoprotein (Raf) isoforms, TPL2, Mos, MEKK1 and MLK2, although many other MAPKKKs can activate MEK1/2 in a cell-specific and stimulus-dependent manner [146].

The better studied Raf kinase isoforms are B-Raf and Raf-1. These kinases are recruited to the cell membrane and are activated by small G-proteins of the Ras family. Ras activation is in turn mediated by several receptor protein tyrosine kinases such as epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor (VEGFR). Additionally, protein kinase C (PKC) activation by phorbol esthers can lead to

a direct activation by phosphorylation of Raf-1[147, 148]. In the context of inflammation in innate immune responses, the MAPKKK TPL2 is a key transducer of signals leading to ERK1/2 activation. During basal conditions, TPL2 is in complex with A20-binding inhibitor of NFκB2 (ABIN2) and with NFκB1 p105 subunit. Interaction with ABIN2 stabilizes TPL2 while the interaction with NFκB1 p105 prevents TPL2 from activating downstream targets. Phosphorylation of p105 by IKKβ leads to its proteolytic degradation, allowing for TPL2 to autophosphorylate and activate MEK1/2 [149].

Upon activation, ERK1/2 phosphorylate hundreds of substrates involved in cell responses such as cell proliferation, survival, morphological adaptations, plasticity, stress responses and cell death. Elk1, Smad1-4, p53, c-Myb, c-Myc, SP1, c-Fos, ER, Ets-1, Stat 1/3, PARP-1 and PARP-γ are among the transcription factors activated by ERK1/2 [140]. In airway epithelial cells exposed to *P. aeruginosa*-derived material, ERK1/2 activation occurs through TPL2, leading to the production of the inflammatory mediators CXCL8 and IL-6 [150].

1.3.4.2.3 JNK MAPKs

c-Jun amino terminal kinases (JNKs) are widely expressed stress response kinases. Three members of this family of kinases have been identified: JNK1, JNK2 and JNK3. These kinases are activated upon dual phosphorylation of tyr and thr residues by MKK4/MKK7. These MKKs are in turn activated by a variety of MKKKs such as TAK1, ASK1 and MAPKKK1. Importantly JNKs play important roles in the development and function of adaptive immune cells. Additionally, JNK1 and JNK2 are activated in macrophages following TLR activation, leading to the production of TNFα. JNK1 and JNK2 also regulate macrophage polarization to the M1 phenotype [126, 151]. JNK

activation leads to the activation of several transcription factors such as p53 and Bcl2 and c-jun, a component of the AP-1 heterodimer [152].

1.3.4.3 Kinase inhibitors used for the study of TLR downstream signaling pathways

Several MAPK pharmacological inhibitors have been developed for both potential clinical use and for the study of signaling pathways implicated in inflammation. BIRB0796 is an allosteric inhibitor of p38α, p38β and p38γ. It induces structural changes in the activation loop, preventing ATP binding. This compound decreases p38 MAPK phosphorylation in a time and dose-dependent manner [153, 154]. PD184352 is a potent allosteric inhibitor of MEK1 and MEK2 isoforms. It binds to a hydrophobic pocket adjacent to the magnesium-ATP binding site. This induces a conformational change in the non-phosphorylated MEKs which locks it in a catalytically inactive form [155, 156]. 5Z-7-oxozaeanol is a fungal-derived product that inhibits TAK1. It acts by covalently binding and alkylating the TAK1-TAB1 complex, which results in an irreversible inhibition of TAK1 kinase activity [157, 158]. BI605906 is a novel inhibitor of IKKβ that can also partially inhibit the activity of IKKε/TBK1 in response to IL-1 stimulation [159]. Compound 1 (C1) is a potent and highly selective small molecule inhibitor of TPL2 kinase activity. The selectivity of C1 is potentially due to the unique structure of the ATP binding loop in TPL2, which contains a proline instead of a conserved glycine in this catalytic motif [160].

1.3.5 Non-TLR pattern recognition receptors

Several other PRRs are involved in the recognition of PAMPs. Such receptors include nucleotide oligomerization domain-like (NOD-like) receptors, c-type lectin

receptors, and RIG-I-like receptors. The study of these receptors is beyond the scope of this thesis, for further review refer to reference [86].

The previous sections covered some aspects of the pathophysiology of CF lung disease, with a focus on proinflammatory pathways activated in response to bacterial-derived products. The following sections focus on CF pulmonary exacerbations and their link to other inflammatory mediators, the damage-associated molecular patterns (DAMPs).

1.4 Cystic Fibrosis Pulmonary Exacerbations

Pulmonary exacerbations are acute changes in respiratory and systemic symptoms, such as increased cough and sputum production, dyspnea and decreased appetite. These episodes require immediate medical intervention and patients suffering from a CF exacerbation are often hospitalized to receive intravenous antibiotics. Studies have correlated CF exacerbations with increased mortality since the decline in pulmonary function that follows these episodes is often irreversible [29, 161, 162]. Furthermore, a recent cohort study revealed that the annual rate of pulmonary exacerbations is the strongest variable associated with risk of death [30]. Changes in bacterial composition are the main components implicated in the pathophysiology of pulmonary exacerbations. Studies have demonstrated that the concentration of *P. aeruginosa* is increased in patients with CF during an exacerbation and then decreased with appropriate antibiotic treatment [163]. It was once believed that the acquisition of a new genetic bacterial strain was responsible for the worsening of symptoms. However, prospective studies have demonstrated that, for the majority of adult patients suffering from CF, a new

exacerbation is not caused by an acquisition of a new strain of *P. aeruginosa* [164]. It has been suggested that viral superinfection liberates planktonic bacteria from biofilms and increases the inflammatory response in airway epithelial cells preinfected with mucoid *P. aeruginosa*, proposing a potential mechanism responsible for the increased virulence of a single bacterial strain [165].

1.4.1 Inflammatory mediators as markers of CF exacerbations

There are no standardized definitions and no specific criteria to diagnose CF exacerbations. Diagnosis is based on the patient's symptoms. Sputum and exhaled breath condensate biomarkers would be useful in the diagnosis and evaluation of therapeutic efficacy of CF exacerbations since they would represent non-invasive approaches to diagnosis and follow up. In the past, studies have reported increased levels of IL-8 and TNFα in both BALF and sputum samples from CF patients suffering from a pulmonary exacerbation [166, 167]. Recently, in an effort to associate the biomarker changes with clinical exacerbations and response to therapy, a multi-parametric biochip array was done in sputum and exhaled breath condensate of CF patients before, during and after the end of treatment. Several inflammatory mediators were found to be increased, including IL-4, IL-6, IL-8, IL-1α, IL-1β, VEGF and TNFα. Among these cytokines, VEGF and IL-1β positively correlated with the respiratory function before treatment and IL-4 and IL-8 negatively correlated with the respiratory function at 15 and 30 days from the start of therapy [168]. Although these mediators could be sensitive markers of pulmonary exacerbations, they might already be present in increased amounts in CF sputum during the chronic phase of the disease and are therefore not specific.

The production of pro-inflammatory cytokines as a consequence of acute or chronic infection in CFLD has been well characterized. However, tissue damage and destruction themselves can lead to the release of different families of mediators that can potentiate the inflammatory response. Among these mediators, High-mobility group box 1 (HMGB1) has been detected in increased amounts in sputum from CF patients during pulmonary exacerbations and in a transgenic mouse model of CF airway disease. In the same study, HMGB1 was found to be chemotactic for human neutrophils *in vitro* and was shown to increase collagen degradation *in vivo* [169]. HMGB1 belongs to the family of damage associated molecular patterns (DAMPs, also called alarmins), a group of molecules that has been recently identified to play important roles in chronic inflammatory diseases.

1.5 Damage Associated Molecular Patterns

Damage Associated Molecular Patterns (DAMPs) are a group of endogenous molecules that are released following tissue injury or cell death and that are capable of activating pro-inflammatory pathways through recognition by PRRs. Under physiological conditions, DAMPs are sequestered intracellularly and are therefore not recognized by the immune system. Following cell death, these molecules can be released extracellularly, triggering diverse inflammatory pathways. The type of cell death affects the ability of cells to release DAMPs. During apoptotic cell death, these molecules are trapped into apoptotic vesicles and phagocytosed by cells of the immune system. However, plasma membrane integrity is lost following necrotic cell death, allowing for the release of intracellular material into the extracellular milieu. High-mobility group box 1 (HMGB1), heat shock proteins (HSPs), purine metabolites, matrix fragments, IL-1α, IL-33 and S100

calcium-binding proteins are among these mediators [170]. IL-33 is of particular interest since it has been recently implicated in chronic inflammatory diseases such as rheumatoid arthritis and systemic lupus erythematosus and in airway diseases such as asthma and allergic rhinitis [171].

1.5.1 HMGB1

HMGBs are highly abundant non-histone DNA-binding proteins that can act as chaperones to regulate transcription, replication, DNA repair and genomic stability. HMGB1 was the first HMGB protein to be discovered and is ubiquitously expressed in most cell types [172]. Structurally, it contains two helical DNA-binding motifs, A and B boxes and an acidic tail with several glutamic and aspartic acid residues. Each of the DNA-binding boxes contains a nuclear localization sequence that is susceptible to acetylation [173]. In the nucleus, HMGB1 binds DNA and promotes the transcription of different target genes. HMGBs can also interact directly with several transcription factors through protein-protein interactions, enhancing their binding to DNA [174]. Acetylation of specific lysine residues leads to the migration of HMGB1 to the cytoplasm where it is packed in vesicles. These vesicles then fuse with the plasma membrane, leading to HMGB1 release [175]. HMGB1 can also be passively released following necrotic cell death. Extracellularly, HMGB1 can act as a cytokine or as a chemokine through its binding to different receptors. Some of the receptors that recognize extracellular HMGB1 are: the receptor for advanced glycation end products (RAGE), TLR2, TLR4, TLR9 and CXCR4 [176]. Importantly, the extracellular redox state of HMGB1 regulates its activity as a cytokine. Three conserved cysteine (cys) residues have to be in the reduced state for HMGB1 to bind and signal through TLR4 [177]. Additionally, all cys-reduced HMGB1

can also signal through CXCR4 to recruit neutrophils and monocytes to sites of infection [178]. In CF, HMGB1 sputum levels correlate with lung function decline and with the number of pulmonary exacerbations. Elevated levels of HMGB1 can predict the time to first pulmonary exacerbation, the number of future exacerbations and the time-to-lung transplantation or death [179].

1.5.2 S100 proteins

The S100 group of proteins is a family of calcium (Ca^{2+}) binding proteins that play diverse roles in cell metabolism, proliferation and differentiation. Structurally, they consist of a helix- Ca^{2+} binding loop-helix motif which is usually packed in pairs. These proteins are usually present as either homo or heterodimers. Intracellularly, S100 proteins act as Ca^{2+} sensors, increases in calcium induce conformational changes that lead to dimerization and favor interaction with target proteins. Additionally, S100 proteins have two metal binding sites at the dimer interface. S100A8 and S100A9 form the heterodimer called calprotectin that binds zinc (Zn^{2+}) and manganese (Mn^{2+}) with high affinity [180]. This ability to sequester metals confers calprotectin its antimicrobial properties during infection through a process called 'nutritional immunity' [181]. Calprotectin is highly expressed in neutrophils and monocytes and its expression can be induced in epithelial cells [182, 183]. Metal chelation by calprotectin reduces the activity of pathogen superoxide dismutase (SOD), increasing the susceptibility of pathogens to ROS produced by neutrophils [184]. Once in the extracellular space, calprotectin binds TLR4, ultimately leading to the translocation of NFκB and the production of TNFα [185]. Additionally, extracellular S100 proteins can bind RAGE in endothelial cells, activating NFκB and the subsequent production of adhesion molecules [186]. Calprotectin levels increase in

sputum and serum from CF patients during exacerbations and decrease following successful treatment [187]. Furthermore, a recent study demonstrated that calprotectin levels can be used as predictors of lung function decline in CF patients [188].

1.5.3 IL-1α

IL-1α is a well characterized member of the IL-1 family of cytokines. The gene coding for IL-1α is found in the long arm of chromosome 2. It codes for a 271 aa precursor with a molecular weight of 33 kDa. IL-1α is constitutively expressed by most non-hematopoietic cell types and is mainly present in the nucleus during resting conditions. This is mediated by the nuclear localization signal present on its N-terminal domain from aa 79 to 86. [189, 190]. The N-terminal domain of IL-1α is also responsible for its association with several components of the RNA splicing and processing pathways. In tumor cells, this interaction with the spliceosome leads to the induction of apoptosis [191]. Another role for the nuclear localization of IL-1α is the transcriptional activation of pro-inflammatory cytokine genes such as IL-6 and IL-8 [192]. Unlike most typical cytokines, IL-1α lacks a signal peptide for secretion through the ER-Golgi pathway. Instead, IL-1α is preferentially released after necrotic cell death. The type of cell death is a key player in the regulation of IL-1α release. Under apoptotic conditions, IL-1α is tightly bound to chromatin, thus preventing its exit from the cell and its actions on the surface of immune cells. On the other hand, necrotic cell death allows for the release of IL-1α to the extracellular space and recruits inflammatory cells to the site of injury [193, 194]. IL-1α binds the MyD88-dependent IL-1 receptor I (IL-1RI) to elicit the inflammatory response. In mice subjected to tissue injury, the interaction of IL-1α with its receptor results in potent neutrophil recruitment [195]. CF patients have significantly

higher levels of serum IL-1α and these levels decrease in response to treatment with anti-inflammatory agents. Moreover, the reduction of IL-1α in serum of CF patients correlates with an improvement of lung function [196, 197].

1.5.4 IL-33

IL-33 is a recently discovered member of the IL-1 family of cytokines. It was first identified in 1999, in a search for upregulated genes in vasospastic cerebral arteries in dogs. In this study, cDNA expression array and mRNA differential display found a previously non-identified gene to be strongly upregulated following subarachnoid haemorrhage, called DVS27 at the time. The full cDNA sequence of DVS27 was obtained by screening a cDNA library and it was found to encode a protein of 263 amino acids with an approximate molecular weight of 30.18 kD. No match for the open reading frame of this sequence was found when searching in different protein databases. The sequence was therefore cloned in an expression vector and transiently transfected into COS-7 cells. The protein was mainly found in the nucleus and in the perinuclear membrane of this monkey kidney cell line. Finally, it was found that DVS27 mRNA was upregulated in human endothelial cells in response to stimulation with IL-1α and IL-1β [198].

The next description of IL-33 was in 2003, where suppression subtractive hybridization (SSH) was used to identify genes preferentially expressed in high endothelial venules endothelial cells (HEVECs). Screening of a cDNA library comparing HEVECs with polyp-derived microvascular endothelial cells (PMECs) yielded a highly upregulated cDNA, which, through *in situ* mRNA hybridization, was found to be

localized in high endothelial venules (HEVs) of tonsils, Peyer's patches and mesenteric lymph nodes. This gene, which we now know encodes for IL-33, was then designated NF-HEV [199].

It was only in 2005 that IL-33 was characterized as a member of the IL-1 family of cytokines. This was done by using a computational approach in which sequence databases were screened to identify distant IL-1 and FGF-like proteins. This search matched the sequence of dog DVS-27 to IL-1/FGF β trefoil fold templates. This new IL-1 family member was then named IL-33. A screening of human cDNA libraries by qRT-PCR revealed constitutive IL-33 expression in human smooth muscle cells of different tissues as well as bronchial and small airway epithelial cells. In primary lung or dermal fibroblasts and keratinocytes, IL-33 expression was induced following incubation of cells with IL-1β and TNFα. It was then discovered that IL-33 binds and signals through the IL-1 receptor ST2, leading to the recruitment of MyD88, IRAK and TRAF6 with the subsequent activation of NFκB and MAPKs p38, ERK1/2 and JNK. Additionally, IL-33 induced the production of IL-5 and IL-13 in polarized T_H2 cells and intraperitoneal injection of mice with IL-33 increased eosinophils, mononuclear cells and plasma cells in the spleen [200].

1.5.4.1 IL-33 structure

In order to get an insight into the function of IL-33, it is important to understand its structure. The gene coding for human IL-33 is located in the short arm of chromosome 9 at 9p24.1. There are three recognized mRNA transcript variants in the NCBI nucleotide database (reference sequences NM_001199641.1, NM_001199640.1 and NM_033439.3)

with a length of 2.340, 2.592 and 2.718 Kbp respectively (www.ncbi.nlm.nih.gov). Interestingly, one study reported 7 different IL-33 splice variants which included variants lacking a single exon (Δ3, Δ4 or Δ5) or variants that lack more than one exon (Δ3-4, Δ4-5, Δ3-5). These variants show differential expression in non-transformed and transformed cells [201]. The longest IL-33 transcript variant contains 8 exons and codes for a 270 amino acid protein (reference sequence NP_254274.1, www.ncbi.nlm.nih.gov).

Similarly to other IL-1 family members, IL-33 has a core β trefoil fold composed of 12 β strands, as shown by NMR spectroscopy. A β4-β5 flexible linker loop is distinct from the structure of the other IL-1 family members [202]. In its N-terminal domain, from amino acids 28 to 68, IL-33 contains a sequence with a low level of homology with the prokaryotic helix-turn-helix (HTH) domain. This homeodomain-like HTH motif is sufficient and necessary for IL-33's nuclear localization and for its association with heterochromatin and mitotic chromosomes. Additionally, this N-terminal motif is thought to mediate IL-33's transcriptional repressor activity [199, 203].

In a later study, deletion mutagenesis experiments demonstrated the presence of a chromatin binding motif (CBM) within the HTH domain, comprising amino acids 40-58. Six of these residues are essential for chromatin binding: M45, L47, R48, S49, G50 and I53. Furthermore, this CBM is conserved between murine and canine IL-33 orthologues and shares homology with the N-terminal CBM of the latency-associated nuclear antigen (LANA) of the Kaposi sarcoma herpervirus (KSHV). Indeed, residues 44-53 of the CBM are responsible for docking of both KSHV and IL-33 to an acidic pocket formed by histones 2A and 2B [204].

In addition to mediating its nuclear localization, the N-terminal domain of IL-33, from residues 66 to 109, binds to NFκB subunits p50 and p65. The binding of IL-33 to p50 is constitutive, both in the nucleus and cytoplasm of cells, whereas its association with p65 in both cell compartments is enhanced by stimulating the cells with rhIL-1β. The latter association leads to the repression of NFκB transactivation and to a decreased expression of the NFκB target genes *IκBα*, *TNFα* and *C-REL* [205].

The C-terminal portion of IL-33 is mainly responsible for its association with the receptor ST2. IL-33 binds to ST2 with high affinity, with a K_D of 4nM. This association is complex and involves a large interface between both proteins [202]. It was initially thought that, similarly to other IL-1 family members, IL-33 requires cleavage by caspase 1 to produce the mature form of the cytokine [200]. Early studies confirmed that *in vitro* translated IL-33 can indeed be cleaved by caspases 1 and 3 to produce 20-22 kDa products. The consensus caspase 3 cleavage site is present in the C-terminal portion, specifically in Asp_{178}. However, caspase cleavage is not necessary to render IL-33 active since full-length IL-33_{1-270} as well as the IL-1-like domain IL-$33_{112-270}$ are able to signal through ST2 and induce NFκB transactivation [206].

Further studies demonstrated that physiological concentrations of caspase 1 fail to efficiently cleave IL-33. Apoptotic caspases 3 and 7 efficiently process IL-33 at physiological concentrations. This study confirmed the consensus caspase cleavage site to be located at Asp_{178} since point mutations at this residue abrogate cleavage from caspases 3 and 7. The cleaved form of the cytokine exhibits decreased activity *in vivo*, suggesting that cleavage by apoptotic caspases decreases the activity of IL-33 as a cytokine [207].

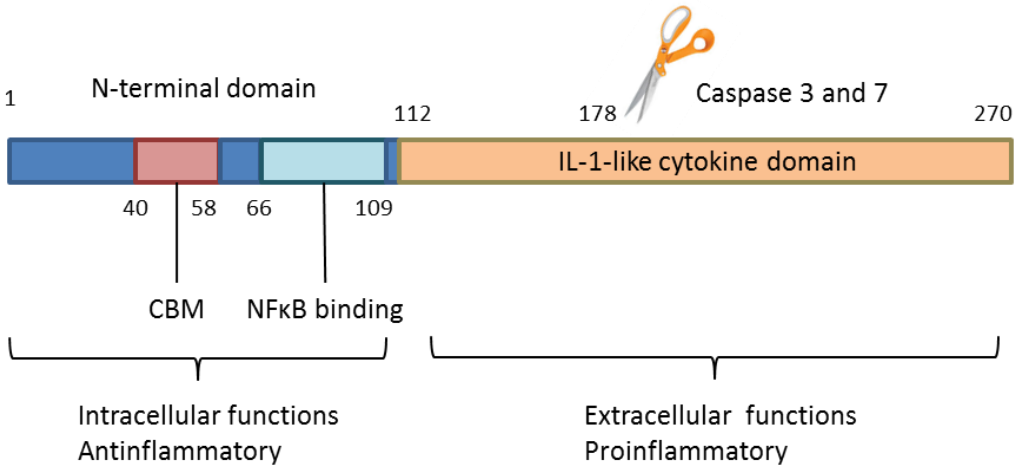


Figure 1.6 : IL-33 structure. IL-33 is a 270 amino acid protein. The N-terminal domain, from residues 40 to 58, contains a chromatin binding motif (CBM) that shares structural homology with the latency-associated nuclear antigen (LANA) of the Kaposi sarcoma herpes virus (KSHV). This domain is responsible for IL-33's association to the acidic pocket formed by histones 2A and 2B. Additionally, the N-terminal domain, from residues 66 to 109, contains a sequence that allows the binding of IL-33 to the p50 and p65 subunits of NFκB. The C-terminal domain shares homology with other IL-1 family members and is responsible for the functions of IL-33 as a cytokine. The Asp_{178} residue is a caspase 3 and 7 cleavage site.

1.5.4.2 IL-33 localization

Studies using fluorescently-tagged IL-33 report its localization to the nucleus of structural cells. This has been shown to be mediated through the CBM, as described in the previous section [204]. However, IL-33's subcellular localization might be cell-type and context dependent. A study reported that endogenous IL-33 is localized in both the nucleus and the cytoplasm of fibroblasts and coronary endothelial cells. This detection was done using a monoclonal antibody against IL-33. Additionally, using a small four-cysteine epitope that can be detected by fluorescent antibodies, IL-33's subcellular localization was mapped to euchromatin in the nucleus and within membrane-bound

vesicles in the cytoplasm by electron microscopy. This is in contrast with previous findings that had demonstrated nuclear IL-33's association to heterochromatin [208].

Also, different IL-33 transcript variants have been reported and this might have an effect on its subcellular localization. Furthermore, different stimuli can alter IL-33's subcellular localization since it was indeed demonstrated that stimulation of airway epithelial cells with the fungus *Alternaria* and Poly I:C induce cytoplasmic IL-33 [209].

1.5.4.3 Signals regulating IL-33 expression

The transcriptional regulation of IL-33 is highly dependent on the cell type and level of differentiation. IL-33 is constitutively expressed in epithelial and endothelial cells of diverse tissues. Pro-inflammatory stimuli such as IL1-α and IL-1β can further increase IL-33 expression in endothelial cells [198]. Similarly, TNFα and IL-1β induce IL-33 expression in human colonic subepithelial myofibroblasts. This induction is mediated through ERK1/2 MAPKs and NFκB [210]. Stimulation of human keratinocytes with IFN-γ increases IL-33 through ERK1/2, p38 and EGFR phosphorylation [211]. Immune cells such as macrophages, express very low levels of IL-33 this expression can be significantly increased by pro-inflammatory stimuli. It has been shown that TLR3 and TLR4 agonists induce IL-33 expression. Furthermore, IL-33 induction in response to the TLR3 agonist Poly(I:C) is dependent on TBK1 and IRF3 in murine peritoneal macrophages [212]. IgE stimulation of mast cells induces IL-33 mRNA upregulation through calcium signaling [213].

1.5.4.4 IL-33 intracellular functions

As mentioned in the 'IL-33 structure' section, IL-33 binds heterochromatin and is potentially involved in transcriptional repression [203]. Additionally, IL-33 contains an NFκB binding motif in its terminal domain, which prevents NFκB transactivation activity [205]. This is supported by the fact that IL-33 knockdown increases the induction of IL-8 by TNFα in human keratinocytes [211]. However, these results contrast with another study that demonstrated that IL-33 enhances the expression of the NFκB target genes ICAM1/VCAM1. This is potentially mediated through the binding of IL-33 to the NFκB p65 promoter region, which in turn enhances NFκB expression [214]. More recently, it was demonstrated that nuclear IL-33 negatively regulates the expression of the NFκB target genes IL-6 and RANTES, as well as the expression of its negative regulator sST2. This is mediated through the interaction of IL-33 with transcriptional repressor proteins [215].

1.5.4.5 IL-33 release

The fact that IL-33 lacks a conventional leader sequence suggests that IL-33 is not secreted through the typical ER-Golgi pathway. Instead, full-length IL-33 is released upon necrotic cell death. Indeed a study demonstrated that cell scraping, repeated cycles of freezing and thawing and treatment with non-ionic detergents release IL-33 to the extracellular space [206]. In this context, IL-33, similarly to HMGB1 and IL-1α, behaves as a prototypical alarmin. On the other hand, certain stimuli can lead to an active secretion of IL-33. It has been demonstrated that the fungus *Alternaria alternata* can induce a rapid release of IL-33 from primary bronchial airway epithelial cells. This is mediated through an increase in extracellular ATP and its action through the $P2Y_2$

purinergic receptors. Binding of ATP to $P2Y_2$ increases intracellular calcium, which induces IL-33 cytoplasmic localization and subsequent release. This release is independent from necrotic cell death, since extracellular LDH levels from cells stimulated with *Alternaria* remained unchanged [209]. However, the exact mechanisms of IL-33 release in this context have to be further characterized. In fibroblasts, mechanical stretch induces IL-33 shuttling from the nucleus to the cytoplasm, followed by IL-33 release that is ATP-dependent [208]. Similarly, IL-33 is released from progenitor bronchial epithelial cells following ATP stimulation [216].

1.5.4.6 IL-33 extracellular functions

Once in the extracellular space, IL-33 binds to the heterodimeric receptor complex consisting on the IL-1 receptor-related protein ST2L (IL-1R4) and the IL-1R accessory protein (IL-1RAcP) [217]. The activation of the ST2L/IL-1RAcp heterodimer results in pathways leading to the activation of NF-κB and MAPKs. This receptor complex is expressed on diverse immune cell types, including basophils, eosinophils, mast cells, T helper (Th)2 lymphocytes, macrophages, dendritic cells, natural killer cells and neutrophils. In mastocytes, the interaction of IL-33 with its receptor results in the production of the Th2 cytokines IL-4, IL-5 and IL-6. In macrophages it induces polarization and induction of the inflammatory phenotype. When in contact with dendritic cells, IL-33 induces the production of IL-6 and the expression of several surface molecules that guide cells to the site of injury [218]. Studies using recombinant IL-33 show that signaling through the ST2 receptor results in Th2 cell polarization, with the subsequent production of IL-5 and IL-13. Activation of eosinophils by IL-33 leads to degranulation and to the release of IL-5 [219-221]. Of relevance to our study, IL-33

potently increased neutrophil migration to the site of infection, significantly reducing the bacterial burden in an *in vivo* model of systemic inflammation. Furthermore, IL-33 played a key role in stabilizing CXCR2 expression in the context of infection. As mentioned in a previous section, CXCR2 is a key receptor required for neutrophil chemoattraction to sites of infection [222]. Neutrophils themselves can also potentiate the actions of IL-33. Full-length IL-33 is a substrate for neutrophil serine proteases, which cleave IL-33 N-terminal domain, generating IL-$33_{95\text{-}270}$, IL-$33_{99\text{-}270}$, IL-$33_{109\text{-}270}$. These products are not only biologically active, but show a 10-fold increased activity in inducing cytokine production by basophils and mast cells when compared to the full length IL-$33_{1\text{-}270}$ protein [223].

Alternative splicing of the ST2 gene can give rise to soluble ST2 (sST2). This isoform of the IL-33 receptor complex lacks the transmembrane and TIR domains and acts as an extracellular decoy receptor for IL-33. The presence of sST2 decreases NFκB activation by IL-33 and hampers the subsequent production of proinflammatory cytokines [224].

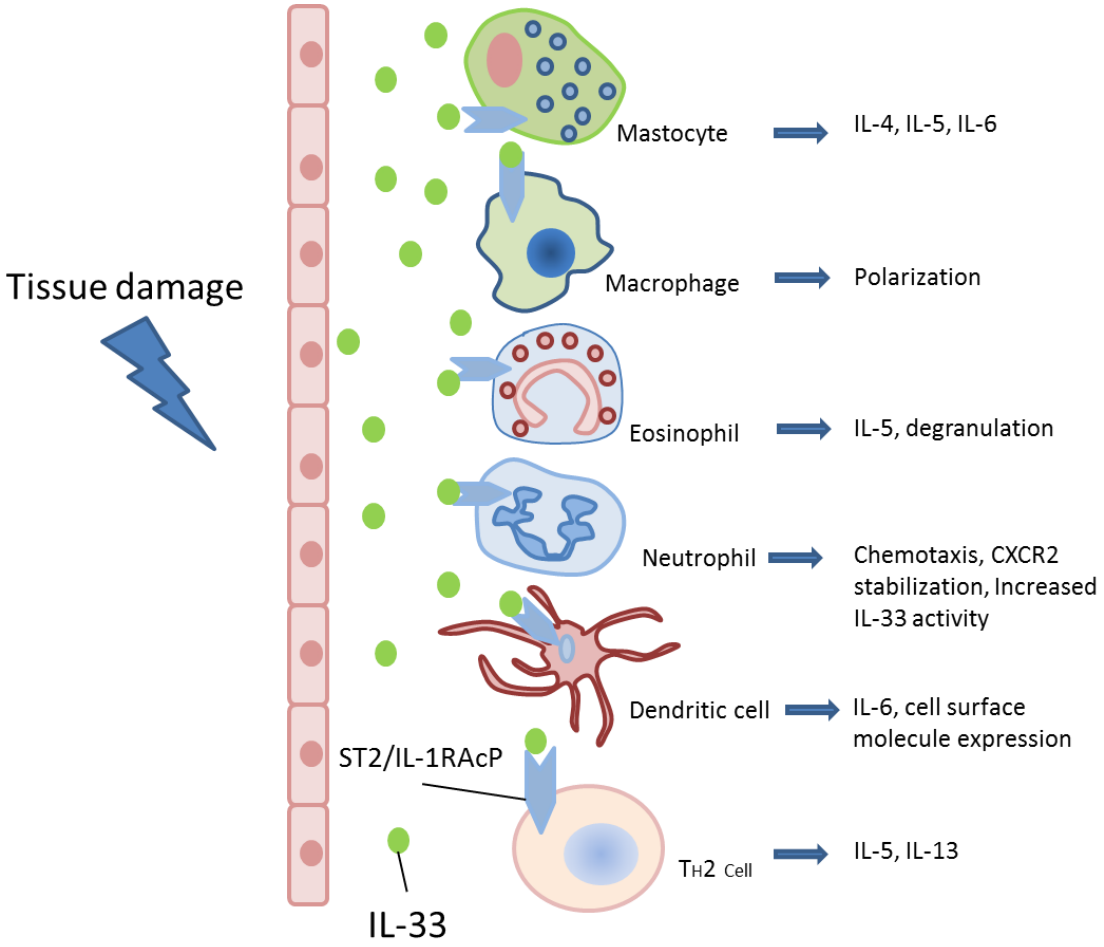


Figure 1.7: Extracellular functions of IL-33. Following tissue damage, IL-33 is released to the extracellular space and binds the ST2/IL-1RAcP heterodimer in immune cells. This leads to release of IL-4, IL-5 and IL-6 by mastocytes; macrophage polarization; eosinophil degranulation and release of IL-5; neutrophil chemoattraction, stabilization of CXCR2 in neutrophil cell surface and release of elastases that increase IL-33 activity; release of IL-6 by dendritic cells and increased expression of key cell surface molecules for antigen presentation and release of IL-5 and IL-13 by T_H2 cells.

1.5.4.7 IL-33 and disease

Given its role as such a potent recruiter and activator of cells of the immune system, IL-33 has been implicated in several inflammatory diseases. Of relevance to our study is the implication of IL-33 in lung diseases, namely asthma, Chronic Obstructive Pulmonary Disease (COPD) and CF. In lung tissue samples from asthmatic patients, IL-

33 protein expression is widespread throughout the epithelium. This is in contrast with samples from non-asthmatics, in which IL-33 expression is mainly limited to the nuclei and cytoplasm of basal cells. Furthermore, IL-33 mRNA expression levels are increased in lung tissue samples from asthmatic patients when compared to non-asthmatic controls [225]. In addition to its increased expression in airway epithelial cells, IL-33 mRNA is increased in airway smooth muscle cells obtained from biopsies of asthmatic subjects [226]. Administration of a neutralizing antibody against IL-33 reduces lung inflammation and mucus hypersecretion [227]. IL-33 levels are also increased in bronchial epithelial cells from COPD patients. Exposure of airway epithelial cells to cigarette smoke increases IL-33 expression in both, peripheral blood lymphocytes and bronchial epithelial cells. Additionally, IL-33 levels are elevated in the serum of COPD patients when compared to controls [228]. Interestingly, IL-33 expression in airway epithelial sections from COPD patients is preferentially increased in basal progenitor cells [216].

Two studies have described the presence of IL-33 in CF, one of them was published by our group in 2013 and will be further discussed in this thesis [229]. A more recent study described significantly higher levels of IL-33 protein in bronchoalveolar lavage (BAL) fluid from stable CF patients compared to non-CF controls. Moreover, this study reported increased IL-33 mRNA expression in both, parenchymal and bronchial epithelial tissue from CF patients [230].

In contrast to its potentially damaging roles in lung disease, extracellular IL-33 is cardioprotective since administration of exogenous IL-33 prevented cardiac hypertrophy and fibrosis in a mouse model of aortic constriction. The decoy receptor sST2 blocked the antihypertrophic effects of IL-33, demonstrating that the cardioprotective effects of

IL-33 are mediated through its actions of ST2L [231]. The presence of extracellular IL-33 is also beneficial in the context of cardiac injury since administration of exogenous IL-33 reduced cardiomyocyte apoptosis in a rat model of ischemia [232]. In addition to its actions in cardiomyocytes, IL-33 also exerts protective effects in the vasculature since administration of IL-33 prevented the formation of atherosclerotic plaque in a mouse model. This effect is mediated through the IL-33-induced Th1 to Th2 switch [233].

1.6 Rationale

During the course of CF, chronic bacterial colonization leads to the release of inflammatory mediators from airway epithelial cells. This, in turn, attracts cells from the innate immune system in an effort to clear the infection. However, this response is excessive and yet inefficient, resulting in an improper clearance of bacteria from the lungs. At the same time, the constant release of proteases such as neutrophil elastase leads to lung tissue damage. Tissue damage itself can lead to the release of Damage Associated Molecular Patterns (DAMPs), which potentiate the recruitment of more inflammatory cells, creating a vicious circle of non-resolving inflammation. IL-33, a recently characterized DAMP, is constitutively expressed in epithelial and endothelial cells. Two studies, including one from our group, have described the increased presence of IL-33 in both, lung tissue and bronchoalveolar lavage fluid from CF patients. Several pieces of evidence support the function of IL-33 as a potent neutrophil recruiter to sites of infection, it is therefore relevant to determine its role in CF lung disease.

1.7 Hypothesis

Our central hypothesis is that decreasing IL-33 levels will decrease inflammation in CFLD.

1.8 Objectives

1) To study the expression profile of IL-33 in CF and non-CF airway epithelial cells in the context of chronic infection as well as during acute bacterial exacerbations.

2) To identify the key signaling pathways involved in up-regulation of IL-33 in response to an acute infection with *P. aeruginosa.*

3) To study the role of IL-33 in our model of CF lung disease.

Chapter 2: Materials and Methods

2.1 Materials

Lung epithelial cell lines of normal phenotype (UNC N1, N3) or expressing the CF mutations G542X or CFTRΔF508 (UNC CF7 and CF2 respectively), were kindly provided by Dr. Scott Randell (The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA). NuLi (normal lung) and CuFi (cystic fibrosis, homozygous for the CFTRΔF508 mutation) cells were kindly provided by Dr. Emanuelle Brochiero (Centre de Recherche, Hôtel-Dieu du CHUM, Université de Montréal, Montreal, QC, Canada). Normal human bronchial epithelial cells (NHBE) were kindly provided by Dr. Qutayba Hamid (Meakins Christie Laboratories, McGill University Health Centre, Montreal, QC, Canada). The human acute monocytic leukemia cells, THP-1 were obtained from Dr. Maziar Divangahi (Meakins Christie Laboratories, McGill University Health Centre, Montreal, QC, Canada). HEK293 cells were obtained from Dr. Elizabeth Fixman (Meakins Christie Laboratories, McGill University Health Centre, Montreal, QC, Canada). Immortalized human bronchial epithelial Beas-2B cells were purchased from ATCC (Manassas, VA, USA).

DMEM (Dulbecco's Modified Eagle Medium: 4.5 g/L D-glucose, (+) L-glutamine, 110 mg/L sodium pyruvate), RPMI 1640, 10,000 Units/mL Penicillin G + 10,000 μg/mL Streptomycin, 0.25% trypsin-0.04% EDTA, HEPES, DPBS (Dulbecco's Phosphate Buffered Saline), qualified foetal bovine serum (FBS) were obtained from Invitrogen, (Carlsbad, CA, USA). CnT-BM.1 and A,B and C supplements (CnT-17.S) were purchased from CELLnTEC (Bern, Switzerland).

5Z-7-oxozeaenol, BI605906, BIRB0796 and C1 were kindly provided by Professor Sir Philip Cohen (MRC PPU, University of Dundee, UK). PD184352 was

bought from USBiological (Swampscott, MA, USA). The CFTR potentiator VX-770 and the CFTR corrector VX-809 were kindly provided by Dr. Emanuelle Brochiero (Centre de Recherche, Hôtel-Dieu du CHUM, Université de Montréal, Montreal, QC, Canada). Flagellin, Pam3CSK4, Poly(I:C), LPS, ODN2006, the polyclonal antibodies against TLR2, TLR4 and TLR5 and the TLR9 antagonist ODNTTAGGG were purchased from InvivoGen (San Diego, CA, USA). Recombinant human IL-1β was purchased from R&D systems (Minneapolis, MN, USA). Nuclear/cytoplasmic extract kit was purchased from Active Motif (Carlsbad, CA, USA, cat# 40010). TNFα was purchased from Enzo Life Sciences (Plymouth Meeting PA, USA) and homoserine lactone from Cayman Chemical (Ann Arbor, MI, USA)

2.2 Methods

2.2.1 Cell culture

NuLi, CuFi, N1, N3, CF7, CF2 and NHBE cells were cultured in CnT-17.S. Cells were plated in 55 cm^2 culture dishes (Sarstedt, Newton, NJ, USA) that had been previously coated as follows: for NuLi and CuFi cells, culture dishes were covered with a solution of collagen (Sigma-Aldrich Type VI Cat#C7521) to a concentration of 60µg/mL for 24 hours, collagen was then aspirated and dishes were dried for 15 minutes under the cell culture hood. For N3, CF7 and CF2 cells, culture dishes were covered with PureCol (Advanced BioMatrix, San Diego, California, USA) to a concentration of 40µg/mL for 24 hours, the solution was aspirated and dishes were dried for 15 minutes under the cell culture hood. All cells were maintained at 37°C in 5 % CO_2, 100% humidity. The medium was changed every 48 to 72 hours until cells reached 100% confluence. Cells were then washed with DPBS and detached with 3 mL of 0.25% trypsin-0.04% EDTA

solution per culture dish for 5 to 10 minutes at 37°C. The previous solution was then collected and resuspended on 7 mL of medium and cells were precipitated by centrifugation for 5 minutes at 700 x *g*. The medium was aspirated, the pellet resuspended and cells counted using a standard haemocytometer. Cells were subcultured on either 12-well (BD Falcon, Franklin Lakes, NJ, USA) or 24-well (Sarstedt, Newton, NJ, USA) pre-coated plates, to a density of 50,000 cells/cm^2. Cells were grown to 100% confluence for Flow cytometry, ELISA and RNA extraction experiments. For immunofluorescence microscopy experiments, sterile microscope glass cover slips (Fisherbrand cat# 12-545-83) were placed on either 12 or 24-well plates and coated with collagen as previously described. Cells were plated at a density of 50,000 cells/cm^2 and grown to 90 to 100% confluence.

Beas-2B cells were cultured in DMEM supplemented with 10% FBS and 100 U/mL of penicillin G, 100 μg/mL of streptomycin (complete DMEM). Cells were maintained in 10 cm petri dishes at 37°C, 5% CO_2, 100% humidity. Medium was changed every 72 hours until cells reached 90% confluence. Cells were then washed with DPBS and incubated with Trypsin 0.25%EDTA at 37°C for 5 minutes. Cells were then resuspended in complete DMEM and harvested by centrifugation at 700 x*g* for 5 minutes. The supernatant was aspirated and cells were resuspended in complete DMEM to reach a cell density of 2 x 10^5 cells/mL. Cells were then plated in 12 well plates and transfected as described in the next section.

THP-1 cells were cultured in RPMI 1640 supplemented with 10% FBS, 2mM L-glutamine and 10mM HEPES. Cells were maintained at 37°C in 5 % CO_2, 100% humidity and kept at a density between 1 x 10^5 and 8 x 10^5. Cells were subcultured when

the cell density reached 9 x 10^5 cells/mL. For subculturing, cells were precipitated by centrifugation at 1,500 rpm for 5 minutes and resuspended in complete RPMI 1640 to attain a final density of 5 x 10^5 cells/mL. Cells were transferred to 12 well plates and were incubated in the presence of different agonists or inhibitors before RNA or protein extraction. To induce differentiation, 5 x10^5 cells/mL were plated in 12 well plates and incubated with PMA to a final concentration of 100 nM for 72 hours before exposure to different agonists or inhibitors. Alternatively, cells were incubated in the presence of PMA in 48 well plates containing sterile microscope cover glass slips (Fisher) precoated with collagen as described above.

2.2.2 Cloning, transient cell transfection and creation of stable cell lines

2.2.2.1 Beas-2B cell stably expressing an NFκB reporter in PGL4.28

Four repeats of the NFκB-response consensus sequence ggggactttcc (System Biosciences, Mountain View, CA, USA) were cloned into the plasmid vector PGL4.28 (Promega, Madison, WI, USA) at the XhoI-BglII sites, upstream the promoter of the luciferase reporter gene *luc2CP*. DH5α competent bacteria were transformed with the resulting plasmid and multiple copies of the plasmid were purified by maxi prep (Invitrogen, Burlington, Ontario). The airway epithelial cell line B2B was transfected with the NFκB luciferase construct and cells expressing the construct were selected with the mammalian antibiotic hygromycin B (Wisent BioProducts, St. Bruno, QC). Single clones were isolated by cell sorting and the stable expression of the plasmid was maintained by culturing the cells in the presence of 200μg/mL of hygromicin.

2.2.2.2 Transient transfection of B2B NFκB cells

Monoclonal Beas-2B NFκB cells were plated into 12-well collagen-coated plates (Purecol, Advanced BioMatrix) at a density of 25,000 cells/cm^2 and grown overnight at 37°C, 5% CO_2, 100% humidity. 1μg of either FL IL-33 or R48 IL-33 in PCMV4a or empty vector was diluted in 100μL of DMEM and 2μL of the TurboFect transfection reagent (Thermo Fisher Scientific) was added and mixed for 5 seconds. The mix was added to each well and incubated for 6 hours; media was then replaced with DMEM supplemented with 1%FBS and cells were further incubated overnight. Cells were then stimulated with flagellin for 3 hours and RNA or proteins were extracted as described in the sections below.

2.2.2.3 Cloning of full length IL-33 and R48 IL-33 in PCMV4a

An arginine to alanine mutation at residue 48 of the IL-33 sequence was created by site directed mutagenesis as previously described [204]. Full length (FL) IL-33 and R48 IL-33 were amplified by PCR from pcDNA3.1 vectors containing the inserts with the following primers: IL-33pcmv eco3'_2 (AAGAATTCAGTTTCAGAGAGCTTAAACA) and IL-33pcmv bam5' (TTGGATCCGCCACCATGAAGCCTAAAATGAAGTA). PCR products were separated by electrophoresis on a 1% agarose gel and purified using the PureLink Quick Gel Extraction Kit (Invitrogen cat K2100-12). Clones were then digested with the restriction enzymes EcoRI and BamH1 (FastDigest, Thermo Scientific) and inserted into PCMV4a (Promega, Madison, WI, USA). DH5α competent bacteria were transformed with the empty vector or with the plasmids containing the FL IL-33 and IL-33 R48

inserts, grown in the presence of ampicilin and several copies of the plasmids were purified by maxi prep (Invitrogen, Burlington, Ontario) and sequenced (Genome Quebec).

2.2.2.4 Generation of Beas-2B cells stably expressing FL IL-33 or R48 IL-33 in PCMV4a

Beas-2B cells were subcultured in collagen-coated (Purecol, Advanced BioMatrix) 6-well plates (Corning) to a density of 25,000 cells/cm^2. 2µg of either FL IL-33 or R48 IL-33 in PCMV4a or empty vector was diluted in 100µL of DMEM and 4µL of the TurboFect transfection reagent (Thermo Fisher Scientific) was added and mixed for 5 seconds. The mix was added to each well and incubated for 6 hours, media was then replaced with complete DMEM and cells were incubated overnight at 37°C, 5% CO_2, 100% humidity. Hygromycin was then added to a final concentration of 400µg/mL for selection of cells expressing the constructs of interest. Media containing 400 µg/mL of hygromycin was replaced every 48 hours and cell viability was assessed using trypan blue staining. Viable cells were maintained in complete DMEM with 200µg/mL of hygromycin and subcultured to a density of 50,000 cells/mL in 12-well plates (Corning) and stimulated with flagellin or infected with *P. aeruginosa* for RNA extraction and qRT-PCR analysis as described in the sections below. For immunofluorescence microscopy, cells were plated into 12-well plates containing sterile, Purecol-coated glass cover slips (Fisher Scientific).

2.2.3 *Pseudomonas aeruginosa* diffusible material and biofilm preparation

Synthetic cystic fibrosis medium (SCFM), a mixture of amino acids, glucose and lactate that mimics the nutritional composition of CF sputum was prepared as previously described [234]. Amino acids were added from 100-mM stocks to a buffered base (6.5 mL 0.2 M NaH_2PO_4, 6.25 mL 0.2 M Na_2HPO_4, 0.348 mL 1 M KNO_3, 0.122 g NH_4Cl, 1.114 g KCl, 3.03 g NaCl, 10 mM MOPS, 779.6 mL deionized water) in the following volumes: l-aspartate, 8.27 mL; l-threonine, 10.72 mL; l-serine, 14.46 mL; l-glutamate·HCl, 15.49 mL; l-proline, 16.61 mL; l-glycine, 12.03 mL; l-alanine, 17.8 mL; l-cysteine·HCl, 1.6 mL; l-valine, 11.17 mL; l-methionine, 6.33 mL; l-isoleucine, 11.2 mL; l-leucine, 16.09 mL; l-tyrosine, 8.02 mL; l-phenylalanine, 5.3 mL; l-ornithine·HCl, 6.76 mL; l-lysine·HCl, 21.28 mL; l-histidine·HCl, 5.19 mL; l-tryptophan, 0.13 mL; and l-arginine·HCl, 3.06 mL. The solution was adjusted to pH 6.8 and filtered through a 0.22µm nitrocellulose membrane (Millipore). Then, the following sterile components were added per liter: 1.754 mL 1 M $CaCl_2$, 0.606 mL 1 M $MgCl_2$, and 1 mL 3.6 mM $FeSO_4 \cdot 7H_2O$.

P. aeruginosa diffusible material (PsaDM) was prepared from the late stationary phase Psa (mucoid strain 508) isolated from sputum of a patient with CF (Hôpital Sainte-Justine, Montréal). Bacteria were grown in peptone (Fisher Scientific) or synthetic CF medium SCFM for 72 hours at 37˚C with shaking at 250 rpm. Bacteria were precipitated by centrifugation at 5,500 x *g* at 4˚C for 30 minutes, the supernatant was filtered through 0.22µM nitrocellulose membranes (Millipore), aliquoted and stored at -20˚C. Diffusible material from static biofilms (Biofilm PsaDM) was prepared from bacteria grown in peptone or SCFM as follows: ~$5x10^7$ log phase cells were used to seed each 9.4 cm^2

polystyrene tissue culture wells (Becton Dickinson). After three hours of initial attachment, the media was removed, replaced with fresh media, and the attached bacteria were incubated statically at 37˚C for an additional 24 hours. After this time, the attached cells were scraped off of the plate and were combined with the cell suspension within the well. Bacteria were centrifuged at 2100 x *g* for 30 minutes and the supernatant was collected and filtered through a 0.22µm nitrocellulose membrane. Total protein content of filtrates was measured by the Bradford method.

2.2.4 Immunofluorescence

Following exposure to agonists and or inhibitors, cells were washed twice with DPBS and fixed by addition of 250 µL of 2.5% formaldehyde in DPBS for 20 minutes. The previous solution was then removed and cells were washed twice with DPBS and incubated with 5 µg/mL of Hoescht 33258 (bis-benzamide) (Sigma cat# B2883) for 5 minutes in darkness for nuclear staining. Cells were washed twice with DPBS, chamber walls removed and the slide was mounted using Permafluor aqueous mounting media (Thermo scientific).

NuLi, CuFi, N3, CF7, THP-1 and Beas-2B cells were plated on pre-coated glass cover slips as described in the cell culture section. Following treatment, supernatants were collected and frozen at -20˚C and individual cover slips were placed in a 12-well plate. Next, cells were washed twice with DPBS and fixed by addition of 2.5% formaldehyde in DPBS for 20 minutes. A low concentration of formaldehyde was used to decrease non-specific background fluorescence. The previous solution was removed and cells were washed twice with DPBS and permeabilized by the addition of 500µL of 0.1%

saponin in DPBS for 15 minutes. Then, a blocking solution of 5% BSA 0.1% saponin was added to prevent non-specific binding. The primary monoclonal mouse antibody to human IL-33 (Nessy-1, Enzo Life Sciences cat# ALX-804-840) was diluted in a solution of 1% BSA 0.1% saponin in DPBS to a concentration of 10μg/mL. The blocking solution was removed and the area around the cover slip was aspirated thoroughly so that no traces of solution were left. 50μL of the primary antibody were added onto each cover glass for an incubation period of 90 minutes. Cells were washed 6 times for three minutes with 1% BSA, 0.1% saponin, 0.05% tween in DPBS, wells aspirated as previously described and 50 μL of secondary antibodies diluted in 1% BSA 0.1% saponin 0.05% tween in DPBS were added as follows: NuLi, CuFi, THP-1 and Beas-2B cells were incubated with 2.6 μg/mL of Alexa Fluor 555 goat anti-mouse IgG (Invitrogen cat# 21424) and N3 and CF7 cells were incubated with a 4 μg/mL of Alexa Fluor 488 goat anti-mouse IgG (Invitrogen cat# A11001) in the dark for 60 minutes at room temperature. Cover slips were washed 5 times for 3 minutes with 1% milk, 0.1% saponin, 0.05% tween in DPBS and nuclei were stained with 5 μg/mL of Hoescht 33258 for 5 minutes in the dark, slips were washed twice with DPBS and mounted on Superfrost Plus glass slides (Fisherbrand cat# 12-550-15) using Permafluor mounting media (Thermo Scientific).

CF2, CF7 and THP-1 cells were visualized under the microscope using 200X to 400X magnification through Olympus BX51 filters and images captured with Image Pro software 6.0.

B2B cells stably expressing empty vector or FL IL-33 and R48 construct were visualized under a laser scanning confocal microscope (LSM 700, Zeiss) and images were captured with the Zen light software (2009 edition).

2.2.5 Cell lysis and immunoblotting

Following stimulation, cells were put on ice and washed with cold PBS. Each well was then lysed in 80 µL buffer containing 50 mM Tris-HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% (v/v) Triton x-100, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.27 M sucrose, 5mM sodium pyrophosphate decahydrate, one complete miniprotease inhibitor mixture (Roche, Mannheim, Germany) and 2 mM DTT. Cell debris were separated from soluble proteins by centrifugation for 5 minutes at 4 °C at 12,000 x *g*. 70 µL of supernatants were then added to 23 µL of loading buffer (0.24 mM Tris-HC, 8% SDS, 40% glycerol and 36% distilled water) containing 1X TCEP (Thermo Scientific). A portion of the supernatant was retained for quantification using the Bradford method.

For subcellular fractionation experiments, a nuclear extract kit was purchased from Active Motif (Carlsbad, CA, USA, cat# 40010). NuLi and CuFi cells were grown to confluence on 55 cm^2 pre-coated culture dishes as previously specified. Following stimulation, medium was aspirated and cells were washed with 5 mL of ice-cold solution of phosphatase inhibitors in PBS to limit further protein modifications. Cells were detached using a 25 cm cell scraper (Sarstedt, Newton, NJ) and transferred to pre-chilled microcentrifuge tube. Cells were precipitated by centrifugation for 5 minutes at 5000 *g* at 4˚C, supernatant was aspirated and the cell pellet was kept on ice. Then, cells were

resuspended in 250 µL of 1X hypotonic buffer and incubated for 15 minutes on ice to induce swelling of the cell membrane. In order to allow for release of cytoplasmic proteins into the supernatant, cells were permeabilized by the addition of 5 µL of detergent and each sample was vortexed for 10 seconds at the highest setting. Cell debris were precipitated by centrifugation for 30 seconds at 14,000 x *g* at 4˚C and supernatants containing cytoplasmic proteins were transferred to pre-chilled microcentrifuge tubes and stored at -80˚C. The pellet containing nuclear proteins was resuspended in Complete Lysis Buffer containing 10 mM DTT and protease inhibitors. To allow for complete lysis of nuclear membrane, the previous suspension was incubated for 30 minutes on ice on a rocking platform set at 100 rpm and vortexed for 30 seconds at the highest setting. Cell debris were then precipitated by centrifugation for 10 minutes at 13,000 x *g* at 4˚C and supernatants were transferred to pre-chilled microcentrifuge tubes and stored at -80˚C.

Quantified and normalized proteins were boiled for 5 minutes at 95 °C. For each gel 30 µgs of protein were deposited per well and separated by SDS-PAGE on a 10 % Pro-pure Next Gel with Pro-Pure Running Buffer (Amresco, Solon, OH) using BioRad Powerpac HC (150 V/3.0 A/300W) at 150 volts for 90 minutes. The proteins were then transferred from the gel to a nitrocellulose membrane using ice cold 1 X NuPAGE transfer buffer (Fisher Scientific) 10% methanol (v/v) for 35 minutes at 100 Volts. The membranes were stained with Ponceau (Sigma) to reveal successful protein transfer, rinsed in PBS and then blocked with either Odyssey Blocking Buffer (Li-Cor Biosciences, Lincoln, NE) or 2% BSA in PBS for one hour at room temperature. Primary antibodies were applied as listed in Table 1 and left overnight to incubate at 4 °C or alternatively at room temperature for 90 minutes. The primary antibody was then washed

off using PBS with 0.05% Tween 3 times for 5 minutes, and secondary antibodies of goat anti-rabbit IgG DyLight^{TM}800 (Thermo Scientific, Cat #35571) and/or goat anti-mouse IgG DyLight^{TM}680 (Thermo Scientific, Cat #35518) were applied for 45 minutes in the dark at room temperature at a dilution of 1:15,000 in blocking buffer 0.05% Tween. The membranes were washed as before and the signal was detected and quantified using a Licor Odyssey imaging system.

TABLE 1. PRIMARY ANTIBODIES

Antibody	Details	Catalogue number	Company
p-38/SAPK2	Monoclonal, mouse dilution: 1:1000 (0.4 ng/mL)	05–454	Millipore
Phospho-p38 MAPK (Thr^{180}/Tyr^{182})	Polyclonal, rabbit dilution : 1 :1000	09-272	Millipore
p44/42 ERK MAPK	Monoclonal, mouse dilution: 1:500 (0.3 ng/mL)	9107	Cell Signaling Technology
Phospho-ERK Thr^{202}/Tyr^{204}	Monoclonal, rabbit dilution: 1:1000 (25	4370	Cell Signaling Technology

	ng/mL)		
Lamin A/C	Monoclonal, rabbit, dilution: 1:2000	MABE481	Millipore
NFκB p65	Monoclonal, rabbit dilution: 1:1000 (1µg/mL)	06-418	Upstate (Millipore)
GAPDH	Monoclonal, mouse dilution: 0.25µg/mL	MAB374	Millipore
IL-33 (Nessy-1)	Monoclonal, mouse dilution: 0.5µg/mL	ALX-804-840/1	Enzo Life Sciences
IL-33	Polyclonal, rabbit dilution: 0.5µg/mL	ALX-210-447-C100	Alexis Biochemicals

2.2.6 Immunoprecipitation

Immunoprecitpitation was performed using recombinant Protein G sepharose 4B beads (Invitrogen). Beads were equilibrated as follows: a suspension of Protein G beads (50% v/v of beads in ethanol) was centrifuged at 13,000 rpm for 1 minute at 4 C. The supernatant was carefully aspirated with a pipette and a 1:1 v/v ratio of ice-cold TBS (pH 7.5) was added to the beads. The suspension was mixed by swirling the tube and beads were precipitated by centrifugation at 13,000 rpm for 1 minute at 4 C. This was repeated twice and beads were finally resuspended in TBS (50% v/v suspension).

Cells were grown in 10cm petri dishes and nuclear and cytoplasmic fractions were separated as described in the previous section. To decrease the sodium concentration in the nuclear extracts, 1 mL of hypotonic buffer solution was added to each tube. To get rid of any non-specific binding, 10 uL of a protein G sepharose pre-equilibrated bead suspension was added to each tube containing either nuclear or cytoplasmic extracts. This suspension of cell lysates and beads was incubated at 4°C for one hour on a rotating platform. Protein-G sepharose beads were then separated from cell lysates by centrifugation at 13,000 rpm at 4°C. Supernatants not containing any beads were carefully aspirated and transferred to microcentrifuge tubes. The cell suspension was then incubated with the respective antibodies (1ug/mg of protein) for 1 hour on a rotating platform at 4°C. A suspension of 10 uL of equilibrated protein-G beads was added to each tube and incubated for 1 hr at 4°C in the rotating platform. The antigen-antibody-bead complexes were precipitated by centrifugation at 13,000 rpm for 1 minute at 4 C. To prevent any other protein contamination, the bead complexes were washed three times with 1mL of ice cold TBS. Then, 10 uL of 2X SDS-PAGE loading buffer was added to the beads, boiled for 5 minutes at 95°C and loaded in a polyacrylamide gel to perform Western Blot analysis.

2.2.7 RNA extraction

Cells were lysed using 0.5 to 1 mL Trizol per well. The solution was transferred to micro-centrifuge tubes and RNA was then extracted by the addition of 100 to 200 µl chloroform (Fisher Scientific, Fairlawn, NJ), the solution was mixed for 3 minutes and the aqueous and organic phase were separated by centrifugation for 15 minutes at 12,000 x g at 4 °C. The aqueous phase was transferred to a new micro-centrifuge tube and 250 to

500 µl isopropanol (Fisher) were added to precipitate the RNA by centrifugation for 10 minutes at 12,000 x g at 4 °C. The supernatant was decanted and the RNA pellet was washed with 0.5 to 1 mL 75% ethanol (Commercial Ethanols, Brantford, Ontario), vortexed briefly and then centrifuged for 5 minutes at 12,000 x *g* at 4 °C. The supernatant was again decanted, samples were centrifuged for 10 seconds and the remaining ethanol was removed via pipette, the sample was then left to dry for 20 minutes at room temperature before being rehydrated in 5 to 10 µl sterile ribonuclease free water (Invitrogen). The diluted RNA was then cooled on ice for 10 minutes, heated for 10 minutes at 65 °C to dissolve and quantified using a nano-drop system (Thermo Scientific).

2.2.8 Reverse transcription and quantitative real-time PCR

To remove any potential DNA contamination, 0.5 to 1 µg of total RNA was incubated with 1 µL of DNAse I RNAse free (Fermentas, Burlington, Ontario, Canada) for 30 minutes at 37°C. 1 µL of 50mM EDTA (Fermentas) was added to each tube and samples were incubated for 10 minutes at 65°C to stop the DNAse reaction. Then, to synthesize the complementary DNA strand, a mixture containing random hexamers, all four deoxynucleotides, 20 U of the RNAse inhibitor Ribolock (Fermentas) and 50 U of Maxima Reverse Transcriptase (Fermentas) was added to each tube and incubated in a BioRad My Cycler as follows: 10 minutes at 25 °C, 30 minutes at 50 °C to achieve full polymerase activity and 5 minutes at 85 °C to inactivate the enzyme.

For quantitative real-time PCR samples were assayed in Fast 96-well reaction plate (Applied Biosystems, Foster City, CA, USA) with each condition containing 50 to 100 ng cDNA in a total volume of 2.5 µL sterile water with 0.3 µM of each forward and

reverse primer (Integrated DNA Technologies, Coralville, IA- see Table 2), 5 µl iTAQ SYBR Green Supermix with Rox (BioRad) as well as 1.9 µl sterile water. The plate was sealed and cycled as follows using a Step-One-Plus machine (Applied Biosystems, Foster City, CA): 95 °C for 10 min, 50 cycles of 95 °C for 10 seconds and 60 °C for 45 seconds. Each condition was normalized to the housekeeping gene GAPDH. Relative fluorescence and therefore gene amplification was interpreted as fold induction from cycle threshold values using the Pfaffl mathematical model. Primer efficiencies were determined using a standard curve generated from a 3 fold serial dilution of cDNA.

TABLE 2.QRT-PCR PRIMER SEQUENCES

Gene	Forward primer (5 prime -3 prime)	Reverse primer (3 prime -5 prime)
GAPDH	AGC AAT GCC TCC TGC ACC ACC	CCG GAG GGG CCA TCC ACA GTC
IL-6	GTG TGA AAG CAG CAA AGA GG	TGC AGG AAC TGG ATC AGG
IL-8	GTG CAG TTT TGC CAA GGA GT	CTC TGC ACC CAG TTT TCC TT
IL-33	CAA AGA AGT TTG CCC CAT GT	AAG GCA AAG CAC TCC ACA GT
IL-1α	ACC TCA CGG CTG CTG CAT TAC ATA	TGG TCT TCA TCT TGG GCA GTC AC
HMGB1	GGA GAT CCT AAG AAG CCG AGA	CAT GGT CTT CCA CCT CTC TGA
S100A9	AAA GAG CTG GTG CGA AAA GA	TCA GCT GCT TGT CTG CAT TT
S100A8	TCA GGA AAA AGG GTG CAG AC	ACG CCC ATC TTT ATC ACC AG
Groα (CXCL3)	AGG GAA TTC ACC CCA AGA AC	TAA CTA TGG GGG ATG CAG GA
Groβ (CXCL2)	GCA GGG AAT TCA CCT CAA GA	GAC AAG CTT TCT GCC CAT TC
IκBα	GCT GAT GTC AAT	CCC CAC ACT TCA ACA

	GCT CAG GA	GGA GT
MYC	AGC GAC TCT GAG GAG GAA CA	CTC TGA CCT TTT GCC AGG AG
IL-10	ACT TTA AGG GTT ACC TGG GTT GC	TCA CAT GCG CCT TGA TGT CTG

2.2.9 Flow cytometry

Annexin V-FITC Apoptosis Detection Kit I was purchased from BD Pharmingen (Mississauga ON, Canada, cat# 556547). Following treatment, supernatants from NuLi and CuFi cells were collected and stored at -80˚C. Cells were washed with sterile DPBS once and 400µL of 0.25% trypsin-0.04% EDTA were added to each well and incubated for 6 to 8 minutes at 36˚C. The solution was collected and transferred to 5mL culture tubes containing 2 mL of DMEM 10% FBS to inactivate trypsin activity and cells were precipitated by centrifugation 5 minutes at 700 x *g*. The medium was then aspirated and the cell pellet was washed twice with cold DPBS to remove any traces of the previous solution and resuspended in 100µL of 1X Binding Buffer. Then, the cell suspension was incubated for 15 minutes at room temperature in the dark with 5 µL of FITC Annexin V, which binds phosphatidylserine on the membrane of cells undergoing apoptosis and 5 µL of propidium iodide (PI), which binds to DNA of cells whose membrane integrity has been lost due to either end stage of apoptosis or necrotic cell death. The reaction was stopped by addition of 400µL of 1X Binding Buffer to each tube, samples were placed on ice and analyzed with the BD LSRII Flow Cytometer using the BD FACSDiva software with compensation. The following controls were used to set up compensation and

quadrants: 1) unstained cells, 2) cells stained with FITC Annexin V only and 3) cells stained with PI only.

2.2.10 Luciferase reporter assays

Following 3 h stimulation, Beas-2B cells were washed once with cold PBS, lysed in 40 μL of 1x reporter lysis buffer (Promega, Madison, WI, USA) and transferred to microcentrifuge tubes. Cell debris were separated from soluble proteins by centrifugation for 5 minutes at 4 °C at 13,000 x *g*. 20 μL of each sample were transferred to a 96 well plate and 25 μL of luciferase reagent (20 mM Tricine, 1.07 mM (MgCO3)·4H2O Mg(OH)2·5H2O, 2.67 mM MgSO4, 0.1 mM ethylenediaminetetraacetic acid, 33 mM dithiothreitol, 270 μM coenzyme A, 0.477 mM d-luciferin, and 0.533 mM adenosine triphosphate) were added to each well with an automatic injector. Chemiluminescence was quantified on a Tecan Infinite M1000 plate reader using the Tecan i-control software.

2.2.11 Statistical Analysis

Differences in means between two groups were compared with independent Student t tests. Differences in means between three or more groups were compared by One-Way ANOVA followed by Dunnet's post-test to compare the means of stimulated conditions to control or by Bonferroni's post-test to compare the differences between selected groups. When two independent variables were hypothesized to influence one outcome, differences in means and interaction between variables were compared with Two-Way ANOVA followed by Bonferroni post-test. For all the experiments shown in this thesis, the level of statistical significance (α) was set to 0.05. Statistical analysis was performed using versions 5.0 and 6.0 of GraphPad Prism software.

Chapter 3: IL-33 expression is increased in CF airway epithelium

Introduction

During the course of cystic fibrosis lung disease (CFLD), chronic infection leads to an imbalance favoring pro-inflammatory mediators to drive excessive neutrophil recruitment to the airways with the subsequent injury to the epithelium. The chronic phase in CFLD is often worsened during pulmonary exacerbations where changes in the colonizing bacteria lead to tissue damage with the subsequent loss of pulmonary function that is often irreversible [235].

Pro-inflammatory cytokines such as IL-6, CXCL-8 and TNFα are increased in sputum samples from patients during pulmonary exacerbations [166]. Non-infectious stimuli can also increase inflammatory signaling through damage-associated molecular patterns (DAMPs), a group of endogenous molecules that are released following tissue injury or cell death [236, 237]. DAMPs such as HMGB1 and IL-1α have been implicated in the pathophysiology of CFLD [169, 238]. IL-33, a member of the IL-1 family of cytokines, is a recently discovered DAMP [200, 206]. Our group has previously shown a marked increase in nuclear staining of IL-33 in airway epithelial cells of explanted CF lungs compared to non-CF lungs[229]. A more recent study revealed increased levels of IL-33 in BALF from stable CF patients [230]. In this chapter we analyze the expression of different DAMPs in an *in vitro* model of chronic infection. We then focus our attention in the study of IL-33, its expression, localization and release in *in vitro* models of both chronic infection and acute exacerbations.

Results

3.1 Exposure of epithelial cells to *Pseudomonas aeruginosa* diffusible material (PsaDM) increases IL-33 mRNA in CF airway epithelial cells

The interaction of airway epithelial cells with *P. aeruginosa* during chronic infection in CF is very complex. It has been demonstrated that bacteria are trapped within exudates and are therefore not in direct contact with the epithelium [239]. To mimic this scenario, we used *P. aeruginosa* diffusible material (PsaDM, for more details refer to materials and methods). Previous work from our group has demonstrated that exposure of airway epithelial cells expressing the CFTRΔF508 mutation to PsaDM increases IL-6 and CXCL8 expression and synthesis [46]. We were therefore interested in studying if a similar response can be observed for the DAMPs IL-33, IL-1α, HMGB1 and S100A9. In order to characterize the expression profile of these cytokines *in vitro*, non CF (NuLi and N3) and CF (CuFi and CF7) cells were incubated with PsaDM at a concentration of 8 ng/mL for 3 h and the mRNA levels of IL-33, IL-1α, HMGB1 and S100A9 assessed by qRT-PCR.

Following exposure to PsaDM, IL-33 mRNA is significantly increased in both CuFi and CF7 cells when compared to non-CF cells and two-way ANOVA correlates the increased IL-33 mRNA levels to the CF phenotype (figure 3.1a). A significant upregulation of IL-1α mRNA can also be observed in CuFi cells but not CF7 after exposure to PsaDM (figure 3.1b). No significant increase in HMGB1 or S100A9 mRNA levels is observed following stimulation (figures 3.1c and 3.1d, respectively). We can therefore conclude that, of the four DAMPs studied, IL-33 is the only one to be

significantly increased in both of the CF cell lines used, potentially implicating this cytokine as an inflammatory mediator in CFLD.

Figure 3.1

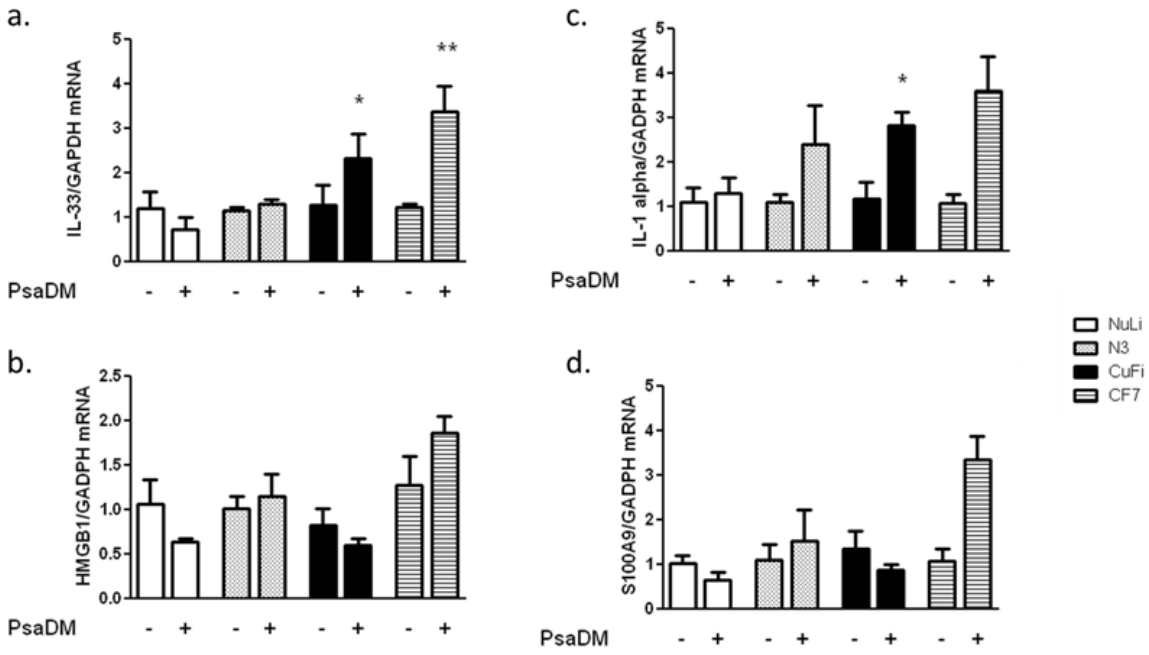


Figure 3.1. Exposure to *Pseudomonas aeruginosa* diffusible material (PsaDM) increases IL-33 mRNA in CF airway epithelial cells. CF airway epithelial cell lines expressing the mutations CFTRΔF508 (CuFi) and G542X (CF7) and their wild-type counterparts (NuLi and N3 respectively) were exposed to PsaDM for 3h and mRNA expression for the alarmins IL-33, IL-1α, HMGB1 and S100A9 was measured by qRT-PCR. Results were analyzed by two-way ANOVA with Bonferroni post-test. a. A significant upregulation in IL-33 mRNA in both, CuFi and CF7 cells can be observed following treatment with PsaDM . b. IL-1α mRNA is upregulated in CuFi cells following incubation with PsaDM. b. and d. No significant difference in HMGB1 or S100A9

mRNA is observed after incubation with PsaDM. n=3 for each condition, * $p<0.05$ **$p<0.01$. Figures a. and b. were published in [229].

3.2 Increased IL-33 levels are localized in the nucleus of CF cells

We next assessed whether increases in IL-33 mRNA in response to PsaDM lead to increased intracellular protein levels. We therefore detected the localization of endogenous IL-33 using a monoclonal antibody and looked at its intracellular localization by immunofluorescence microscopy. Two non-CF (NuLi and N3) and two CF (CuFi and CF7) airway epithelial cell lines were exposed to PsaDM for 24 hours, followed by fixation with formaldehyde and immunofluorescence staining for IL-33.

As shown in figure 3.2, very little to no staining for IL-33 can be detected in the absence of treatment (left panels under 'Ctl', first two columns). Incubation with PsaDM for 24 hours did not increase IL-33 staining in neither NuLi nor N3 cells (right panels under 'PsaDM', first and third rows). In contrast, 24h exposure to PsaDM increased IL-33 in the nuclei of both, CuFi and CF7 cells (right panels under 'PsaDM' second and fourth rows). These results are in accordance with previous immunohistochemical findings [229] suggesting that CF cells respond to bacterial derived products by increasing IL-33 protein levels in the nucleus.

Figure 3.2

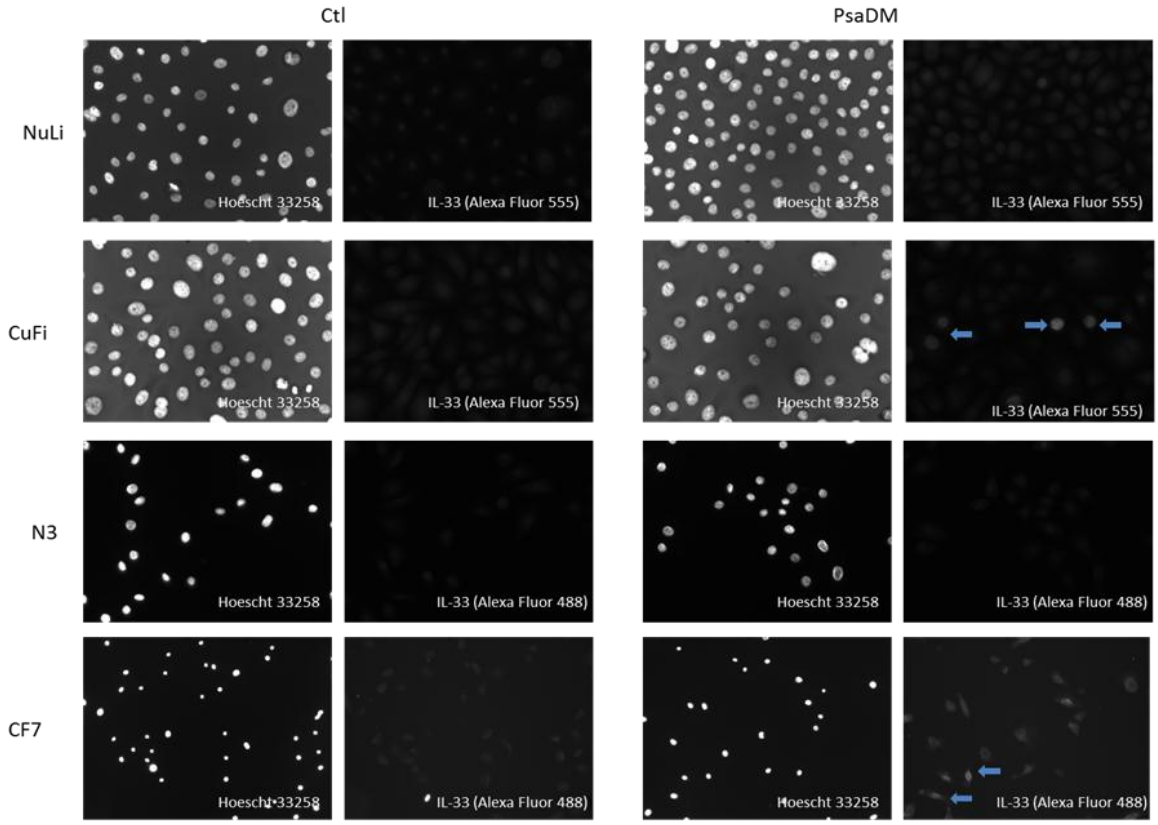


Figure 3.2. Increased IL-33 levels are localized in the nucleus of CF cells. Non CF (NuLi and N3) and CF (CuFi and CF7) airway epithelial cell lines were incubated with PsaDM for 24 h, fixed with formaldehyde, nuclei were stained with Hoescht 33258 and IL-33 levels were assessed using the monoclonal antibody Nessy-1. Very low levels of IL-33 are observed for all of the untreated controls (left panels). Following treatment with PsaDM, increased IL-33 staining can be observed in the nucleus of both, CuFi and CF7 cells (right panels). Images from control and PsaDM stimulated CuFi cells were published in [229].

3.3 IL-33 is not released following exposure to PsaDM

To determine if IL-33 is released in our model, supernatants from NuLi and CuFi cells that had been exposed to PsaDM for 24 h were collected and IL-33 levels were measured by ELISA.

As shown in figure 3.3, no increase in extracellular IL-33 levels can be observed in either one of the cell lines following 24h exposure to PsaDM. This might be due to the fact that PsaDM induces the pathways leading to IL-33 upregulation but is not strong enough to cause cell damage leading to IL-33 release. Therefore, we developed an *in vitro* model of chronic infection with *Pseudomonas aeruginosa* (Psa) followed by a model of acute bacterial exacerbations CF cell lines to study IL-33 signaling.

Figure 3.3

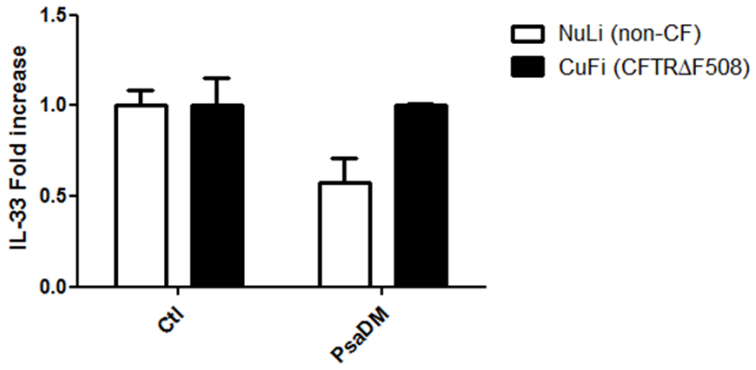


Figure 3.3. IL-33 is not released following exoposure to PsaDM. Supernatants from NuLi (white bars) and CuFi (black bars) cells that had been exposed to PsaDM for 24 hours were collected and IL-33 levels were quantified by ELISA. IL-33 protein levels in supernatants are not increased following stimulation for either one of the cell lines. A slight, but not significant decrease can be observed in NuLi cells following stimulation.

3.4 Exposure to *Pseudomonas* biofilms leads to IL-6 secretion

In our previous experiments, we used PsaDM to simulate the interaction of planktonic bacteria with airway epithelial cells. In this context, bacteria are grown at 37°C with shaking, as described in the Materials and Methods. However, biofilm formation is essential for the survival of *Pseudomonas* in the airways of CF patients, leading to chronic infection and inflammation [240]. Since the way bacteria are cultured can affect their gene expression and phenotype, as well as the factors they secrete, we grew bacteria under static conditions to induce biofilm formation *in vitro.* This model served to more accurately recreate the chronic phase of CF. We then used non CF and CFTRΔF508 airway epithelial cells (NuLi and CuFi respectively) and exposed them to *Pseudomonas* biofilm (Bcf) material. We first wanted to establish the concentrations of Bcf needed to induce an inflammatory response in our model. To do so, cells were exposed to concentrations of 10, 28, 86, 261 and 783 ng/mL every 24h for 72h and IL-6 levels in the culture supernatants were measured by ELISA. We decided to measure IL-6 since previous studies done by our group have identified it as a key cytokine in the inflammatory response in CF [46]. Since biofilms might contain proteases that could lead to cytokine degradation, the Bcf used for stimulations was either heat inactivated or not. Synthetic Cystic Fibrosis Media (SCFM, described in Materials and Methods) was used as a control. In NuLi cells, only a small increase in IL-6 secretion can be observed following treatment with 86 ng/mL of heat-inactivated Bcf (figure 3.4a). A stronger effect can be observed in CuFi cells, where concentrations of Bcf greater than 86 ng/mL increase IL-6 secretion more than 2-fold as compared to controls (figure 3.4b). We can only observe a mild effect of heat inactivation in the prevention of IL-6 degradation and

more replicates would be needed to achieve significance. Since a concentration of 261 ng/mL of heat inactivated Bcf is sufficient to induce a 2.5 fold increase in IL-6 secretion in CuFi cells, we chose this concentration to simulate the chronic phase of bacterial infection in cystic fibrosis in future experiments.

Figure 3.4

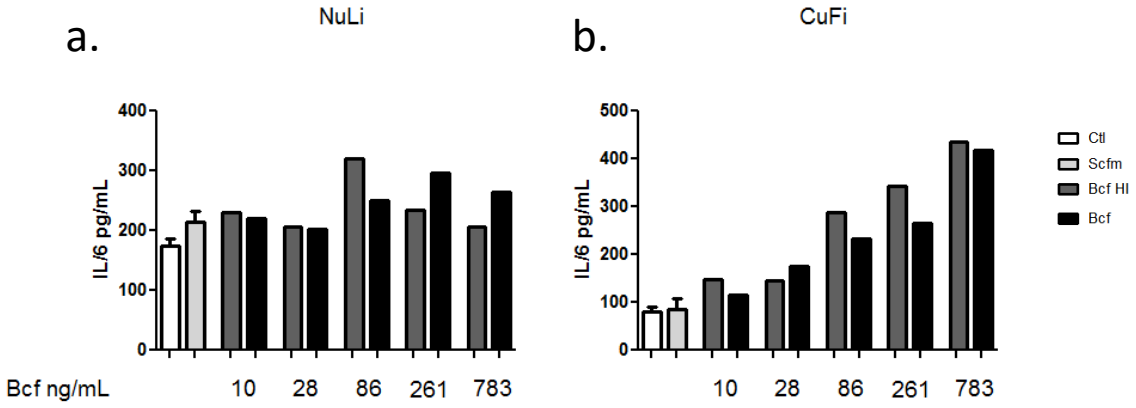


Figure 3.4. Chronic exposure to *Pseudomonas* biofilms induces IL-6 secretion from CF cells. Non-CF (panel a., NuLi) and CFTRΔF508 airway epithelial cells (panel b., CuFi) were exposed to *Pseudomonas* biofilm (Bcf) concentrations of 10, 28, 86, 261 and 783 ng/mL every 24 h for 72 h and the levels of IL-6 in the supernatants were measured by ELISA. White bars represent the unstimulated controls. Light grey bars represent cells stimulated with Synthetic Cystic Fibrosis Media (SCFM), a mixture of different amino acids used for Bcf preparation. Dark grey bars represent cells stimulated with Bcf that had been previously heat inactivated at 95°C for 10 minutes. Black bars represent cells treated with non-heat inactivated Bcf. n=4 for controls and scfm treated samples. n=1 for samples incubated with Bcf.

3.5 Exposure to *Pseudomonas* biofilm increases IL-6 and IL-33 mRNA in CF cells

We then wanted to look at the effects of a chronic exposure to Bcf on IL-33 and IL-6 mRNA expression. For this, NuLi and CuFi cells were exposed to 261 ng/mL of Bcf every 24 h. mRNA was extracted at 6, 12, 24, 48 and 72 h and gene expression for IL-33 and IL-6 was assessed by qRT-PCR.

As seen in figure 3.5a, IL-33 mRNA is significantly upregulated in CuFi cells after 6 h and 72h incubation with Bcf. As expected and in line with cytokine secretion results, IL-6 mRNA is significantly upregulated after 48 h and 72 h exposure to Bcf (3.5b).

Figure 3.5

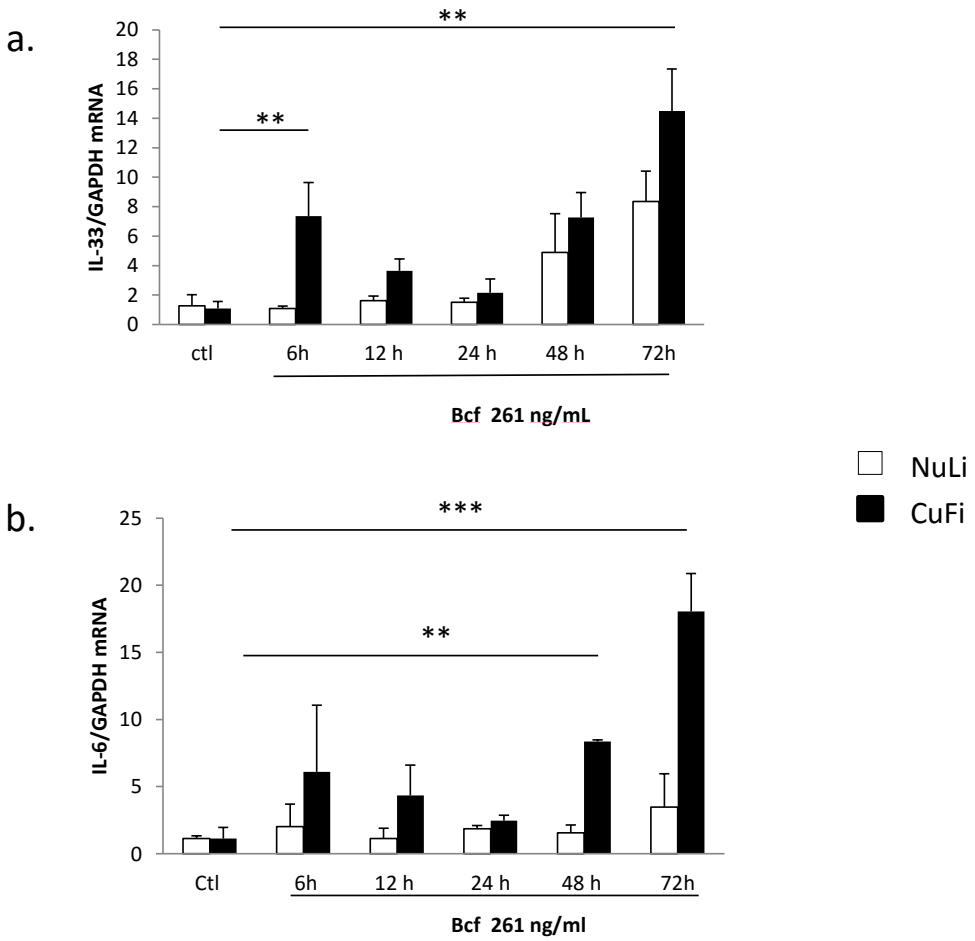


Figure 3.5. Chronic exposure to Bcf significantly increases IL-33 and IL-6 mRNA. NuLi (white bars) and CuFi (black bars) cells were exposed to 261 ng/mL of Bcf every 24 h and mRNA was extracted at 6, 12, 24, 48 and 72 h. IL-33 and IL-6 mRNA levels were quantified by qRT-PCR. a. IL-33 mRNA is significantly upregulated after 6 and 72 h of exposure to Bcf in CuFi cells. b. A significant upregulation of IL-6 mRNA can be observed after 48 and 72 h exposure of CuFi cells to Bcf. Data were analyzed by two way ANOVA followed by Bonferroni post-test, ***$p<0.001$, **$p<0.01$.

3.6 Stimulation of airway epithelial cells with homoserine lactone (HSL) increases IL-6 and IL-1α mRNA in CF cells

N-(3-oxododecanoyl)-l-homoserine lactone (3O-C_{12}-HSL) is an extracellular signal involved in quorum sensing and is necessary for *Pseudomonas* biofilm differentiation and formation *in vivo* [241]. Previous research has identified 3O-C_{12}-HSL as a virulence factor that disrupts the epithelial barrier through the activation of p38 and ERK1/2 MAPKs and is known to induce IL-6 synthesis in CF cells [242, 243]. In order to study the possibility that HSL is the element in Bcf leading to the upregulation of DAMP genes, NuLi and CuFi cells were exposed to HSL concentrations of 11, 33 and 100 μM over 3 h and IL-33 and IL-1α mRNA levels were measured by qRT-PCR. IL-6 mRNA was also quantified and used as a control. As shown in figure 3.6, IL-6 and IL-1α mRNA are significantly upregulated after a 3 h exposure to 11μM 3O-C_{12}-HSL in CuFi cells (figures 3.6a and 3.6b, respectively). IL-33 mRNA is not significantly upregulated in neither NuLi nor CuFi cells following treatment with either of the concentrations used. These results implicate 3O-C_{12}-HSL as one of the components in Bcf leading to the upregulation of proinflammatory cytokine genes such as IL-6 and IL-1α, but not IL-33. However, incubation with 3O-C_{12}-HSL for longer time points might be needed to determine if IL-33 mRNA is upregulated.

Figure 3.6

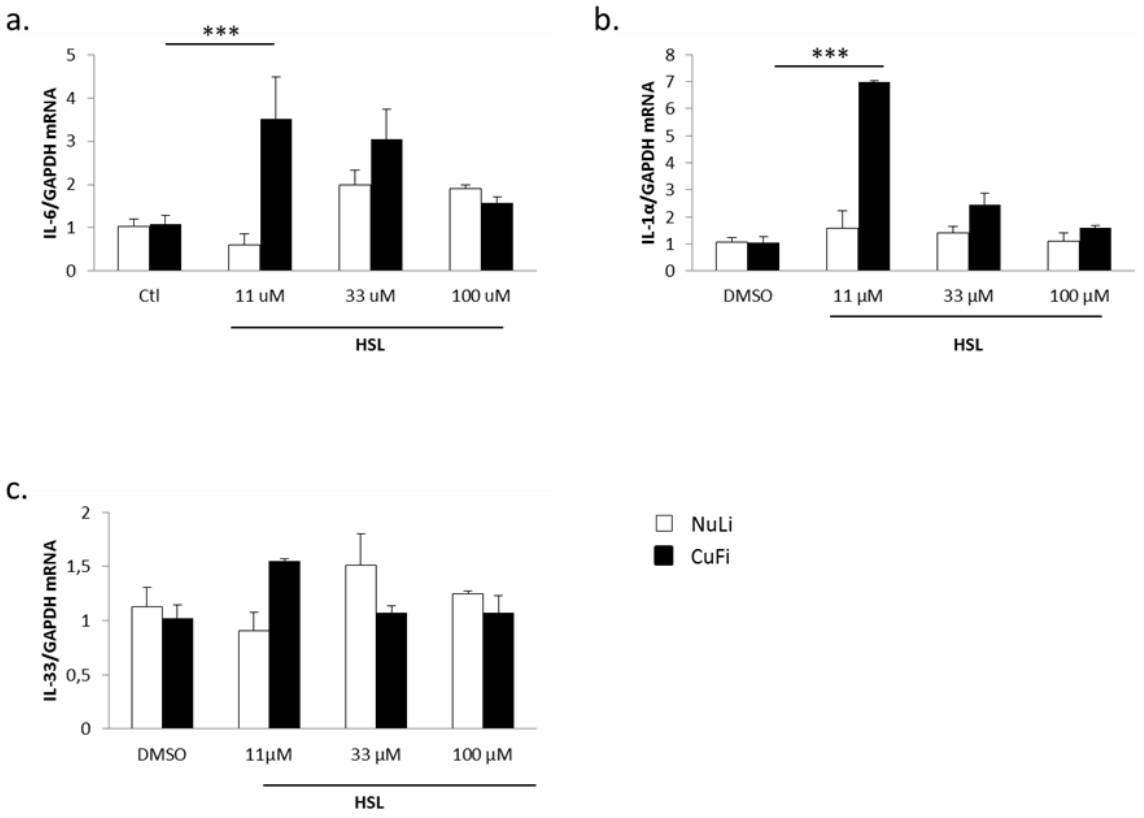


Figure 3.6. Stimulation of airway epithelial cells with homoserine lactone (HSL) increases IL-6 and IL-1α mRNA in CF cells. NuLi (white bars) and Cufi cells (black bars) were incubated with either DMSO or with HSL concentrations of 11, 33 and 100 µM for 3 h. RNA was extracted and IL-6, IL-1α and IL-33 gene expression was analyzed by qRT-PCR. a and b. IL-6 and IL-1α are significantly upregulated following exposure to a concentration of HSL of 11 µM. c. No significant upregulation of IL-33 mRNA is observed following treatment with the different concentrations of HSL. Results were analyzed by two-way ANOVA followed by Bonferroni post-test, n=3, ***$p<0.001$.

3.7 Chronic exposure to *Pseudomonas* biofilm significantly increases IL-8 secretion but not IL-33

Previous results show an increase in IL-33 and IL-6 mRNA in CF cells after long term incubation with Bcf. To determine whether IL-33 is secreted in this model, NuLi and CuFi cells were incubated with 261 ng/mL of heat inactivated Bcf every 24 h for 72 h. Supernatants were then collected and IL-33 levels were measured by ELISA. IL-8 levels were also measured and used as a control. As shown in figure 3.7a, incubation with Bcf does not significantly increase IL-33 release in neither NuLi nor CuFi cells. IL-33 levels in untreated controls are higher in supernatants from CuFi cells and, contrary to what was expected, there is a tendency for IL-33 levels to decrease following exposure to Bcf. This effect might be explained by the fact that treatment with Bcf is not sufficient to induce necrotic cell death and therefore IL-33 is not released in the supernatants. Exposure to Bcf significantly increases IL-8 secretion in both NuLi and CuFi cells. The effect is stronger in CuFi cells, where incubation with Bcf leads to an IL-8 secretion that is 180-fold greater when compared to controls (figure 3.7b).

Figure 3.7

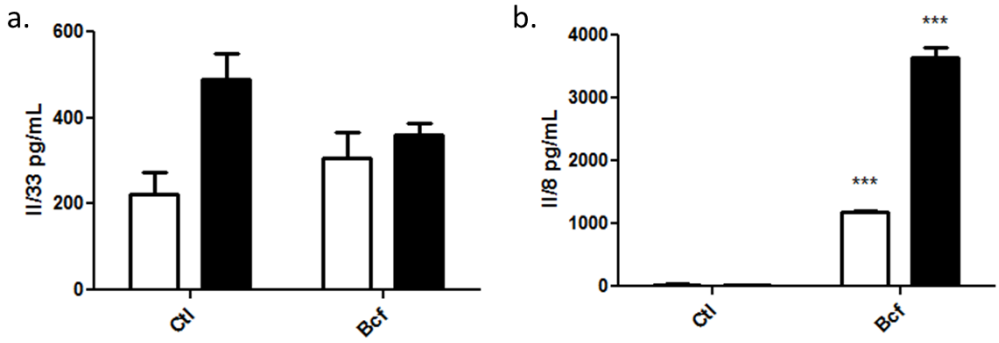


Figure 3.7. Chronic exposure to Pseudomonas biofilm does not increase IL-33 release. NuLi (white bars) and CuFi cells (black bars) were incubated with 261 ng/mL of Bcf every 24 h for 72 h. Supernatants were then collected and IL-8 and IL-33 levels were measured by ELISA. a. No significant difference in IL-33 release is observed in either one of the cell lines following incubation with Bcf. b. IL-8 secretion significantly increases following exposure of both cell lines to Bcf. Results were analyzed by two-way ANOVA followed by Bonferroni post-test, n=6, *** $p<0.001$.

3.8 Acute infection of airway epithelilal cells with *Pseudomonas aeruginosa* increases cell death

Our previous experiments have failed to demonstrate an increase in IL-33 release following stimulation with either PsaDM or Bcf. This might be explained by the fact that, even though IL-33 is constitutively expressed in the nucleus of structural cells, the biologically active full-length IL-33 is only released extracellularly after cell damage has occurred. It has been demonstrated that cleavage of IL-33 by pro-apoptotic caspases 3 and 7 leads to its inactivation, suggesting that it might act as a danger signal that is released following necrotic but not apoptotic cell death [207]. Therefore, a stronger stimulus might be needed to induce cell death with subsequent IL-33 release. A model recreating the chronic phase of CFLD with Bcf followed by an acute bacterial exacerbation would be appropriate for this purpose.

To determine whether chronic exposure to Bcf increases secondary necrotic cell death and if this is potentiated by an acute Psa infection, NuLi and CuFi cells were exposed to 261 ng/mL of Bcf every 24 h for 72 h. Then, cells were either untreated or infected with live *P. aeruginosa* ($1.6x10^6$ CFU) for 3 h. Cells were washed and detached

with trypsin, collected and incubated with Annexin V and Propidium Iodide (PI). Annexin V binds to membrane phospholipids of cells undergoing apoptosis and PI binds to DNA. Therefore, cells that stain positive for Annexin V and negative for PI are considered to be undergoing apoptosis and cells that stain positive for both, Annexin V and PI are considered to be undergoing necrosis. This includes cells that were apoptotic and underwent secondary necrosis. Staining was analyzed by flow cytometry (see Materials and Methods).

The graphs in figure 3.8 represent the percentage of cells in each population. Treatment with Bcf alone does not decrease cell viability, however, a strong decrease in the percentage of live cells can be observed following acute infection with Psa (figure 3.8a). Treatment with Bcf alone does not increase apoptosis in either cell line but the percentage of apoptotic cell death increases in both, NuLi and CuFi cells following acute infection with Psa (figure 3.8b). Necrotic cell death is increased in both cell lines when infected with Psa (figure 3.8c). We can therefore conclude that exposure to Bcf is not sufficient to cause cell death in our model and that acute infection of airway epithelial cells with Psa increases apoptotic and necrotic cell death in both, CF and non-CF cells.

Figure 3.8

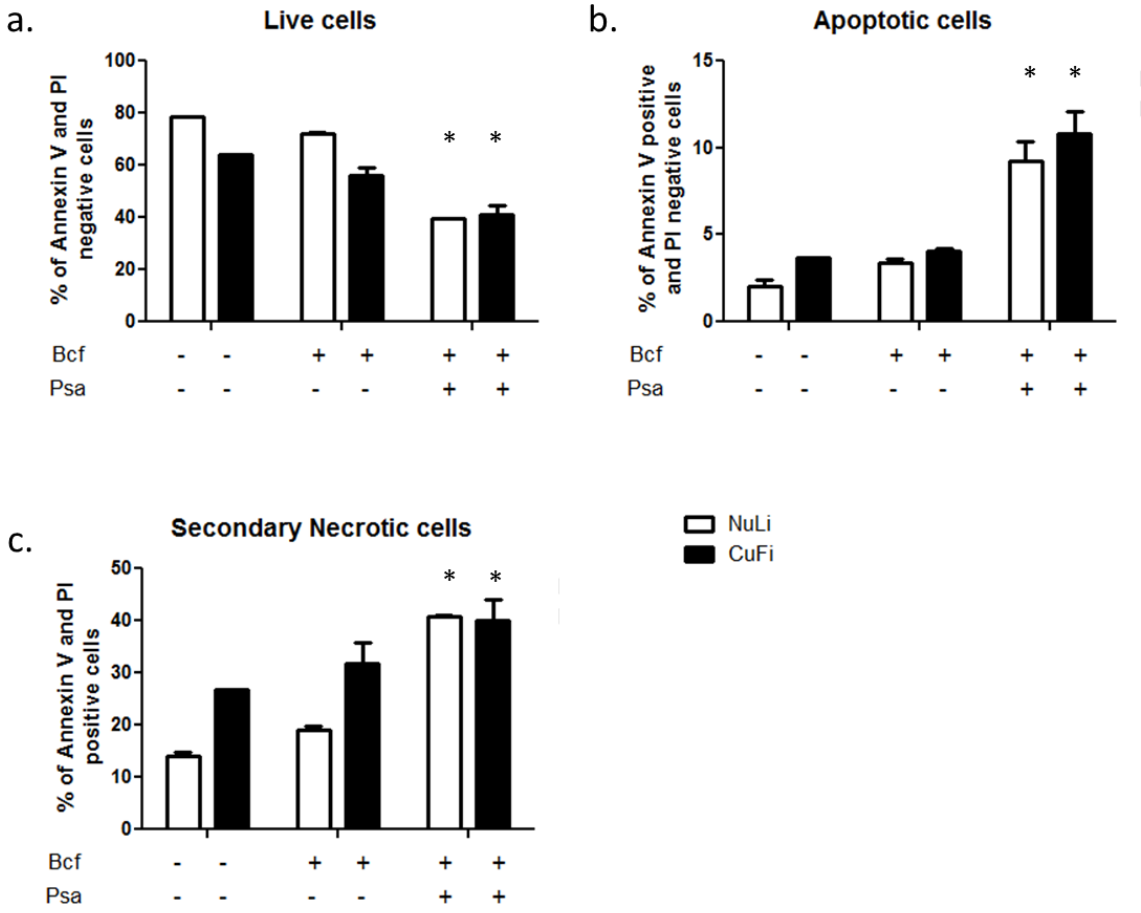


Figure 3.8 Acute infection of airway epithelilal cells with *Pseudomonas* aeruginosa increases cell death. NuLi (white bars) and CuFi cells (black bars) were exposed to Bcf every 24 h for 72 h. Then, cells were either left untreated or infected with live Psa, $1.6x10^6$ CFU for 3 h. Cells were washed and detached with trypsin 0.25% EDTA, collected, washed and incubated with Annexin V and Propidium Iodide (PI) and staining was analyzed by flow cytometry. a. Cells that were negative for both, Annexin V and PI staining represent live cell population. Nearly 80% of the control NuLi cell population is viable, whereas only close to 60% of CuFi cells are alive in the untreated group.

Incubation with Bcf for 72 h did not significantly decrease cell viability in either one of the groups. Acute infection *with P. aeruginosa* markedly decreases cell viability in both cell lines. b. Cells that stain positive for Annexin V and negative for PI represent the percentage of apoptotic cells. Even in the absence of stimulation, CuFi cells have a higher tendency to undergo apoptotic cell death. Chronic exposure to Bcf does not significantly increase apoptotic cell death in either one of the cell lines. Infection with Psa significantly increases apoptotic cell death in both cell lines, p=0.0007 for row factor. c. Cells that stain positive for both, Annexin V and PI represent the population of cells undergoing necrotic and/or secondary necrotic cell death. Even in the absence of stiumulation, CuFi cells show significantly increased secondary necrotic cell death compared to NuLi cells (p=0.014 for column factor). However, treatment with Bcf does not significantly increase cell death compared to controls. Acute infection with *P. aeruginosa* significantly increases secondary necrotic cell death in both cell lines (p=0.0018 for row factor). Results were analyzed by two-way ANOVA followed by Bonferroni post-test and represent two independent experiments.

3.9 Acute infection of CF cells with *Pseudomonas aeruginosa* leads to IL-33 mRNA upregulation

Our previous results show an increase in cell death in response to an acute bacterial infection, therefore, it would be relevant to determine whether IL-33 mRNA is increased following this stimulus. To study the inflammatory response resulting from the direct interaction of live *P. aeruginosa*, two CFTRΔF508 airway epithelial cell lines (CuFi and CF2) and one non-CF cell line (N3) were infected with $1.6x10^6$ (MOI of 18) CFU of Psa for three hours and IL-33 mRNA were measured by qRT-PCR.

As shown in figure 3.9, infection with *P. aeruginosa* significantly increased IL-33 mRNA in CF2 and CuFi cells (3.9a and 3.9b respectively). The infection did not significantly increase IL-33 mRNA in N3 cells.

Figure 3.9

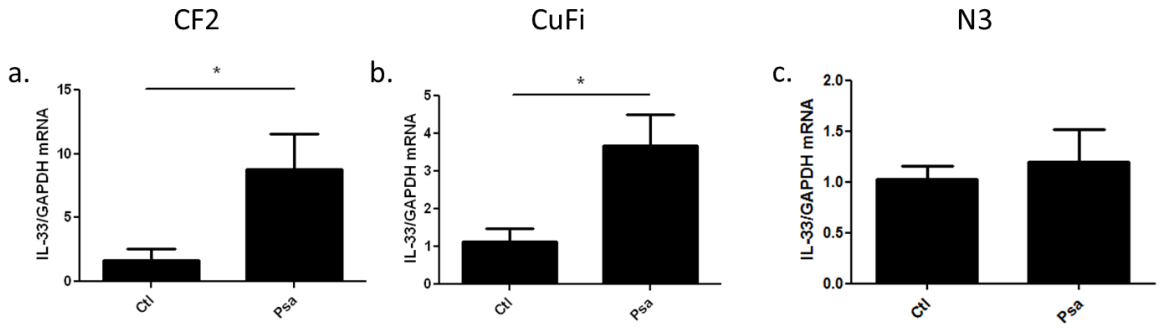


Figure 3.9. Acute infection with *P. aeruginosa* increases IL-33 mRNA in CF airway epithelial cells. CF airway epithelial cells expressing the CFTRΔF508 mutation (CF2, panel a. and CuFi, panel b.) and non-CF airway epithelial cells (N3, panel c.) were infected with $9x10^6$ CFU *of P. aeruginosa* for 3 h (MOI of 18), RNA was extracted and relative IL-33 gene expression was measured by qRT-PCR. An acute infection with *P. aeruginosa* significantly upregulated IL-33 mRNA in CF2 and CuFi cells. No significant difference can be observed between control and infected non-CF cells. Results were analyzed by one-tailed t tests, n=3, *p<0.05, **p<0.01. Figures 3.9a and 3.9c have been publilshed in *Frontiers in Cell and Developmental Biology* [244].

3.10 IL-33 mRNA upregulation in response to infection is dependent on CFTR channel function in CF2 airway epithelial cells

In previous results, we have only observed a significant increase of IL-33 mRNA expression in CF airway epithelial cells. This effect could be directly related to CFTR

malfunction and inadequate membrane expression or might be independent of the function of CFTR as an ion channel. Therefore we used a pharmacological approach to determine whether the increase in IL-33 mRNA that we observe in CF airway epithelial cells is dependent on CFTR. For this purpose, we potentiated CFTR channel function in CF2 cells with VX-770 (Ivacaftor) or corrected CFTR expression to the cell membrane with VX-809 (Lumacaftor). Additionally, a combination of both VX-770 and VX-809 was used to assess a potential additive effect. The vehicle DMSO was used as a control. CF2 cells were then infected with *P. aeruginosa* for 3 h and IL-33 mRNA expression was measured by qRT-PCR (figure 3.10a). IL-8 expression was measured as a control (figure 3.10b). As observed previously, an acute infection with *P. aeruginosa* significantly increased IL-33 mRNA. The potentiation of CFTR channel activity with VX-770 prevented the increase in IL-33 mRNA in response to infection. CFTR correction with VX-809 also abrogated the increase in IL-33 mRNA in response to *P. aeruginosa*, although to a lesser extent when compared with VX-770. Incubation with both, the CFTR potentiator and the CFTR corrector also prevented the increase of IL-33 mRNA, although an additive effect could not be observed. Finally, neither the corrector nor the potentiator alone decreased baseline IL-33 expression in uninfected conditions. A similar trend is observed for IL-8 mRNA expression. These results suggest that the increase in IL-33 mRNA in CFTRΔF508 airway epithelial cells in response to infection is due to CFTR malfunction.

Figure 3.10

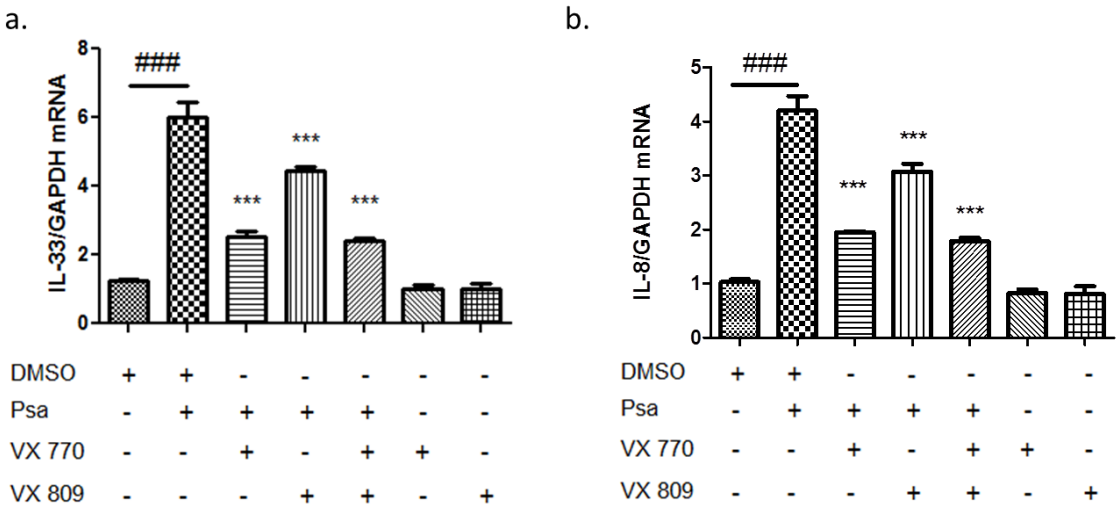


Figure 3.10. IL-33 mRNA upregulation in response to infection is dependent on CFTR channel function in CF2 airway epithelial cells. CF2 cells were incubated in the presence of the CFTR corrector VX-770 (1μM), the CFTR potentiator VX-809 (5μM) or a combination of both for 24 h. Cells were then infected with *P. aeruginosa* (MOI of 18) for 3 h and IL-33 (panel a) and IL-8 expression (panel b) were measured by qRT-PCR. A. An acute infection significantly increased IL-33 mRNA. This can be prevented by incubating the cells with VX-770 and, to a lesser extent, with VX-809. The combination of both, VX-770 and VX-809 also prevented the increase in IL-33 expression by *P. aeruginosa* although no synergism or potentiation is observed. B. An acute infection with *P. aeruginosa* increased IL-8 mRNA expression. This could be prevented by incubating the cells with VX-770 or VX-809. Results were analyzed by one-way ANOVA followed by Bonferroni post-test comparing control vs infected (#), infected vs the corrector or potentiator (*) and control vs the corrector or potentiator alone. ### $p<0.001$, ***

$p<0.001$. Figure 3.10 has been published in *Frontiers in Cell and Developmental Biology* [244].

Summary of Results

We have demonstrated that, out of the four DAMPs studied, IL-33 is the only one to be significantly upregulated in two different CF cell lines, CuFi and CF7, *in vitro*. Increased mRNA levels translate to increased intracellular protein levels as demonstrated by immunofluorescence microscopy. Staining with a monoclonal antibody against IL-33 reveals that most of the protein is localized in the nucleus of CuFi and CF7 cells following 24 h incubation with PsaDM. This same stimulus is not sufficient to induce IL-33 release from neither NuLi nor CuFi cells.

We then created a model representing the chronic phase of CF using *Pseudomonas* biofilm material (Bcf). In this model, IL-33 mRNA is significantly upregulated after 6 h and 72 h exposures to Bcf in CF cells. As expected, IL-6 mRNA is also significantly upregulated after 48 h and 72 h exposures to the same stimulus. We then hypothesized that 3O-C_{12}-HSL might be the component in Bcf driving IL-6, IL-33 and potentially other DAMP upregulation. According to our results, IL-6 and IL-1α mRNA are significantly upregulated following exposure to the lowest concentration of 3O-C_{12}-HSL. In contrast, IL-33 mRNA was not increased in response to any of the concentrations of 3O-C_{12}-HSL used in this experiment. Exposure for different time points might be needed to study the IL-33 mRNA expression profile in response to 3O-C_{12}-HSL. Alternatively, a wider range including lower concentrations might be needed since the response might not be monotonic and the effect could only be observed at lower doses. Despite the increase in IL-33 mRNA, we cannot observe an increase in IL-33

release in response to a chronic stimulation with Bcf. We know, however, that the stimulus is strong enough to significantly increase IL-8 secretion.

Even though our previous results show an increase in IL-33 mRNA following treatment with either PsaDM or Bcf, we have not yet observed an increase in IL-33 release to the extracellular medium. This might be due to the fact that neither one of the latter stimuli are sufficient to drive necrotic cell death and therefore IL-33 release. We therefore decided to create a model that integrates both, the chronic and acute phase of CFLD using Bcf followed by an acute infection with live *P. aeruginosa*. We first studied the type of cell death occurring in response to either Bcf alone or to Bcf followed by a 3 h infection with *P. aeruginosa*. Flow cytometry results demonstrate that exposure to Bcf alone does not decrease cell viability. However, infection of NuLi and CuFi cells with *P. aeruginosa* increases both, apoptotic and secondary necrotic cell death. We then studied IL-33 mRNA expression profile in the CFTRΔF508 cell lines CF2 and CuFi and in the non-CF cell line N3 following three hour exposure to *P. aeruginosa*. A significant upregulation of IL-33 mRNA is observed in CF2 and CuFi cells in response to an acute infection. No significant IL-33 mRNA increase is observed in N3 cells following infection. Finally, we sought to determine whether the increase in IL-33 expression observed in CF airway epithelial cells is related to CFTR malfunction. Pharmacological potentiation and correction of CFTR with VX-770 and VX-809 prevented the increase of IL-33 mRNA in CF airway epithelial cells infected with *P. aeruginosa*, potentially correlating CFTR channel malfunction with the increased IL-33 mRNA expression.

Chapter 4: Signaling Pathways Regulating IL-33 expression

Introduction

In the previous chapter, we demonstrated that a direct infection with *P. aeruginosa* increases IL-33 mRNA expression in CF airway epithelial cells. We were therefore interested in studying the signaling pathways that regulate IL-33 expression in this model of acute infection. It is known that TLR2 and TLR4 agonists increase IL-33 mRNA in the human acute monocytic leukemia THP-1 cells [245]. On the other hand, our group has demonstrated that TLR2 and TLR5 mediate key signaling pathways that increase inflammation in the CF airway epithelium in response to bacterial products [246]. We therefore started by measuring IL-33 expression in response to different TLR ligands and looked at the elements downstream TLR signaling implicated in the regulation of IL-33 in both, airway epithelial cells and human monocytic-like cells. We were then interested in studying the protein levels, localization and release of IL-33 in response to this model of acute infection.

Results

4.1 Signaling through TLR2 and TLR5 regulates IL-33 mRNA in response to *P. aeruginosa* infection

To determine if extracellular TLRs regulate IL-33 expression, we exposed CF airway epithelial cells to the TLR1/2, TLR4 and TLR5 agonists Pam3CSK4, LPS and flagellin, respectively. We used the IL-1R agonist IL-1β as a positive control for IL-33 upregulation [198]. As shown in figure 4.1a, exposure to the TLR5 agonist flagellin significantly upregulated IL-33 mRNA and a trend towards an increase can be observed with the TLR1/2 agonist Pam3CSK4. Incubation with the TLR4 ligand LPS did not increase IL-33 mRNA. As expected, incubation with IL-1β significantly increased IL-33

mRNA. We then wanted to validate the effect of TLR receptor signaling in our model of acute infection. For this purpose, CF airway epithelial cells were pre-incubated in the presence of neutralizing antibodies towards TLR2, TLR4, TLR5 and a combination of TLR2 and TLR5. These neutralizing polyclonal antibodies bind to extracellular TLRs preventing their activation by their ligands. Cells were then infected with *P. aeruginosa* for 3 hours and IL-33 mRNA was measured by qPCR. Neutralization of both TLR2 and TLR5 prevented IL-33 mRNA upregulation in response to infection (figure 4.1b).

Figure 4.1

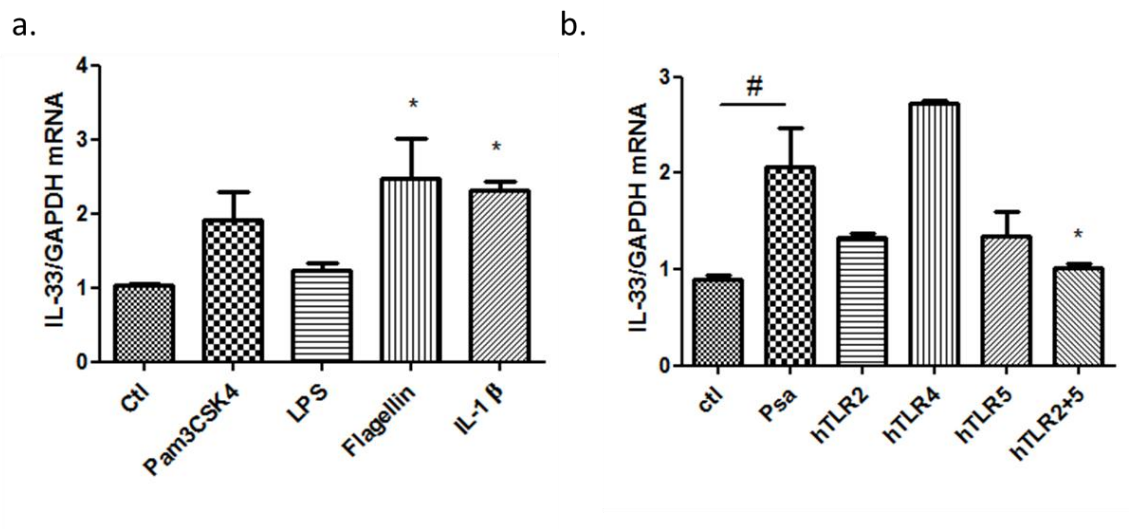


Figure 4.1. IL-33 expression is regulated through TLR2 and TLR5-activated signaling following an acute infection with *Pseudomonas aeruginosa* . A. CF2 cells were stimulated with the following TLR receptor agonists for 6 h: Pam3CSK4 (TLR1⁄2), 1µg/mL; LPS (TLR4), 1µg/mL; flagellin (TLR5), 400 ng/mL and IL-1 β, 100 ng/mL. IL-33 mRNA expression was measured by qRT-PCR. B. CF2 cells were incubated with of the neutralizing antibodies against TLR2, TLR4, TLR5 and a combination of TLR2 and TLR5 (20 µg/mL of each antibody) for 30 minutes. Cells were then infected with $9x10^6$ CFU of *P.aeruginosa* (MOI of 18) for 3 h. IL-33

mRNA expression was measured by qPCR. Results from three independent experiments were analyzed by one-way ANOVA followed by Dunnett (Fig 2A) or Bonferroni (Fig 2B) post-tests, #,*$p<0.05$. Figure 4.1 has been published in *Frontiers in Cell and Developmental Biology* [244].

4.2 TLR3 and TLR9 agonists increase IL-33 mRNA in CF airway epithelial cells

We have shown that signaling through extracellular TLR2 and TLR5 increase IL-33 mRNA expression in CF airway epithelial cells in response to infection. We were then interested in determining whether intracellular TLR signaling could also regulate IL-33 expression in our model of infection. Indeed, it has been shown that stimulation with the TLR3 agonist Poly I:C increases IL-33 mRNA in murine peritoneal macrophages *in vitro*. On the other hand, incubation with the TLR9 agonist did not induce IL-33 mRNA increase in this model [212]. Non-methylated CpG DNA from virus and bacteria acts as a ligand for TLR9 [247] and could play a role in our model of infection. We therefore incubated CF airway epithelial cells with the TLR3 and TLR9 agonists Poly(I:C) and ODN2006 and measured IL-33 expression by qRT-PCR. Incubation with both Poly(I:C) and ODN2006 significantly increased IL-33 mRNA (figure 4.2a). We were interested in further studying the involvement of TLR9 in our model of infection. First, we validated the effect of ODN2006 in TLR9 by preventing its activation with the nucleotide antagonist ODNTTAGGG. As expected, TLR9 neutralization prevented IL-33 mRNA upregulation in response to ODN2006 (figure 4.2b). We then wanted to look at the effect of TLR9 signaling in our model for which we preincubated CF airway epithelial cells with ODNTTAGGG and then infected them with *P. aeruginosa*. Interestingly, TLR9 antagonism prevented IL-33 mRNA upregulation in response to this acute infection,

suggesting that non methylated bacterial DNA can induce inflammation in CFLD (figure 4.2c).

Figure 4.2

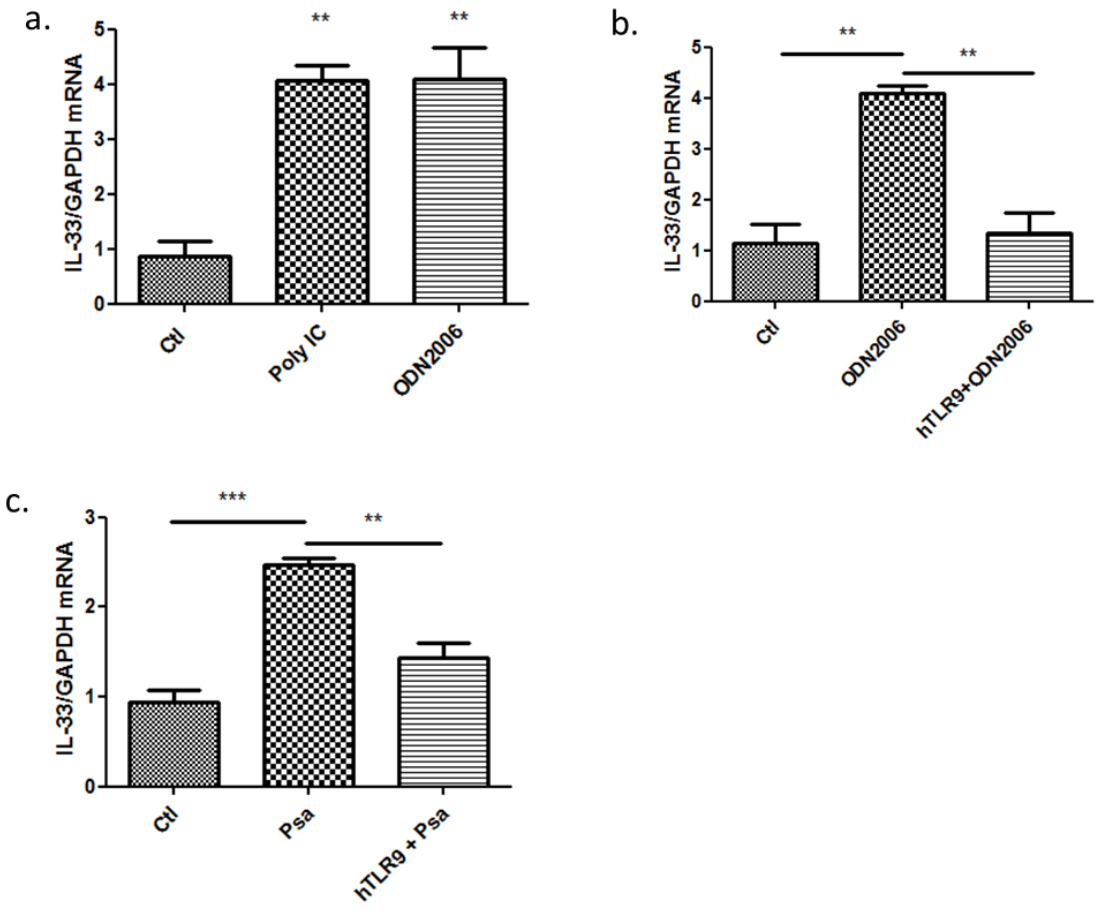


Figure 4.2. TLR3 and TLR9 agonists increase IL-33 mRNA in CF airway epithelial cells. A. CF2 cells were stimulated with the TLR3 agonist Poly(I:C) (1 μg/mL) and with the TLR9 angonist ODN2006 (2 μg/mL) for 6 h and relative IL-33 mRNA expression was quantified by qRT-PCR. Incubation with both agonists significantly increased IL-33 mRNA. B. Cells were pre-incubated with the TLR9 antagonist ODNTTAGGG (4 μg/mL) and stimulated with ODN2006 for 6 h. IL-33 expression was assessed by qRT-PCR.

Incubation with the TLR9 antagonist prevents the increase of IL-33 expression induced by the TLR9 ligand. C. Cells were pre-incubated with the TLR9 antagonist and infected with live *P. aeruginosa* for 3 hours. IL-33 mRNA expression was determined by qRT-PCR. Neutralization of TLR9 partially prevents IL-33 mRNA upregulation in response to *P. aeruginosa.*

4.3 The TAK1-IKKβ-TPL2-MKK1/2 and p38 MAPK Signaling Pathway Regulates IL-33 Expression in Response to *P. aeruginosa* in Airway Epithelial Cells

In order to identify the signaling pathways regulating IL-33 expression in our model, we used pharmacological inhibitors of kinases downstream TLR. TAK1 activity was inhibited with 5Z-7-oxozeaenol (250 nM); IKKβ activity was blocked with BI605906 (7.5μM); TPL2 kinase was inhibited using C1 (2μM); MKK1/MKK2 activity was prevented with PD184352 (2μM) and p38 MAPK activity was blocked by using BIRB0796 (0.1μM), as previously reported [150]. Then CFTRΔF508 (CF2, panel A) and CFTRG542X (CF7, panel B) airway epithelial cells were infected with *P. aeruginosa.* As shown in previous experiments, an acute infection with *P. aeruginosa* upregulated IL-33 mRNA in both cell lines, although induction was greater in CFTRΔF508 airway epithelial cells. Incubation of CFTRΔF508 cells with the different kinase inhibitors revealed that IL-33 mRNA is regulated through the TAK1, IKKβ, TPL2, MKK1/MKK2 MAPK signaling pathway as well as the TAK1, MKK3/MKK6, p38 MAPK pathway (figure 4.3A). In CFTRG542X cells the kinases IKKβ, TPL2 and MKK1/MKK2 regulate IL-33 expression in response to infection (figure 4.3B).

Figure 4.3

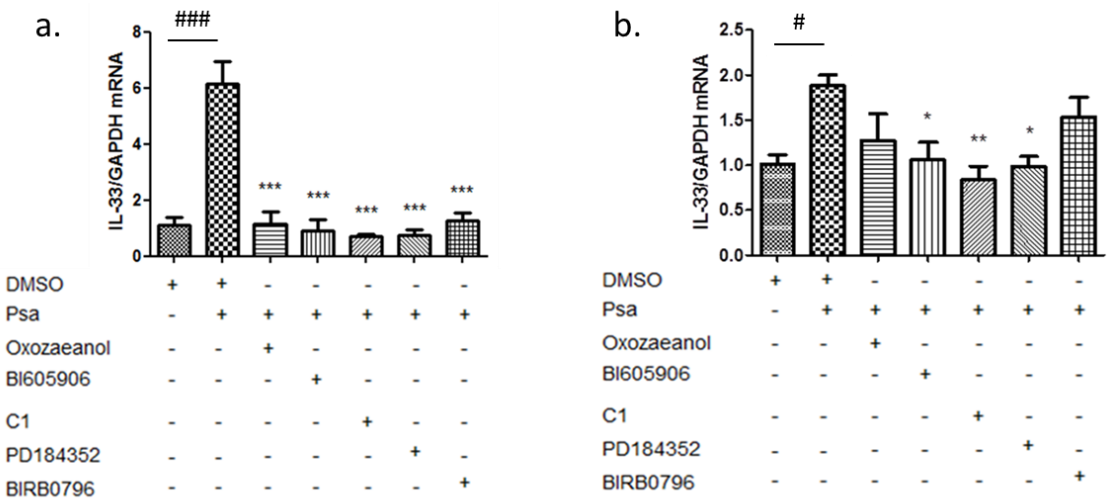


Figure 4.3. *P. aeruginosa*-driven IL-33 mRNA expression is dependent on the TAK1→IKKβ→TPL2→MKK1/MKK2 and the TAK1→MKK3/6→p38 MAPK pathways in CF airway epithelial cells. CF2 (ΔF508 mutation, panel a) and CF7 airway epithelial cells (G542X mutation, panel b) were pre-incubated for 1 hour with DMSO (vehicle) or with one of the following inhibitors: BIRB0796, 0.1 µM; PD184352, 2µM; BI605906, 7.5µM; C1, 2µM or 5Z-7-oxozeaenol, 250nM. Then, cells were infected with $9x10^6$ CFU of *P. aeruginosa* (MOI of 18) for three hours. IL-33 mRNA expression was analyzed by qRT-PCR. A. An acute infection increases IL-33 mRNA in CF2 cells. This can be prevented by blocking the kinases TAK1, IKKβ, TPL2, MKK1/2 and p38. B. An acute infection of CF7 cells with *P. aeruginosa* increases IL-33 mRNA expression. This can be prevented by inhibiting IKKβ, TPL2 and MKK1/2. Results from three independent experiments were analyzed by one-way ANOVA followed by Bonferroni post-test comparing control vs infected (#) or infected vs the different inhibitors

(*),*p<0.05, **p<0.01, ***p<0.001. Figure 4.3a has been published *Frontiers in Cell and Developmental Biology* [244].

4.4 *P. aeruginosa* induces ERK1/2 phosphorylation through TAK1-IKKβ-TPL2-MEK1/2 and p38 MAPK phosphorylation through TAK1 in CF airway epithelial cells

It has been previously shown that ERK1/2 MAPK regulate IL-33 expression in colonic subepithelial myofibroblasts in response to TNFα and IL-1β [210]. Furthermore, our group has demonstrated that the ERK1/2 MAPK is a key mediator of inflammation in response to bacterial virulence factors [46, 150]. The role for p38 MAPK in the regulation of IL-33 has been described in an *in vitro* model of human keratinocytes, where INF-γ induced IL-33 mRNA upregulation through p38 MAPK activation [211]. To demonstrate the involvement of ERK1/2 and p38 MAPKs in our model of infection, we looked at their phosphorylation in response to an acute infection with *P. aeruginosa.* Additionally, to look at the effects of different kinase inhibitors in this context, we pre-incubated CF2 cells with the following inhibitors or vehicle (DMSO): the TAK1 inhibitor 5Z-7 Oxozaeanol, 0.25μM; the TPL2 inhibitor C1, 2μM; the MKK1/2 inhibitor PD184352, 2μM and the p38 MAPK inhibitor BIRB0796, 0.1μM. In figure 4.4A we can observe that an acute infection with *Pseudomonas* increases ERK1/2 MAPK phosphorylation and that this can be prevented by blocking TAK1, TPL2 and MKK1/2 activity. Interestingly, incubation with the p38 inhibitor BIRB0796 also decreased ERK1/2 MAPK phosphorylation in response to an infection. This could be due to non-specific effects of the inhibitor or to the effects of p38 MAPK in the regulation of ERK1/2 MAPK activity. Indeed, it has been previously reported that p38γ and p38δ

isoforms play crucial roles in the stabilization of TPL2/ABIN2 complex, allowing for ERK1/2 MAPK phosphorylation in the context of TLR activation [142]. Figure 4.4B shows a relative intensity quantification of ERK1/2 MAPK phosphorylation from two independent experiments. As shown in figure 4.4C, p38 MAPK phosphorylation is also increased by an acute infection with *P. aeruginosa*. This effect can be prevented by blocking TAK1 and also partially by incubating the cells with the p38 allosteric inhibitor BIRB0796. Figure 4.4D shows a relative intensity quantification of two independent experiments.

Figure 4.4

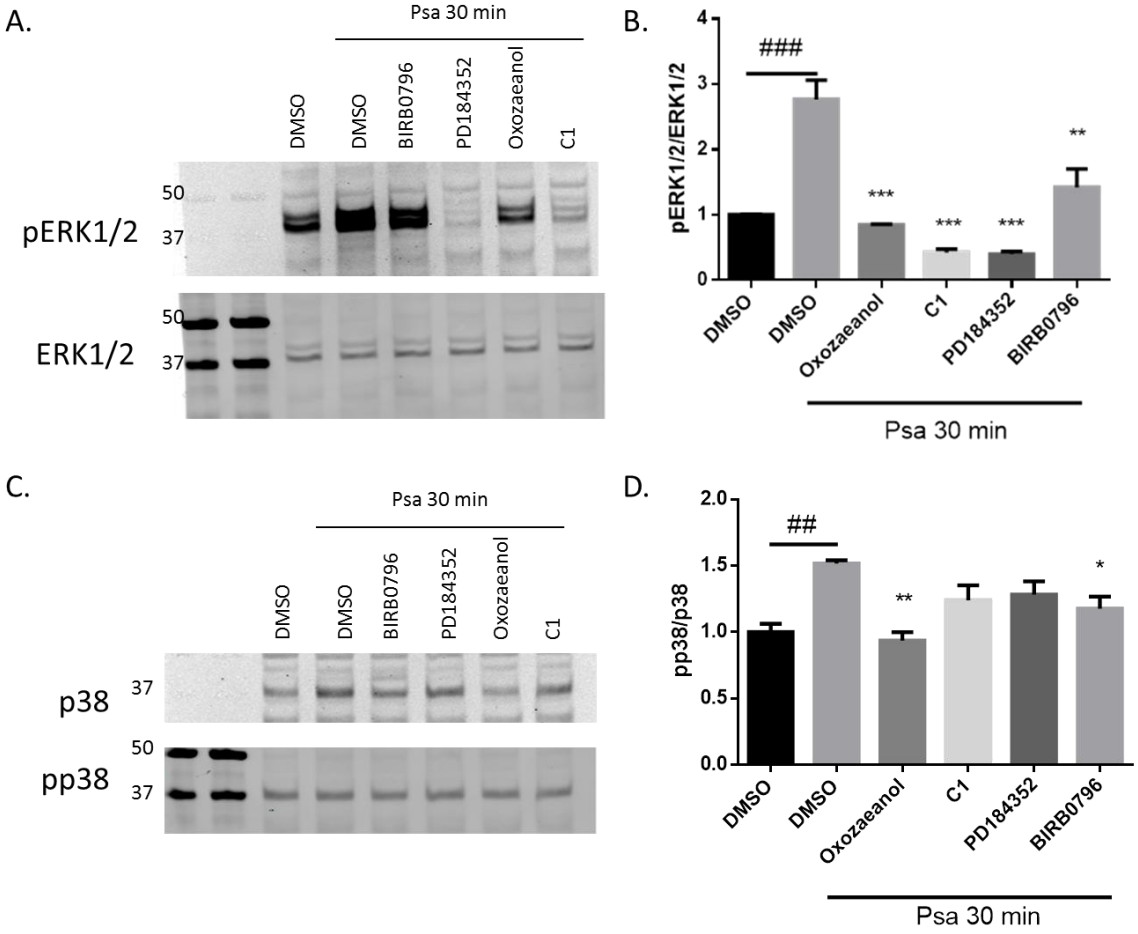


Figure 4.4. *P. aeruginosa* induces ERK1/2 phosphorylation through TAK1-IKKβ-TPL2-MEK1/2 and p38 MAPK phosphorylation through TAK1 in CF airway epithelial cells. CF2 cells were pre-incubated in the presence of the following inhibitors or vehicle (DMSO) for 1 hour at the following concentrations: the TAK1 inhibitor 5-7 oxozaeanol, 0.25μM; the TPL2 inhibitor C1, 2μM; the MKK1/2 inhibitor PD184352, 2μM and the p38 MAPK inhibitor BIRB0796, 0.1μM. Cells were then infected with *P. aeruginosa* (MOI of 18) for 30 minutes and p38 and ERK1/2 phosphorylation was measured by Western Blot. A. An acute infection with *P. aeruginosa* increases ERK1/2 MAPK phosphorylation and this can be prevented by blocking TAK1, TPL2 and MEK1/2. The p38 MAPK inhibitor also partially decreases ERK1/2 MAPK phosphorylation. B. Relative intensity quantification of ERK1/2 phosphorylation from two independent experiments. C. p38 MAPK phosphorylation is increased by *P. aeruginosa* and this can be prevented by pre-incubating the cells with the TAK1 inhibitor oxozaeanol. The allosteric p38 MAPK inhibitor BIRB0796 also prevents its phosphorylation in response to infection. D. Relative intensity quantification of p38 MAPK phosphorylation from two independent experiments. Results were compared by one-way ANOVA ($p<0.0001$ for ERK1/2 and $p=0.0034$ for p38) followed by Bonferroni post-hoc test, comparing control vs infected (#) or infected vs the different inhibitors (*),*$p<0.05$, **$p<0.01$, ***$p<0.001$. Figure 4.4a has been published in *Frontiers in Cell and Developmental Biology [244]*.

4.5 *P. aeruginosa* increases IL-1α and S100A9 mRNA expression

We then analyzed the expression patterns of other alarmins in our model of infection in the presence of the different kinase inhibitors downstream TLR. IL-1α expression is strongly increased when cells are exposed to *P. aeruginosa* and its upregulation in this context can be prevented by blocking IKKβ, TPL2 and MKK1/2 kinases (figure 3.5a). The calcium-binding protein S100A9 is also significantly increased upon an infection, however its expression is regulated through a different pathway since none of the inhibitors used significantly prevented this upregulation (figure 3.5b). Despite showing high levels of transcripts constitutively, the DNA binding protein HMGB1 seems to be decreased in response to an infection with *P. aeruginosa* and no significant effect can be observed upon incubation with the different kinase inhibitors (figure 3.5c).

Figure 4.5

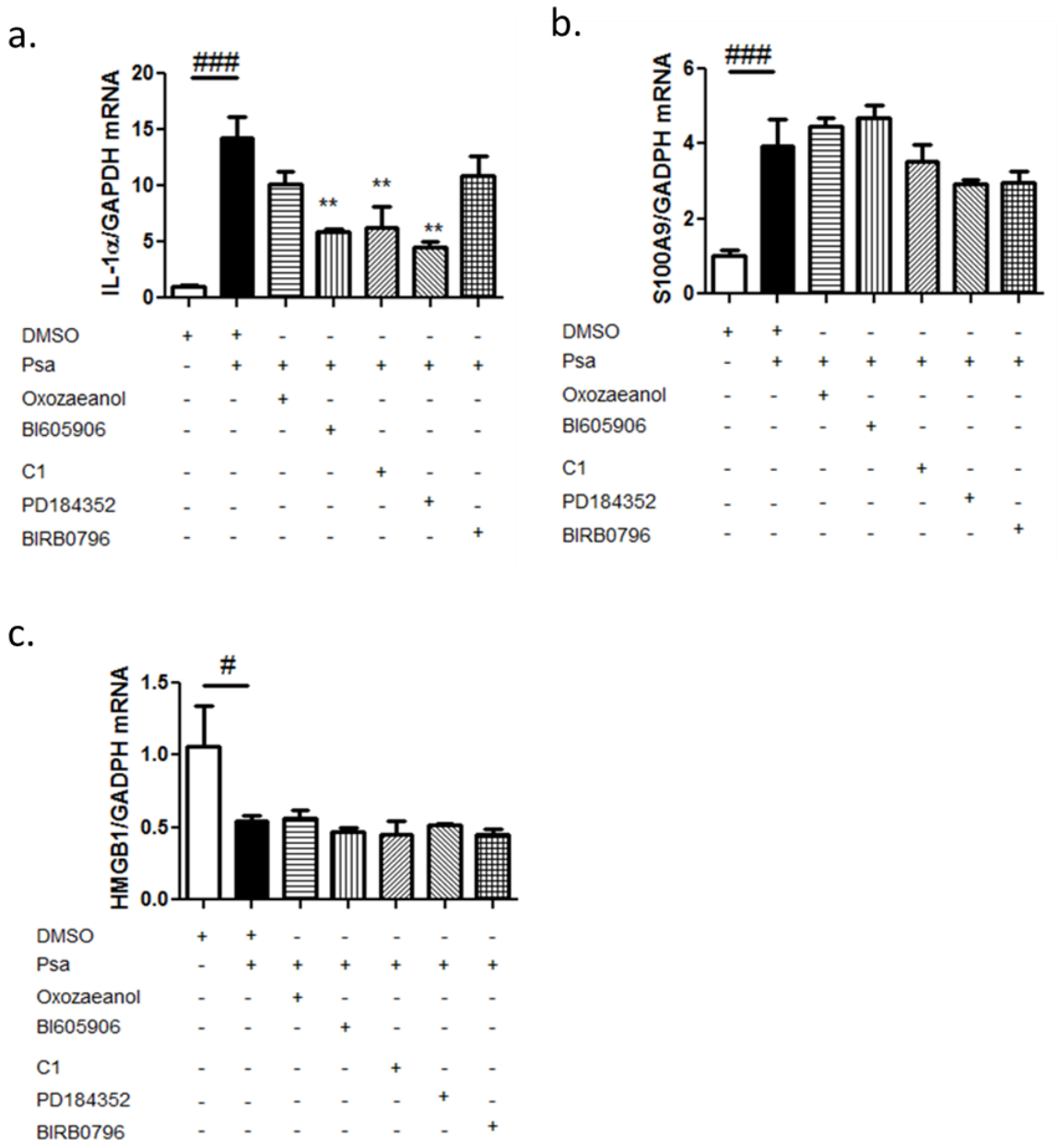


Figure 4.5. *P. aeruginosa* infection increases IL-1α and S100A9 mRNA expression but not that of HMGB1. CF airway epithelial cells were pre-incubated with different kinases inhibitors as described in figure 3. Cells were then infected with *P. aeruginosa* (MOI of 18) for three hours. IL-1α (panel A), S100A9 (panel B) and HMGB1 (panel C)

gene expression was measured by qRT-PCR. Results were analyzed by one-way ANOVA followed by Bonferroni post-test comparing control vs infected (#) or infected vs the different inhibitors (*), *p<0.05, **p<0.01, ***p<0.001. Figure 4.5 has been published in *Frontiers in Cell and Developmental Biology* [244].

4.6 IL-33 is increased in the cytoplasm of CF and non CF cells following a bacterial infection with *P. aeruginosa*

We previously showed that a 24 hour exposure of airway epithelial cells to PsaDM increases IL-33 in the nucleus of cells. We wanted to determine if an acute bacterial infection has a similar effect on nuclear IL-33 protein levels. For this purpose, we separated the nuclear and cytoplasmic fractions from control and infected non-CF (N3) and CF (CF2) cells and analyzed IL-33 levels in both cellular compartments by Western Blot. We used the antibody against lamin A/C as a nuclear marker and GAPDH as a marker of both nuclear and cytoplasmic proteins. In figure 4.6a we can observe the presence of multiple bands in the nuclear extracts from both untreated cell lines when immunoblotting against IL-33 with the polyclonal antibody. Similarly, at least two bands of approximate molecular weights of 37 and 45 kDa can be seen in the cytoplasmic extracts of both untreated cell lines. An acute infection with *P. aeruginosa* strongly increases the cytoplasmic band at approximately 45 kDa. We quantified the relative intensity of the cytoplasmic bands from three independent experiments and we can consistently observe this increase of the 45 kDa cytoplasmic band upon infection (figure 4.6b). These results contrast with the results obtained in the model of chronic infection, in which PsaDM increases nuclear IL-33. We wanted to confirm these results in two more cell lines that had undergone the same transformation process for which we took the

cytoplasmic fractions of N1 (non-CF) and CF7 (CF) airway epithelial cells infected with *P. aeruginosa*. We could also observe an increase of the 45 kDa cytoplasmic band following an infection in both cell lines (figure 4.6c). Whether, the 45 kDa band is a post-translationally modified form of IL-33 remains to be confirmed, as preliminary mass spectrometry data was inconclusive.

Figure 4.6

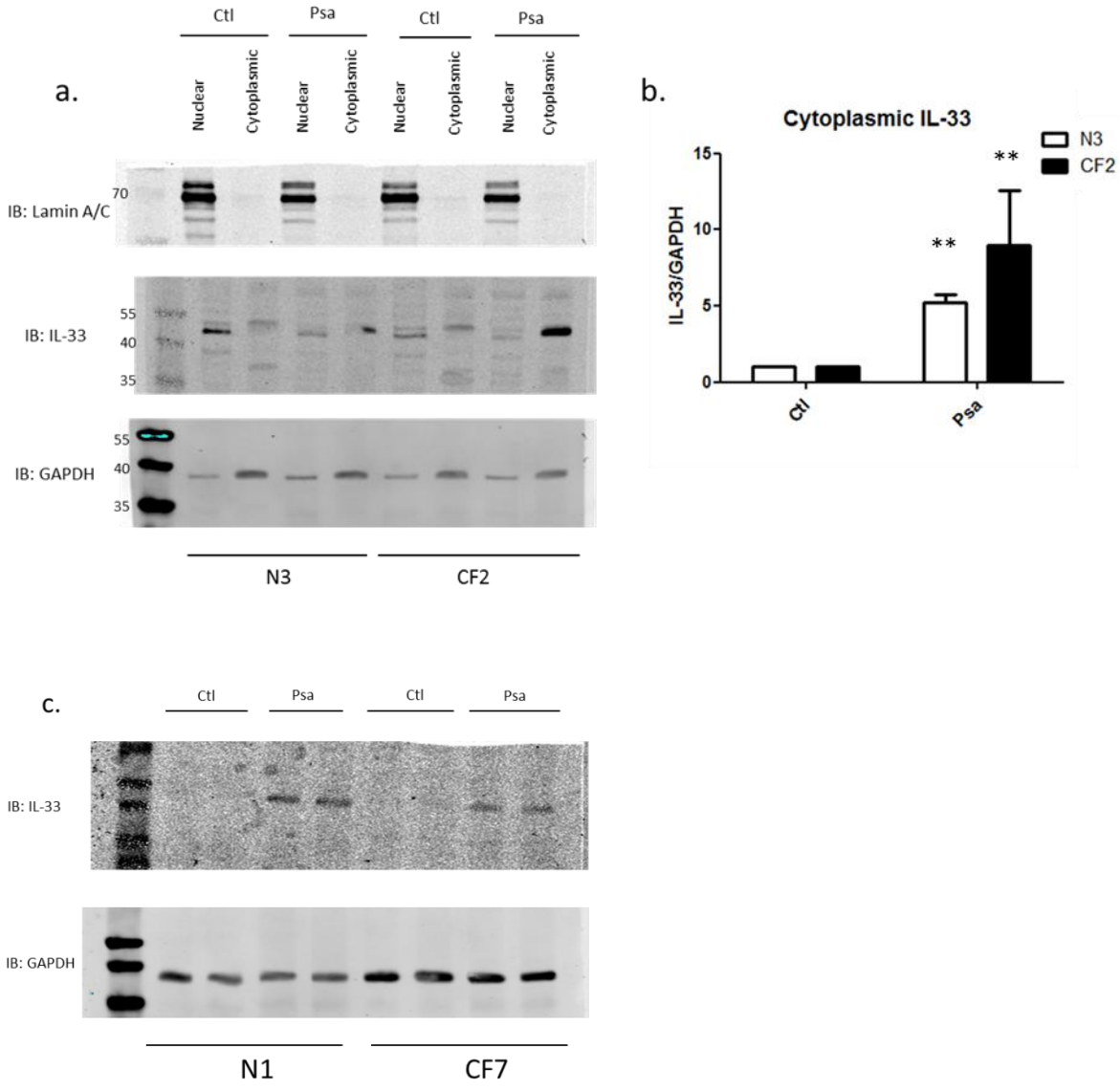


Figure 4.6. Intracellular IL-33 levels are increased following an acute infection with *P. aeruginosa.* A. Non-CF (N3) and CFTRΔF508 (CF2) airway epithelial cells were infected with live *Pseudomonas aeruginosa* (MOI of 2) for 6 h. Cells were then harvested and protein extracts from nuclear and cytoplasmic fractions were separated and analyzed by Western Blot. Lamin A/C was used as a marker of nuclear protein. GAPDH was used to normalize protein contents from cytoplasmic extracts. IL-33 was detected with a polyclonal antibody. B. Quantification of cytoplasmic IL-33 from 3 independent experiments was analyzed by two-way ANOVA followed by a Bonferroni post-test, **$p<0.001$ for row factors and non-significant for column factors and interaction. C. Non-CF (N1) and CFTRG542X (CF7) cells were infected with *P. aeruginosa* for 6 hours. Cytoplasmic IL-33 was detected by Western Blot.

4.7 *P. aeruginosa* infection increases cytoplasmic IL-33 in primary airway epithelial cells

In light of the previous findings, we wanted to look at the effect of an acute *P. aeruginosa* infection in non-transformed airway epithelial cells. For this purpose, we took normal bronchial epithelial cells (NHBE), infected them with *P. aeruginosa* and measured IL-33 expression by qRT-PCR and protein levels by Western Blot. Although a trend can be observed, infection with *P. aeruginosa* does not significantly increase IL-33 mRNA in NHBE cells (figure 4.7a). Similarly to what was found with transformed cells, an infection with *P. aeruginosa* increases the abundance of 45kDa cytoplasmic band whose identity has yet to be confirmed. (figure 4.7b).

Figure 4.7

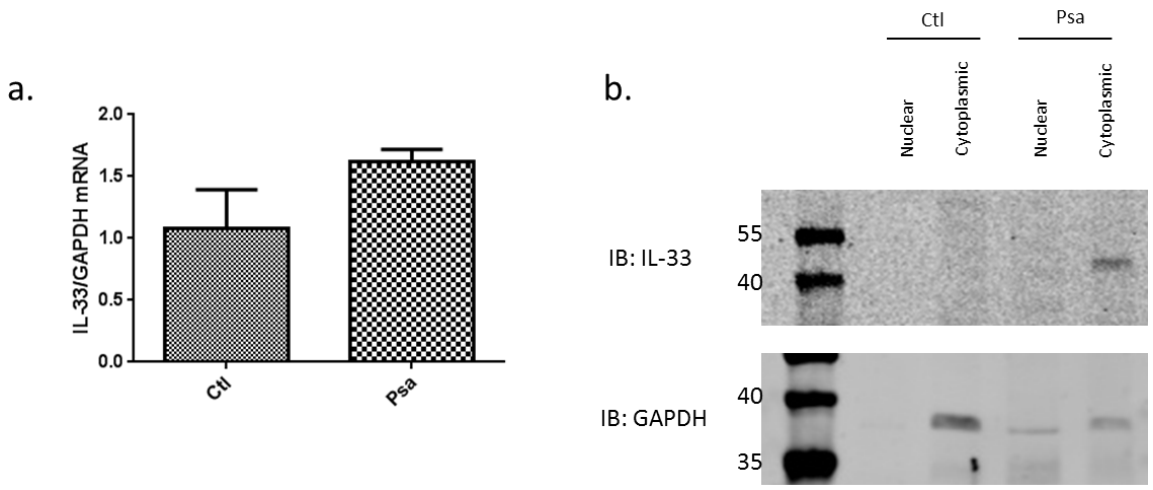


Figure 4.7. *P. aeruginosa* infection increases cytoplasmic IL-33 in primary airway epithelial cells. A. NHBE cells were infected with *P. aeruginosa* (MOI of 18) for 3 hours and IL-33 expression was measured by qRT-PCR. B. NHBE cells were infected with *P. aeruginosa* (MOI of 2) for 6 hours, nuclear and cytoplasmic proteins were isolated and intracellular IL-33 was detected by Western Blot.

4.8 Overexpression of IL-33 drives its localization to the cytoplasm of HEK293 cells

The localization of endogenous IL-33 in both the nucleus and the cytoplasm has been previously reported [208]. Therefore, IL-33's localization in different sub-cellular compartments could be cell-type and stimulus dependent. Moreover, upregulation of IL-33 might drive its localization to the cytoplasm of cells that normally contain it in the nucleus. In order to determine if the presence of cytoplasmic IL-33 is dependent on its expression level, we overexpressed IL-33 in HEK293 cells. We used HEK293 cells because they endogenously express low levels of IL-33 [201]. While endogenous IL-33 is mainly localized in the nucleus of HEK293 cells, overexpression of full-length IL-33

increases its presence in the nucleus, as well as driving its localization into the cytoplasm (figure 4.8). The band corresponding to IL-33 can be observed at a molecular weight of approximately 37 kDa, in contrast with the higher molecular weight bands observed in airway epithelial cells.

Figure 4.8

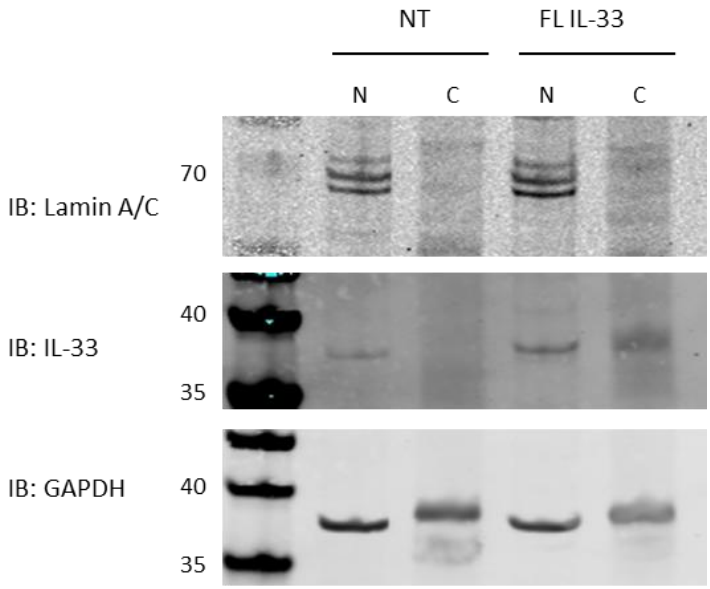


Figure 4.8. Overexpression of IL-33 drives its localization to the cytoplasm of HEK293 cells. HEK293 cells were either not transfected (NT) or transiently transfected with the plasmid vector PCMV4a carrying full-length IL-33 (FL IL-33). Nuclear and cytoplasmic fractions were isolated and intracellular IL-33 was detected by Western Blot. Lamin A/C was used as a marker of nuclear protein. Low levels of nuclear IL-33 are observerd in non-transfected cells. IL-33 overexpression increases nuclear IL-33 and drives its localization to the cytoplasm.

4.9 IKKβ and TPL2 regulate the levels of intracellular IL-33 following an infection with *P. aeruginosa*

We then wanted to assess the role of IKKβ and TPL2 in regulating IL-33 cytoplasmic protein levels. To do so, CF2 cells were preincubated in the presence of the IKKβ inhibitor BI605906 and the TPL2 inhibitor C1. Cells were then infected with *P. aeruginosa* for 6 hours. To release cytoplasmic contents, cells were permeabilized with a hypotonic buffer solution and IL-33 levels in these cell lysates were measured by ELISA. In accordance with previous results, an acute infection with *P. aeruginosa* significantly increased cytoplasmic IL-33. This increase can be prevented by blocking IKKβ and TPL2 kinases (figure 4.9).

Figure 4.9

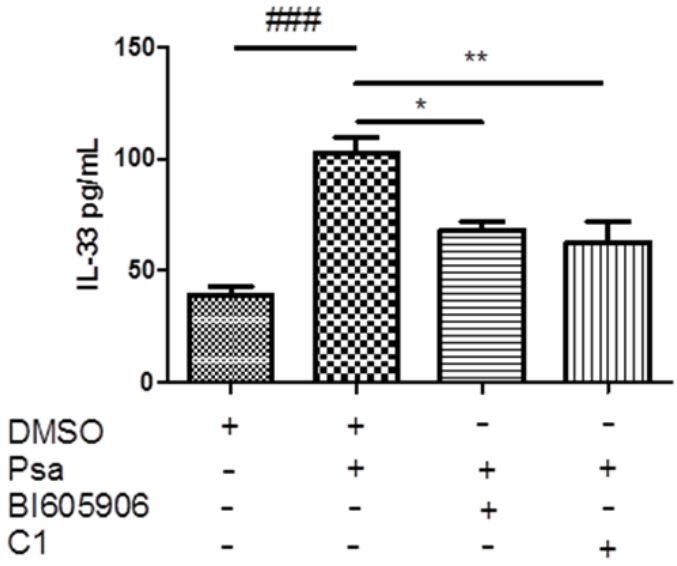


Figure 4.9. IKKβ and TPL2 regulate the levels of intracellular IL-33 following an infection with *P. aeruginosa*. CF airway epithelial cells were pre-incubated in the presence of either DMSO (vehicle) or one of the following inhibitors: the IKKβ inhibitor

BI605906 (7.5 μM) or the TPL2 inhibitor C1 (2μM). Cells were then infected with *Pseudomonas aeruginosa* (MOI of 2) for 6 hours and lysed in a hypotonic solution as described in the methods section. IL-33 levels were quantified by ELISA. Results were analyzed by One-way ANOVA followed by Bonferroni multiple comparison test, n=6 for control and stimulated and n=3 for the different inhibitors, *p<0.05, **p<0.01, ### p<0.0001. A modified version of figure 4.9a has been published in *Frontiers in Cell and Developmental Biology* [244]

4.10 IL-33 is not released in our *in vitro* model of acute infection

We next wanted to measure extracellular IL-33 levels in CF2 cells following a 6 hour infection with *P. aeruginosa*. For this purpose, we took the supernatants of control and infected cells and measured IL-33 release by ELISA. Although a trend towards an increase can be observed, extracellular IL-33 is not significantly increased in our model of infection (figure 4.10a).

The danger signal ATP, through its actions on P_2Y_2 purinergic receptors, is known to release IL-33 extracellularly in fibroblasts and airway epithelial cells [208, 209, 216]. We wanted to determine whether addition of ATP would release the intracellularly accumulated IL-33 in our model of infection. For this purpose, cells were infected with *P. aeruginosa* and incubated in the presence of ATP for 6 hours. IL-33 release was then measured in the supernatants by ELISA. In contrast with previous studies, addition of ATP did not release IL-33 in our *in vitro* model of infection (figure 4.10b). This might be due to the level of cell differentiation, since the airway epithelial cells known to release IL-33 have been characterized as basal progenitor cells [216].

Figure 4.10

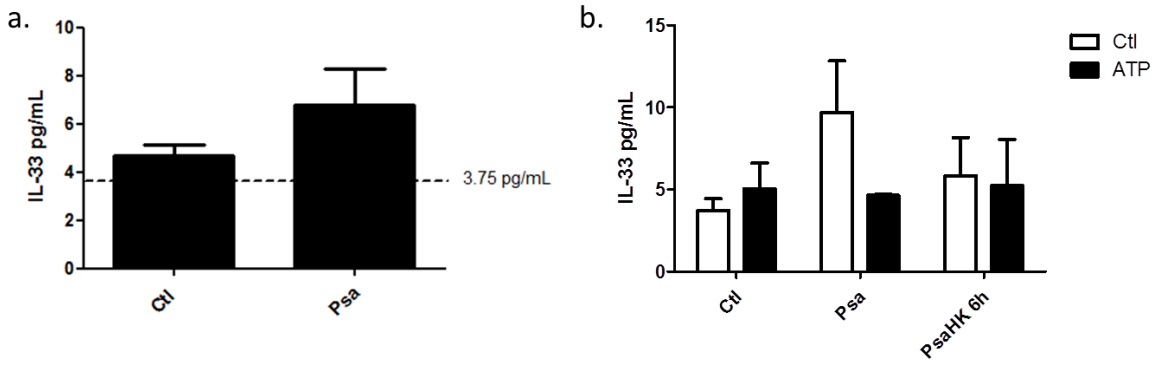


Figure 4.10. IL-33 is not released in our *in vitro* model of acute infection. A. CF airway epithelial cells were infected with live *P. aeruginosa* (MOI of 2) for 6 hours. Supernatants were collected and extracellular IL-33 levels were measured by ELISA. Although a trend can be observed, an acute infection with *P. aeruginosa* does not increase release of IL-33 to the extracellular space. B. CF cells were incubated with live *P. aeruginosa* or with heat-killed bacteria (PsaHK) and 1 hour later ATP was added to the culture media to a final concentration of 100μM. Supernatants were collected and IL-33 was measured by ELISA. Figure 4.10a has been published in *Frontiers in Cell and Developmental Biology* [244].

4.11 Summary of signaling pathways regulating IL-33 expression in CF airway epithelial cells

Figure 4.11 summarizes the signaling mechanisms regulating IL-33 expression in CF airway epithelial cells in response to an acute infection with *P. aeruginosa*. The contact of bacterial derived products with TLR2 and TLR5 induces the recruitment of adaptor molecules that activate TAK1. TAK1 can activate the IKK complex and the activation of IKKβ induces the TPL2-MKK1/2-ERK1/2 signaling pathway which

increases IL-33 expression. TAK1 is also a MAP3K that can phosphorylate and activate MKK3/6 which in turn activate p38 MAPK to upregulate IL-33 mRNA. The effects of NFκB and the MAPK JNK were not assessed in the present study.

Figure 4.11

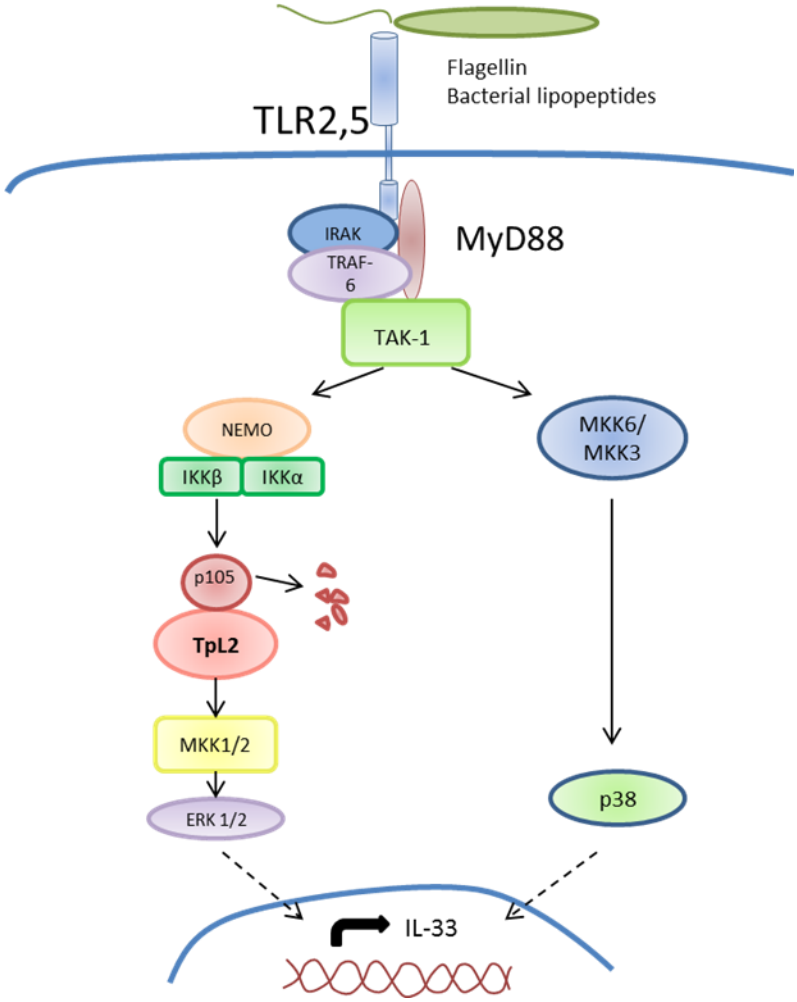


Figure 4.11. Signaling pathways regulating IL-33 expression in airway epithelial cells following an acute bacterial infection with *P. aeruginosa*. Binding of the TLR2 and TLR5 agonists to their receptors leads to the recruitment of the adaptor molecules MyD88, IRAK and TRAF6, which in turn activate TAK-1. TAK-1 activates the IKK

complex formed by NEMO, IKKα and IKKβ. Activation of IKKβ leads to the degradation of the p105 and the subsequent activation of TPL2. TPL2 in turn phosphorylates MKK1/MKK2 which phosphorylate and activate ERK1/2 MAPK. ERK1/2 activates downstream transcription factors that upregulate IL-33 mRNA expression. TAK-1 is also a MAP3K and on a parallel pathway phosphorylates and activates MKK3/MKK6 which in turn phosphorylate p38 MAPK. Phosphorylation of p38 MAPK activates downstream effectors ultimately increasing the expression of IL-33 mRNA.

4.12 PsaDM increases IL-33 expression in undifferentiated monocytic cells

Monocytes and macrophages are key immune cells involved in the innate response against *P. aeruginosa* [61, 62]. Furthermore, it has been described that TLR3 and TLR4 agonists upregulate IL-33 mRNA in murine peritoneal macrophages [212]. We therefore wanted to study the expression patterns of IL-33, the alarmins IL-1α, HMGB1, S100A8 and S100A9 in the human monocytic THP-1 cells in response to PsaDM and the TLR4 and TLR5 agonists LPS and flagellin, respectively. For this purpose, non-differentiated THP-1 cells were incubated in the presence of PsaDM (8ng/mL), LPS (100ng/mL) and flagellin (100ng/mL) for 6 hours and mRNA expression of the alarmins was measured by qPCR. As shown in figure 4.11, PsaDM strongly induced mRNA upregulation of both IL-33(4.11a.) and IL-1α (4.11b.). This strong induction might be due to the high level of TLR expression in monocytes and to the fact that several TLR ligands are present in PsaDM. Although non-significant by our statistical analysis, an approximate 5-fold increase in IL-33 and IL-1α expression is observed following exposure to the TLR4 or TLR5 ligands. HMGB1 expression levels remain unchanged in

response to the latter stimuli (figure 4.11c). PsaDM strongly induced S100A8 (figure 4.11d.) and S100A9 (figure 4.11e.) mRNA upregulation, whereas neither LPS nor flagellin had an effect in the mRNA levels of these alarmins at this particular time point.

Figure 4.12

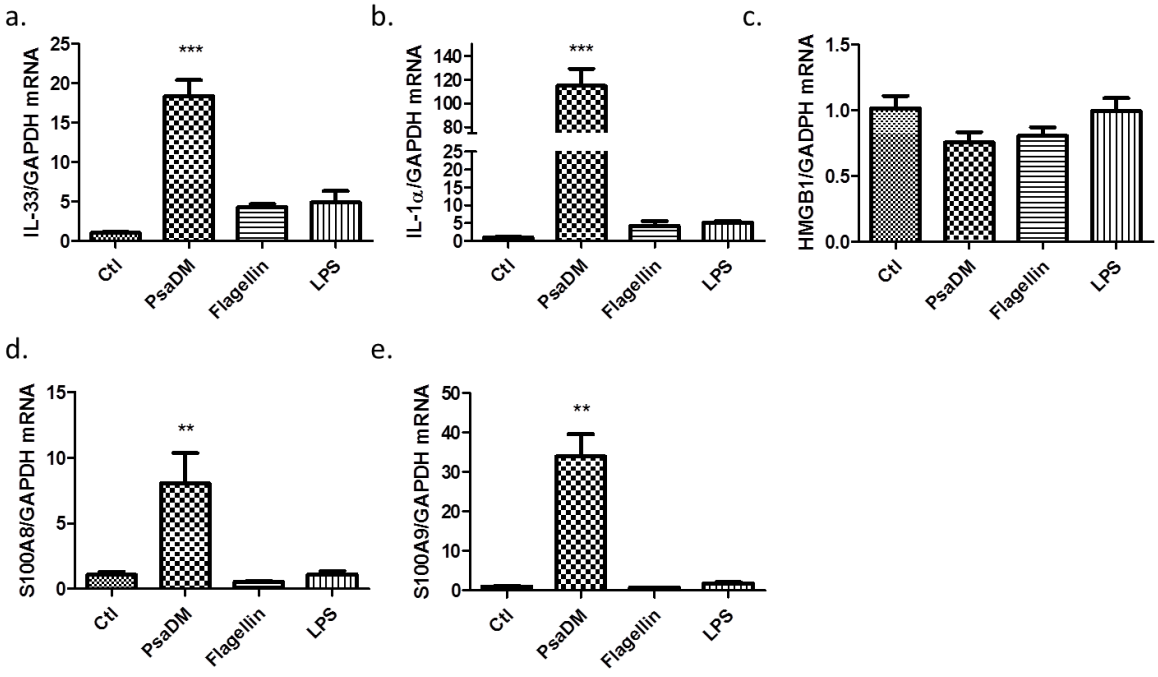


Figure 4.12: PsaDM increases IL-33 mRNA in undifferentiated THP-1 cells. THP-1 cells were incubated with PsaDM (8ng/mL), the TLR4 agonist LPS (100ng/mL) or the TLR5 agonist flagellin (100ng/mL) for 6 h. IL-33, IL-1α, HMGB1, S100A8 and S100A9 expression was measured by qRT-PCR (a, b, c, d and e, respectively). A. PsaDM strongly induces IL-33 mRNA upregulation. Although not significant, a 5-fold increase in IL-33 mRNA can be observed with the TLR4 and TLR5 agonists. B. IL-1α mRNA is significantly increased with PsaDM. Similar to IL-33, a trend can be observed with the

TLR4 and TLR5 agonists. C. HMGB1 mRNA expression remains unchanged following exposure to PsaDM, LPS and flagellin. D. and E. S100A8 and S100A9 expression are significantly increased with PsaDM whereas exposure to the TLR4 or TLR5 agonists does not change the expression levels of these alarmins. Analyzed by One-Way ANOVA followed by Dunnet post-test, n=3. **p<0.01, *** p<0.0001.

4.13 PsaDM and TLR1/2, TLR4 and TLR5 agonists increase IL-33 mRNA expression in PMA-differentiated THP-1 cells

In the previous experiment we showed an increase in IL-33 mRNA in response to PsaDM in undifferentiated THP-1 cells. Since the level of differentiation can affect gene expression, we wanted to look at the response of macrophages to different TLR agonists and PsaDM. We therefore differentiated THP-1 cells into macrophage-like cells with PMA and incubated them in the presence of the TLR1/2 agonist Pam3CSK4, the TLR3 agonist Poly(I:C), the TLR4 agonist LPS, the TLR5 agonist flagellin and PsaDM for 6 hours. Incubation with PsaDM and with agonists for TLR1/2, TLR4 and TLR5 significantly increased IL-33 (Figure 4.12a) and IL-1α (Figure 4.12b) mRNA. The TLR3 agonist Poly(I:C) did not significantly upregulate IL-33 or IL-1α mRNA in this model.

Figure 4.13

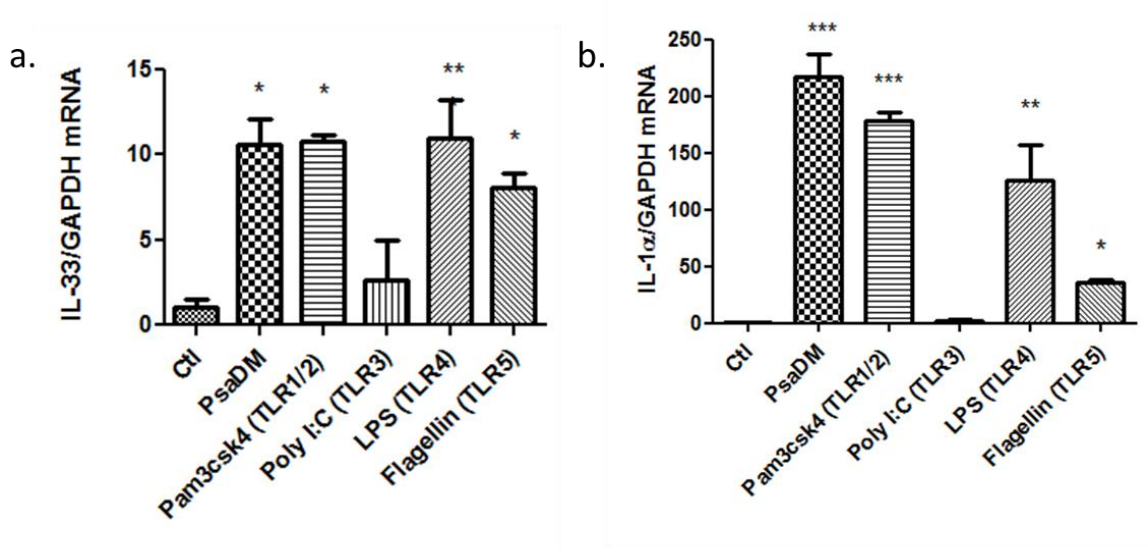


Figure 4.13: PsaDM and TLR1/2, TLR4 and TLR5 agonists increase IL-33 mRNA in PMA-differentiated THP-1 cells. THP-1 cells were differentiated with PMA (100 nM) and incubated with PsaDM, the TLR1/2 agonist Pam3CSK4 (1μg/mL), the TLR3 agonist Poly(I:C) (1μg/mL), the TLR4 agonist LPS (100 ng/mL) and the TLR5 agonist flagellin (100 ng/mL) for 6 h. IL-33 (a.) and IL-1α (b.) expression was measured by qRT-PCR. PsaDM as well as the TLR1/2, TLR4 and TLR5 agonists significantly increase IL-33 and IL-1α mRNA expression in differentiated THP-1 cells. Analyzed by One-Way ANOVA followed by Dunnett's post-test, n=3. * $p<0.05$, **$p<0.01$, *** $p<0.0001$.

4.14 The TLR5-TAK1-IKKβ-MKK1/2 signaling pathway regulates IL-33 expression in macrophage-like cells

We next wanted to look at the pathways regulating IL-33 expression in differentiated THP-1 cells in response to TLR5 activation. For this purpose, cells were pre-incubated for one hour in the presence of vehicle or one of the following: the TAK1

inhibitor 5Z-7-oxozeaenol, the IKKβ inhibitor BI605906, the MKK1/2 inhibitor PD184352 or the p38 MAPK inhibitor BIRB0796. Cells were then stimulated with flagellin for 6 hours and IL-33 expression was analysed by qRT-PCR. As shown in figure 4.13a, inhibition of TAK1, IKKβ and MKK1/2 prevents IL-33 mRNA upregulation in response to flagellin. Inhibition of p38 MAPK did not prevent this IL-33 mRNA increase, suggesting that p38 MAPK does not modulate IL-33 expression in response to inflammatory stimuli in this cell line. We then wanted to assess the signaling pathways regulating IL-33 in response to PsaDM. In addition to the inhibitors mentioned above, cells were pre-incubated in the presence of the TPL2 inhibitor C1, exposed to PsaDM for 6 hours and IL-33 expression was measured by qRT-PCR. As shown in figure 4.13b, inhibition of TAK1, IKKβ and MKK1/2 prevented the increase of IL-33 mRNA induced by PsaDM. Inhibition of TPL2 could not prevent IL-33 upregulation, suggesting that ERK1/2 activation in differentiated macrophages is mediated through a different signaling pathway. This could be due to the effect of PMA in intracellular signaling, since it is a known inducer of PKC, an activator of Raf kinases, which in turn can activate MKK1/2 independently from TPL2 [248, 249] signaling and has been shown to induce IL-33 expression Similar to the previous experiment, the p38 MAPK inhibitor had no effect in IL-33 expression in response to PsaDM.

Figure 4.14

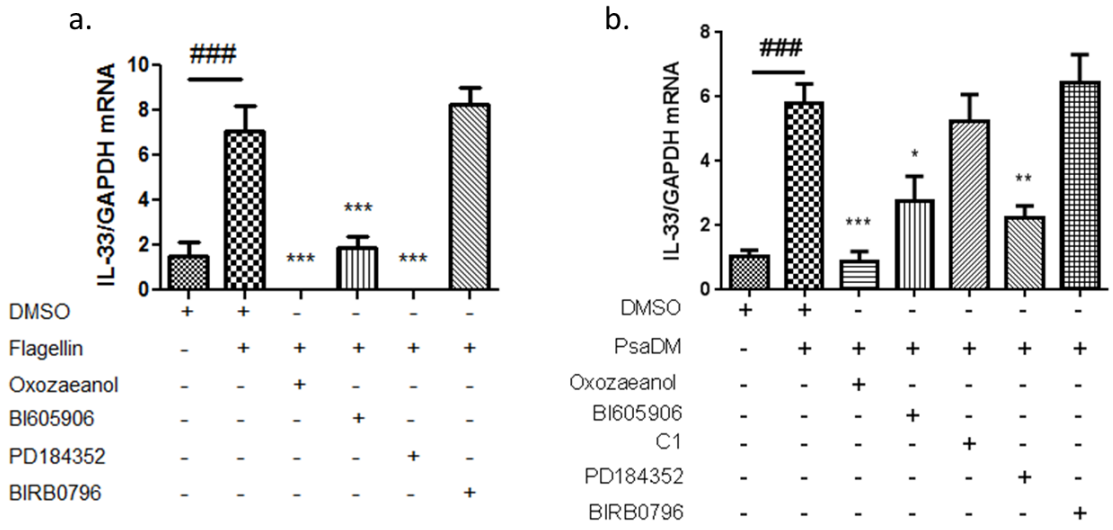


Figure 4.14: The TAK1-IKKβ-MKK1/2 signaling pathway regulates IL-33 expression in macrophage-like cells. A. PMA-differentiated THP-1 cells were incubated for 1 h in the presence of vehicle (DMSO) or each one of the following inhibitors: 5Z-7-oxozeaenol, 250nM; BI605906, 7.5μM; BIRB0796, 0.1 μM or PD184352, 2μM. Cells were then stimulated with the TLR5 agonist flagellin (100 ng/mL) for 6 h and IL-33 mRNA was measured by qRT-PCR. Stimulation with flagellin significantly increases IL-33 mRNA and this can be prevented by inhibiting TAK1, IKKβ and MKK1/2 kinases. Inhibition of p38 MAPK had no effect in IL-33 expression in response to flagellin. B. In addition to the inhibitors mentioned in A., differentiated THP-1 cells were incubated with the TPL2 inhibitor C1, 2 μM for 1 h. Cells were then incubated with PsaDM for 6 h and IL-33 mRNA expression was assessed by qRT-PCR. PsaDM increased IL-33 mRNA and this can be prevented by blocking TAK1, IKKβ and MKK1/2. Inhibition of TPL2 and p38 MAPK did not affect IL-33 mRNA expression in response to PsaDM. Results were analyzed by One-Way ANOVA followed by

Bonferroni post-test comparing controls vs stimulated (#) or stimulated vs the different inhibitors (*), n=3. * $p<0.05$, **$p<0.01$, *** or ### $p<0.0001$.

4.15 The TLR5 agonist flagellin increases p38 and ERK1/2 MAPK phosphorylation in THP-1 cells

We wanted to assess the effect of the TLR5 agonist flagellin in p38 and ERK1/2 MAPK phosphorylation in THP-1 cells. Additionally, we wanted to look at the effects of the kinase inhibitors used in the previous experiment in p38 and ERK1/2 MAPK phoshorylation. We therefore pre-incubated THP-1 cells for one hour in the presence of vehicle (DMSO) or one of the following inhibitors: 5Z-7-oxozeaenol (TAK1), C1 (TPL2), PD184352 (ERK1/2) or BIRB0796 (p38). Cells were then stimulated with flagellin for 30 minutes and p38 and ERK1/2 MAPK phosphorylation was assessed by Western blot. As shown in figure 4.13a, Flagelllin induces p38 MAPK phosphorylation and this can be prevented by blocking either TAK1 or p38 MAPK itself. The TPL2 and the MKK1/2 inhibitors did not affect p38 MAPK phosphorylation in response to TLR5 activation. ERK1/2 MAPK shows high levels of phosphorylation even in control conditions and the response to flagellin is difficult to assess in this context (figure 4.13b). However, we can observe that the inhibitors of TAK1, MKK1/2 and TPL2 kinases prevent ERK1/2 MAPK phosphorylation in these conditions.

Figure 4.15

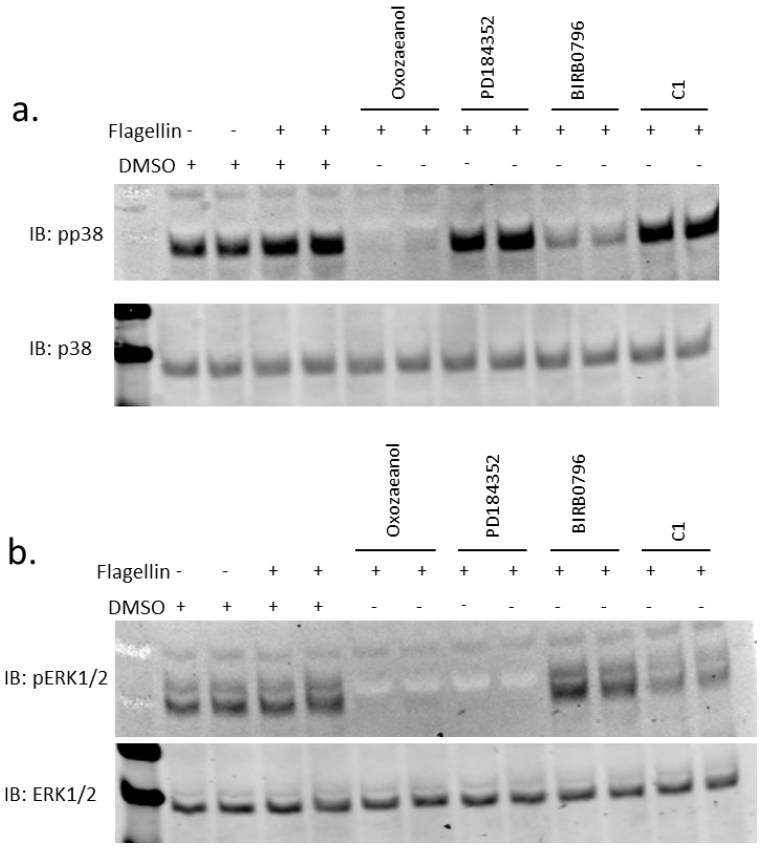


Figure 4.15: p38 and ERK1/2 MAPK phosphorylation in response to the TLR5 agonist flagellin in THP-1 cells. THP-1 cells were pre-incubated for 1 h in the presence of DMSO or the following kinase inhibitors: 5Z-7-oxozeaenol, 250nM; BIRB0796, 0.1 µM; PD184352, 2µM or C1 2µM. Cells were then incubated for 30 min with the TLR5 agonist flagellin, 100ng/mL and p38 and ERK1/2 MAPK phosphorylation was assessed by Western Blot. A. Incubation with flagellin increases p38 MAPK phosphorylation and this can be prevented by blocking TAK1 and p38 MAPK itself. The MKK1/2 inhibitor PD184352 or the TPL2 inhibitor C1 did not have an effect on p38 MAPK phosphorylation. B. High levels of constitutive ERK1/2 MAPK phosphorylation are present in THP-1 cells and it is therefore difficult to assess the effect of flagellin in this

kinase. However, the TAK1 inhibitor 5Z-7-oxozeaenol as well as the TPL2 and MKK1/2 inhibitors (C1 and PD184352, respectively) prevented ERK1/2 MAPK phosphorylation.

4.16 IL-33 is increased in the nucleus of THP-1 cells after 24h incubation with PsaDM

We finally wanted to look at the effects of PsaDM in intracellular IL-33 in THP-1 cells by immunofluorescence microscopy. Differentiated THP-1 cells were exposed to PsaDM for 24 hours and IL-33 was detected with the monoclonal antibody Nessy-1 (see Materials and Methods). In unstimulated cells, a faint staining for IL-33 can be observed in the cytoplasm (figure 4.14, upper panels). Incubation with PsaDM increases intracellular IL-33 which is mainly localized in the nucleus (figure 4.14, lower panels). Supernatants from this experiment were collected to measure extracellular IL-33 by ELISA, however IL-33 levels were below the limit of detection (data not shown).

Figure 4.16

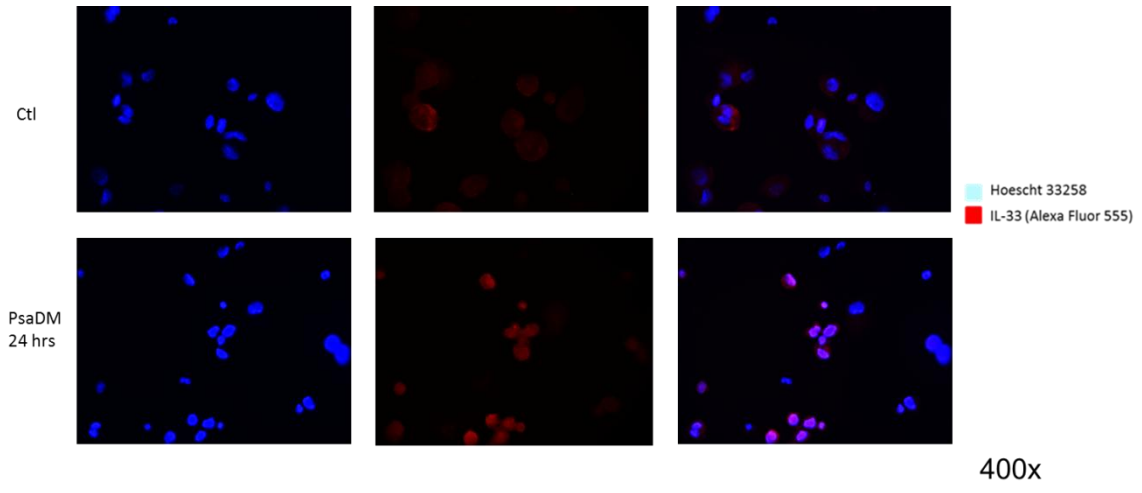


Figure 4.16 IL-33 is increased in the nucleus of differentiated THP-1 cells following 24 hour incubation with PsaDM. THP-1 cells well plated in 12 well plates

containing sterile glass coversilps and differentiation to macrophage-like cells was induced with PMA for 72 h. Cells were left untreated or incubated in the presence of PsaDM for 24 h and IL-33 was detected by immunofluorescence microscopy with a monoclonal antibody (Nessy-1). Nuclei were counterstained with Hoescht 33258. In control conditions, IL-33 observed diffusely in both, the nucleus and the cytoplasm of cells (upper panels). Following incubation with PsaDM, IL-33 is increased in the nucleus of cells (lower panels).

Summary of Results

In this chapter we described the signaling pathways regulating IL-33 expression in the context of infection in CF airway epithelial cells and monocytes.

In CF airway epithelial cells, TLR2, TLR5 and TLR9 are the main TLRs increasing IL-33 expression in response to an acute infection with *P. aeruginosa*. In this context, the TAK1-IKKβ-TPL2-MKK1/2-ERK1/2 and TAK1-MKK3/6 and p38 MAPK signaling pathways regulate the increase in IL-33 expression (figure 4.16). The expression of the alarmin IL-1α is also increased in the context of infection and its transcriptional regulation is similar to that of IL-33. The expression of the calcium-binding protein S100A9 is also increased following an infection with *P. aeruginosa*, however, it is regulated through different signaling pathways since the kinase inhibitors used could not prevent the increase in expression induced by *P. aeruginosa.* The expression of the alarmin HMGB1 is surprisingly decreased in the presence of *P. aeruginosa* and its regulation is not affected by any of the kinase inhibitors used in the experiment.

IL-33 protein seems to be increased in the cytoplasm of both, CF and non-CF airway epithelial cells following an infection with *P. aeruginosa.* Surprisingly, a band at approximately 45 kDa is increased in cytoplasmic extracts from cells infected with the bacteria. This change in molecular weight could be due to post-translational modifications affecting the molecular weight of the protein. However the identity of this cytoplasmic band has to be further confirmed. Similarly, intracytoplasmic IL-33 is increased in N3, CF7 and NHBE cells following an infection with *P. aeruginosa* and the band observed appears at the same molecular weight as in N3 and CF2 cells. The presence of IL-33 in the cytoplasm is probably due to increased IL-33 transcript levels since overexpression of IL-33 in HEK293 cells- a cell line that constitutively expresses IL-33 in the nucleus- increases IL-33's expression in the nucleus and drives its localization to the cytoplasm. Increases in intracellular IL-33 in response to infection are mediated through IKKβ and TPL2 signaling. Finally, IL-33 is not released from airway epithelial cells in our model of infection and the danger signal ATP does not have an effect in the release of IL-33 in *P. aeruginosa*-infected cells.

IL-33 expression is increased in response to PsaDM in both monocytes and macrophage-like cells. Similar to what was observed for airway epithelial cells, the TLR1/2 and TLR5 agonists significantly increase IL-33 expression in macrophage-like cells. Additionally, the TLR4 agonist LPS increased IL-33 expression in differentiated THP-1 cells. The TAK1-IKKβ-MKK1/2-ERK1/2 signaling pathway regulates IL-33 expression in response to PsaDM and the TLR5 agonist flagellin in differentiated THP-1 cells. IL-33 is increased in the nucleus of THP-1 cells following 24 hour incubation with PsaDM.

Chapter 5: Intracellular IL-33 Attenuates Inflammation

Introduction

In the previous chapters we have demonstrated that IL-33 is kept intracellularly in response to an acute bacterial infection. Very interestingly, an acute infection with *P. aeruginosa* seems to drive IL-33's localization to the cytoplasm of airway epithelial cells. This led us to explore the intracellular functions that IL-33 might be playing in our model of infection. Although the role of intracellular IL-33 has not been extensively studied, structural analysis of its interaction with heterochromatin has led to the hypothesis that it acts as a transcriptional repressor [203]. Furthermore, it has been demonstrated that IL-33 interacts with the p50 and p65 subunits of NFκB, repressing NFκB's transactivation of *IκBα* and *TNFα* in response to IL-1β [205]. This led us to hypothesize that intracellular IL-33 might be acting as a transcriptional repressor in our model. In order to clarify the roles of nuclear and cytoplasmic IL-33, we overexpressed either wild type full-length IL-33 (FL IL-33) or a mutant in with an arginine to alanine substitution at residue 48 (R48). The R48 mutation is sufficient to prevent IL-33's localization to the nucleus [204], therefore, this mutant was used as a tool to study IL-33's functions in the cytoplasm. In this chapter we studied the effects of nuclear and cytoplasmic IL-33 on NFκB transactivation in airway epithelial cells. Additionally we looked at the effects of nuclear and cytoplasmic IL-33 in the expression of NFκB and non-NFκB target genes in the context of acute infection.

Results

5.1 Transient transfection of IL-33 represses NFκB transactivation

It has been shown that the N-terminal domain of IL-33 interacts with the p65 subunit of NFκB in the nucleus and cytoplasm of HEK293 and mouse embryonic

fibroblasts. This interaction is enhanced by proinflammatory stimuli and results in a decreased transactivation of the NFκB target genes IκBα, TNFα and C-REL [205]. Conversely, in human umbilical vein endothelial cells (HUVECs), IL-33 increases the expression of ICAM-1 and VCAM-1, both NFκB target genes [250]. We first wanted to determine the role of nuclear and cytoplasmic IL-33 in the regulation of NFκB transactivation. For this purpose, we used B2B cells that stably express an NFκB reporter in PGL4.28 (Figure 5.1a, see materials and methods) and transiently transfected them with either empty vector (PCMV4a), FL IL-33 or with the mutant IL-33 R48. Cells were then left untreated or stimulated with the TLR5 agonist flagellin for 3 hours and luciferase activity was measured by chemiluminescence. Incubation with flagellin induces an 8-fold increase in luciferase activity. Transfection of FL IL-33 inhibits 50% this response and the R48 mutant exerts a stronger effect, preventing this increase by approximately 60% (figure 5.1b). Two-way ANOVA analysis of results shows statistical significance with $p=0.001$ for interaction, $p=0.0025$ for column factor (phenotype) and $p<0.0001$ for row factor (stimulation). This indicates that the overall means of control and stimulated conditions are significantly different and that the cell phenotype (empty vector, FL IL-33 or R48) affects the level of NFκB activity. For stimulated conditions, Bonferroni post-test shows statistical significance between empty vector and FL IL-33 ($p<0.001$) and between empty vector and R48 ($p<0.001$), indicating that transfection of FL IL-33 or the chromatin binding mutant affects the response of airway epithelial cells to flagellin. No statistical significance is observed between FL IL-33 and R48 in stimulated conditions.

Figure 5. 1

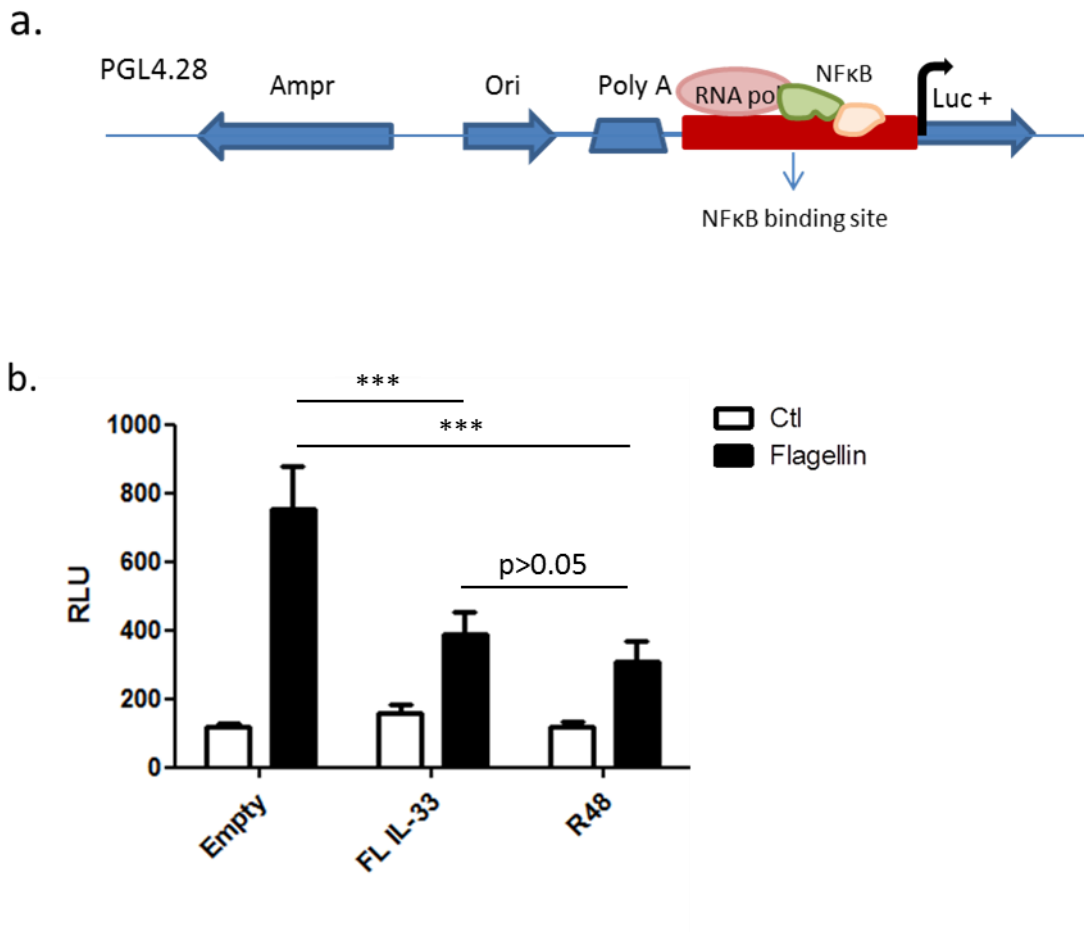


Figure 5.1. Transient transfection of IL-33 represses NFκB transactivation. A. Schematic representation of Beas-2B cells stably expressing the NFκB reporter driving luciferase expression. B. Beas-2B NFκB cells were transfected with the plasmid vector PCMV4a containing either wild-type, Full-length IL-33 (FL IL-33) or a mutant with an arginine to alanine substitution at position 48 (R48). Transfection with empty PCMV4a was used as a control. 24hours after transfection, cells were incubated with the TLR5 agonist flagellin (100ng/mL) for 3 hours, proteins were extracted and luciferase activity was measured by a chemiluminescene assay. Stimulation with flagellin significantly

increases luciferase activity in Beas-2B NFκB cells transfected with the empty vector. Transfection with FL IL-33 prevents the increase in luciferase activity and a stronger inhibition can be observed upon transfection with R48 IL-33. Results were analyzed by two-Way ANOVA; interaction $p=0.0011$; Column factor $p<0.0001$; row factor $p=0.0025$. This was followed by Bonferroni post-test comparing stimulated groups ($p<0.001$ when comparing both empty vector vs FL IL-33 and empty vector vs R48 and $p>0.05$ when comparing FL IL-33 vs R48).

5.2 Transient transfection of IL-33 decreases IL-8 expression in B2B NFκB cells

IL-8 is an NFκB-target gene and it is well known for its role as an inflammatory mediator in CFLD [46, 251]. Since previous results showed that NFκB transactivation is decreased in the presence of IL-33, we wanted to explore the possibility that this effect results in the decreased expression of IL-8. To do so, we transiently transfected B2B NFκB cells with either an empty vector, FL IL-33 or R48 IL-33, stimulated them with flagellin and measured IL-8 expression by qRT-PCR. In figure 5.2 we can observe that incubation with flagellin increases IL-8 mRNA expression. Overexpression of FL IL-33 decreases baseline IL-8 levels, however, there is still an increase in IL-8 mRNA in response to stimulation with flagellin. Overexpression of IL-33 R48 further decreases IL-8 expression at baseline levels and, similar to what was observed with WT FL IL-33, we can still observe an increase of IL-8 mRNA in response to flagellin. When analyzing by two-way ANOVA, interaction is non-significant ($p=0.14$). This can be explained by the fact that transfection of Beas-2B cells with FL IL-33 and the R48 mutant decreases IL-33 baseline expression even in non-stimulated conditions. However, there is statistical

significance in the response to flagellin when comparing between cell phenotypes ($p<0.0001$) and when comparing overall IL-8 expression ($p<0.0001$).

Figure 5.2

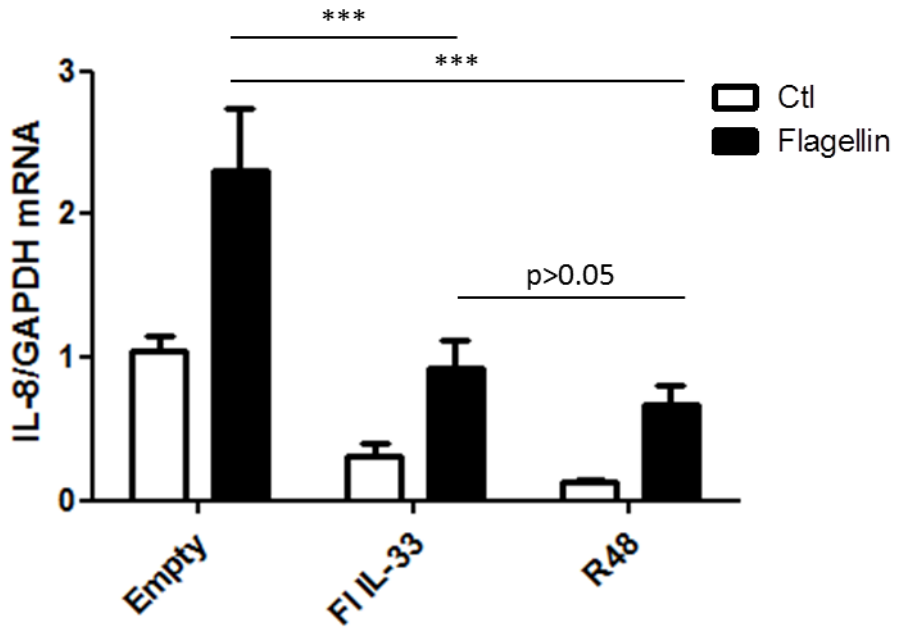


Figure 5.2. Transient transfection of IL-33 decreases IL-8 mRNA. Beas-2B NFκB cells were transfected with FL IL-33 or R48 constructs in PCMV4a. Transfection with empty vector was used as a control. Cells were incubated with 100ng/mL of flagellin for 3 hours, RNA was extracted and IL-8 expression was quantified by qRT-PCR. Stimulation of empty vector-transfected cells with flagellin significantly increased IL-8 mRNA. Transfection with both, FL IL-33 and R48 IL-33 decreased baseline IL-8 mRNA expression. However, an approximately two-fold increase in IL-8 mRNA is still observed when stimulating FL IL-33 or R48-transfected cells with flagellin. Results were analyzed

by two-way ANOVA, showing non-significant interaction between rows and columns ($p=0.14$) and significant difference between column factors and row factors, $p<0.0001$ for both. This was followed by Bonferroni post-test that showed statistical significance between the empty vector (control) and FL IL-33 ($p<0.001$) and between empty vector and R48 ($p<0.001$) when stimulated with flagellin. No significant difference in IL-8 expression was observed when comparing FL IL-33 and R48 constructs under stimulated conditions ($p>0.05$). Additionally, statistical analysis shows significant difference in IL-8 expression when comparing empty vector and FL-IL33 ($p<0.05$) and when comparing empty vector with R48 ($p>0.01$) in unstimulated conditions.

5.3 IL-33 is localized in the nucleus of airway epithelial cells stably expressing FL IL-33 and in the cytoplasm of airway epithelial cells stably expressing the R48 IL-33 construct

In previous experiments, transient transfection was used to assess the effect of WT IL-33 and the R48 mutant on NFκB activity and IL-8 expression in B2B NFκB cells. Transfection efficiency might be variable in transient conditions and this could affect the levels of expression of FL IL-33 and the R48 mutant. We therefore selected for the stable expression of PCMV4a, FL IL-33 and R48 in B2B cells with hygromycin. We then looked at the localization of intracellular IL-33 in each cell line by immunofluorescence microscopy. IL-33 is barely detectable in B2B cells expressing the empty vector (figure 5.3 upper panels). Stable overexpression of IL-33 mainly drives its expression to the nucleus (arrow) although IL-33 can also be detected in the cytoplasm of airway epithelial cells (figure 5.3, middle panel). As expected, the R48 mutant is mainly localized in

vesicles in the cytoplasm of cells (arrowhead) and only faint levels can be observed in the nucleus (figure 5.3, lower panel).

Figure 5.3

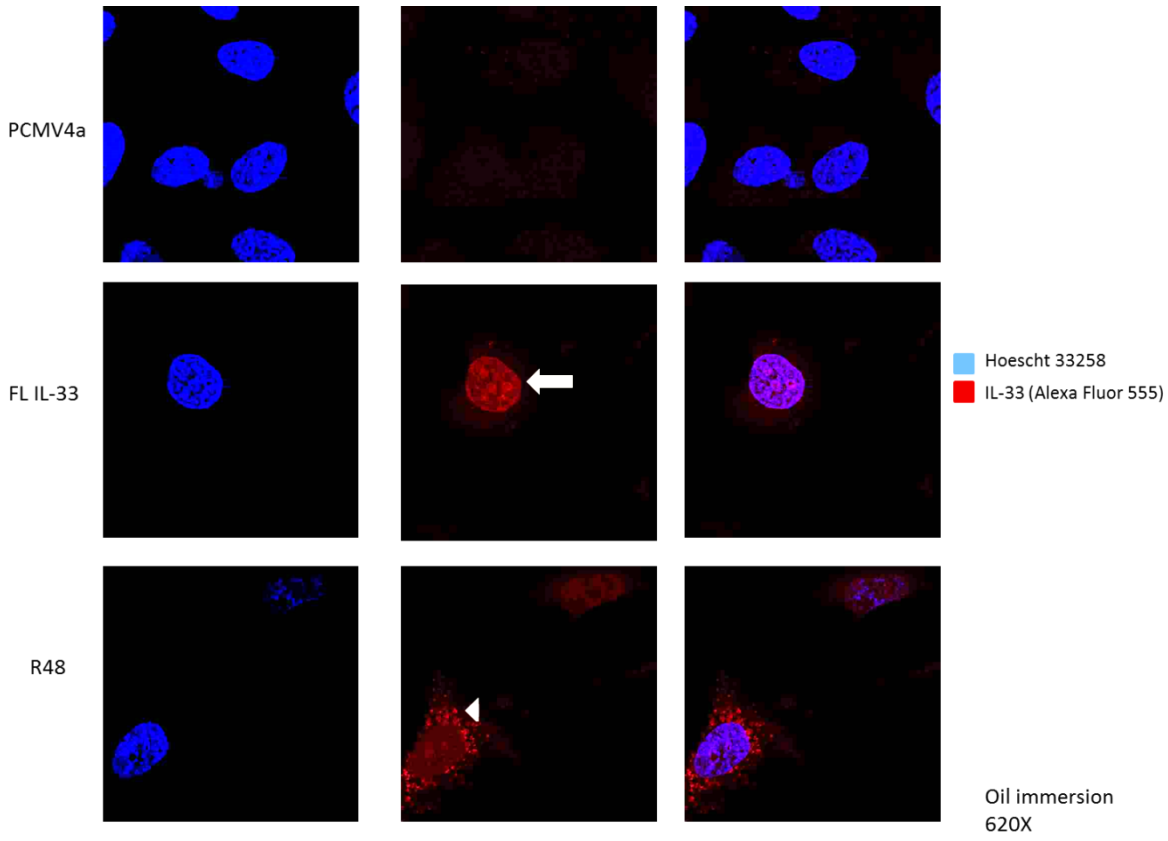


Figure 5.3. Intracellular localization of stable FL IL-33 and R48 IL-33 constructs. Beas-2B cells stably expressing an empty vector (PCMV4a), FL IL-33 and R48 IL-33 constructs were visualized by confocal microscopy. IL-33 is almost undetectable in cells stably expressing the empty vector PCMV4a (top rows). Overexpression of FL IL-33 results in a strong expression of IL-33 in the nucleus of Beas-2B cells (middle rows).

Overexpression of the R48 IL-33 mutant leads to increased expression of IL-33 in the cytoplasm of cells, apparently contained in vesicles (bottom rows).

5.4 Stable overexpression of IL-33 in airway epithelial cells prevents upregulation of NFκB target genes

Previous results have shown a decrease in IL-8 mRNA expression when transiently transfecting FL IL-33 or R48 IL-33 in Beas-2B NFκB cells. We then wanted to assess the effects of a stable expression of nuclear and cytoplasmic IL-33 (FL IL-33 and R48 IL-33, respectively) in pro-inflammatory gene expression. For this purpose, polyclonal cell lines stably expressing PCMV4a, FL IL-33 and the R48 mutant were exposed to flagellin or to live *P. aeruginosa* for three hours and the expression of NFκB and non NFκB dependent genes was assessed by qRT-PCR. We analyzed the expression of the NFκB dependent genes IL-8, IκBα, GROα and GROβ and of the non-NFκB dependent genes MYC, IL-10 and ATF3. The expression of these genes was increased in response to PsaDM in an array performed in our laboratory (unpublished data) and was therefore considered relevant in our model. Figure 5.4 shows the expression of the genes mentioned above in response to the TLR5 agonist flagellin and to an acute infection with *P. aeruginosa.* White columns represent cells stably expressing the empty vector, PCMV4a, blue columns represent cells stably expressing wild-type full-length IL-33 (FL IL-33) and grey columns represent cells stably expressing the IL-33 mutant expressing an arginine to alanine substitution at the residue 48 (R48). Each set of three columns represents cells that were untreated (Ctl), stimulated with flagellin (100 ng/mL) or infected with *P. aeruginosa* (Psa) for three hours. Incubation with flagellin induces a 10-fold increase in IL-8 mRNA and this can be prevented by overexpressing both, FL IL-33

and the R48 mutant. Infection with *P. aeruginosa* strongly induces IL-8 expression and the presence of FL IL-33 completely prevents this increase. Overexpression of the R48 mutant partially prevents the increase of IL-8 mRNA in response to *P. aeruginosa*. This could be due to the fact that IL-8 is regulated by several transcription factors and the preferential presence of IL-33 in the cytoplasm is not sufficient to block its expression in the context of an acute infection (figure 5.4a). IκBα is increased in response to both flagellin and *P. aeruginosa.* FL IL-33 prevents the increase of IκBα expression in response to both stimuli and the presence of cytoplasmic IL-33 with the R48 mutant exerts a stronger effect in repressing IκBα expression in the context of inflammation (figure 5.4b). GROα mRNA is increased following exposure to flagellin and this increase is still observed in cells stably expressing IL-33. On the other hand, the presence of IL-33 in the cytoplasm prevents this significant increase in GROα expression. An infection with *P. aeruginosa* strongly increases GROα mRNA and, similar to what is observed upon stimulation with flagellin, the presence of IL-33 in the nucleus is not sufficient to block GROα mRNA upregulation in response to an acute infection. The presence of IL-33 in the cytoplasm reduces GROα mRNA upregulation but does not completely abrogate the increase of its expression in the context of acute infection (figure 5.4c). GROβ mRNA is significantly increased following exposure to both flagellin and *P. aeruginosa.* Stable expression of IL-33 in the nucleus with FL IL-33 partially prevents the increase in GROβ expression in response to both stimuli. Overexpression of IL-33 in the cytoplasm of cells exerts a stronger effect in preventing GROβ mRNA upregulation in response to both flagellin and *P. aeruginosa* (figure 5.4d).IL-10 expression is increased in response to flagellin but not after infection with *P. aeruginosa*. Stable overexpression of FL IL-33

and R48 can prevent the increase of IL-10 mRNA induced by flagellin. No significant changes in IL-10 expression are observed when cells are infected with *P. aeruginosa* (figure 5.4e). MYC expression is not significantly increased following stimulation of Beas-2B cells with flagellin or infection with *P. aeruginosa*. The presence of nuclear IL-33 decreases constitutive MYC expression following an acute infection with *P. aeruginosa* (figure 5.4f). Statistical analysis of the genes analyzed in figure 5.4 is summarized in tables 5.1 and 5.2.

Figure 5.4

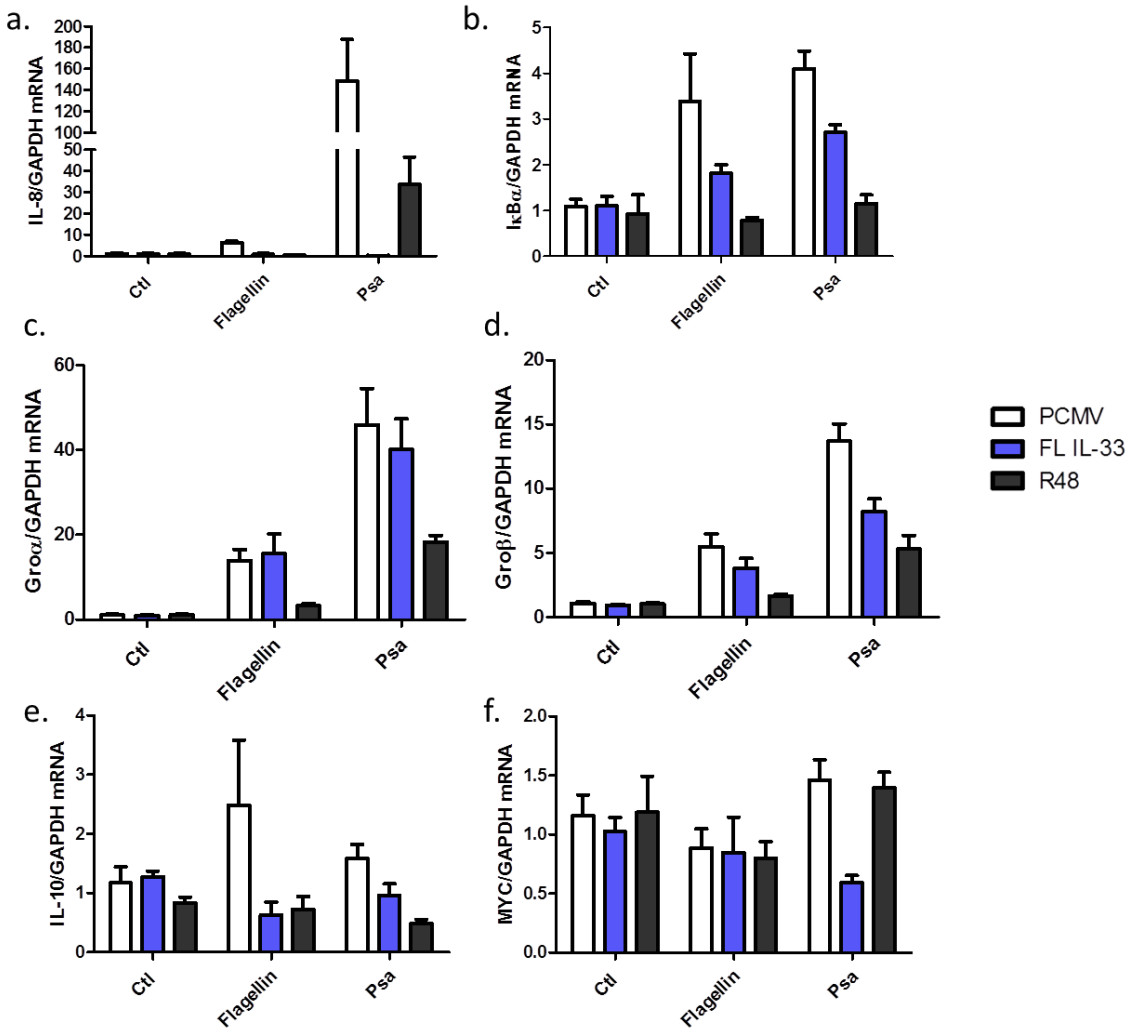


Figure 5.4. Stable overexpression of IL-33 in airway epithelial cells prevents upregulation of NFκB target genes. Beas-2B cells stably expressing FL IL-33 (blue columns) or R48 IL-33 (grey columns) were either stimulated with flagellin (100 ng/mL) or infected with *P. aeruginosa* (MOI of 18) for 3 hours. Cells stably expressing the empty vector PCMV4a were used as controls (white columns). Each set of columns represents either untreated cells (Ctl), cells incubated with flagellin or cells infected with *P.*

aeruginosa. The expression of the NFκB-dependent genes IL-8, IκBα, Groα and Groβ and of the NFκB-independent genes IL-10 and MYC was analyzed by qRT-PCR.

Table 5.1 Two-way ANOVA p values for interaction, row factor and column factor for each gene in figure 5.4

	Interaction	**Row Factor (stimulus)**	**Column factor (phenotype)**
IL-8	p<0.0001***	p<0.0001***	p<0.0005***
IκBα	0.0222*	p=0.0003***	p<0.0001***
GROα	0.053	p<0.0001***	p=0.0033**
GROβ	p=0.0003***	p<0.0001***	p<0.0001***
IL-10	p=0.09	p=0.618	p=0.0023
MYC	p=0.14	p=0.128	p=0.71

Table 5.2 Bonferroni post-test comparing the effect of stable overexpression of the different constructs in the genes analyzed in figure 5.4 under stimulated conditions (flagellin or *P. aeruginosa*).

	Flagellin			***P. aeruginosa***		
	Empty v. vs FL IL-33	Empty v. vs R48	FL IL-33 vs R48	Empty v. vs FL IL-33	Empty v. vs R48	FL IL-33 vs R48
IL-8	p>0.05	p>0.05	p>0.05	p<0.001***	p<0.001***	p>0.05
IκBα	p<0.05*	p<0.001***	p>0.05	p>0.05	p<0.001***	p<0.05*
GROα	p>0.05	p>0.05	p>0.05	p>0.05	p<0.001***	p<0.01**

GROβ	p>0.05	p<0.01**	p>0.05	p<0.001***	p<0.001***	p<0.05*
IL-10	p<0.01**	p<0.01**	p>0.05	p>0.05	p>0.05	p>0.05
MYC	p>0.05	p>0.05	p>0.05	p<0.01**	p>0.05	p<0.05*

5.5 Intracellular IL-33 associates with NFκB in CF airway epithelial cells

We finally wanted to determine whether IL-33 associates with NFκB in our model of acute infection. For this purpose, CF2 cells were infected with *P. aeruginosa* for 6 hours and endogenous IL-33 was immunoprecipitated with a monoclonal antibody. Immunoprecipitated IL-33 and its association with NFκB was assessed by Western Blot with a polyclonal antibody against IL-33 and a monoclonal antibody against NFκB p65. Lamin A/C was used as a marker for nuclear protein. GAPDH was used as a marker for total protein content. IL-33 is present in the cytoplasm of control and infected cells. Two cytoplasmic IL-33 bands can be detected at approximately 35 and 37 kDa upon pulldown of IL-33 with the monoclonal antibody (left panel, middle row). The band of an approximate molecular weight of 68 kDa represents NFκB p65 and is present in cytoplasmic extracts of control and stimulated cells. A band of approximately 56 kDa appears when immunoblotting against NFκB p65 upon pulldown of IL-33 in uninfected cells. This could represent the association of IL-33 and an NFκB isoform or subunit (left panel, top row). This band is considerably decreased in the cytoplasm of *P. aeruginosa* - infected cells. GAPDH is present in the total cytoplasmic extracts and is not detectable upon IL-33 pulldown (left panel, bottom row). Nuclear IL-33 can only be detected upon pulldown with the monoclonal antibody (right panel, middle row). NFκB p65 translocates

to the nucleus of cells infected with *P. aeruginosa*. The band at approximately 56 kDa appears upon pulldown of IL-33 in the nuclear fractions of *P. aeruginosa*-infected cells, indicating a possible association of an NFκB subunit or isoform with IL-33 in the nucleus (right panel, top row). The nuclear marker lamin A/C is present in nuclear extracts and its presence can still be detected upon nuclear IL-33 immunoprecipitation (right panel, bottom row).

Figure 5.5

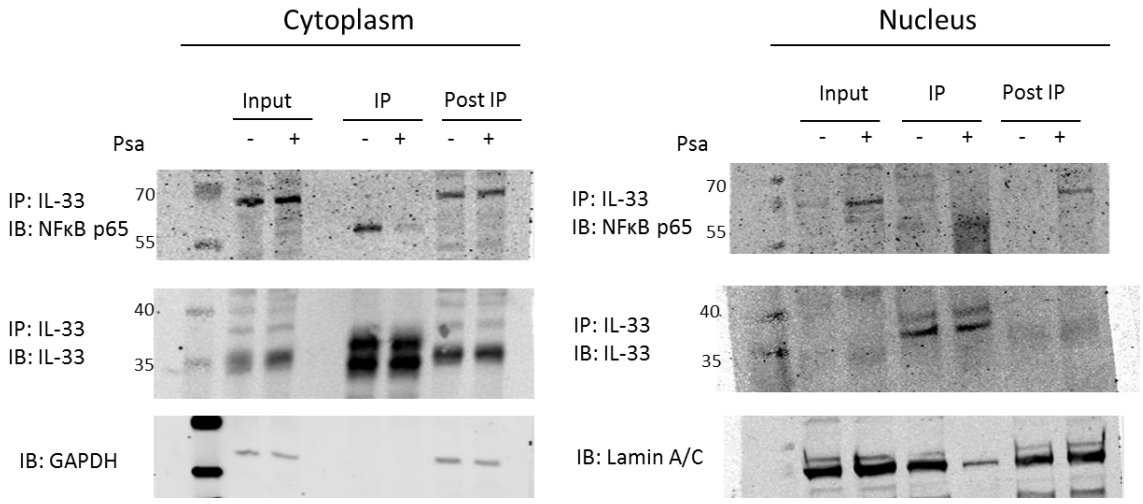


Figure 5.5. Intracellular IL-33 associates with NFκB p65 in CF airway epithelial cells. CF2 cells were infected with *P. aeruginosa* (MOI of 2) for 6 hours. Nuclear and cytoplasmic fractions were isolated and IL-33 was immunoprecipitated with a monoclonal antibody bound to Protein G Sepharose beads. Total protein contents and immunoprecipitated fractions of nuclear and cytoplasmic extracts were separated by SDS-PAGE and IL-33 and NFκB p65 were detected by immunoblotting. GAPDH was used as a marker of total protein contents and lamin A/C was used as a marker of nuclear

proteins. Input=cell fraction before immunoprecipitation, IP=immunoprecipitated fraction.

Summary of Results

Chapter 5 describes the potential roles for nuclear and cytoplasmic IL-33 in airway epithelial cells. We have demonstrated that overexpression of FL IL-33 represses NFκB transactivation in response to the TLR5 agonist flagellin. Overexpression of the R48 IL-33 mutant, which localizes in the cytoplasm, further decreases NFκB activity in response to flagellin. IL-8 baseline expression is decreased upon overexpression of FL IL-33 and R48 IL-33. However, transfection with this construct is not sufficient to prevent the two-fold increase in IL-8 mRNA in response to flagellin.

Intracellular IL-33 protein is almost undetectable in Beas-2B cells stably expressing the empty PCMV4a vector. IL-33 is mainly localized in the nucleus of cells stably expressing FL IL-33. Stable expression of the R48 IL-33 mutant drives IL-33's localization to intracytoplasmic vesicules in airway epithelial cells.

Stable overexpression of FL IL-33, which mainly localizes in the nucleus, prevents the increase of the NFκB-dependent genes IL-8, IκBα and GROβ in response to flagellin. The presence of IL-33 in the cytoplasm further prevents the increase in expression of the NFκB-dependent genes IL-8, IκBα, GROα and GROβ in response to flagellin. An infection with *P. aeruginosa* leads to a strong induction of IL-8, IκBα, GROα and GROβ. Stable expression of FL IL-33 completely prevents the increased expression of IL-8 in response to infection and partially blocks the increase of IκBα and GROβ mRNA in the same conditions. The presence of R48 IL-33 in the cytoplasm only

partially blocks the increase of IL-8, GROα and GROβ mRNA in response to infection. However, R48 IL-33 completely blocked the increase of IκBα mRNA in response to *P. aeruginosa.* The non-NFκB-dependent genes IL-1 and MYC are not significantly increased in response to flagellin or *P. aeruginosa* and the presence of FL IL-33 or R48 IL-33 does not exert a significant effect in their expression.

IL-33 can be immunoprecipitated from nuclear and cytoplasmic extracts of CF2 cells and it associates with an NFκB isoform in the cytoplasm of uninfected cells. Infection with *P. aeruginosa* induces the translocation of NFκB to the nucleus and its association with IL-33.

Chapter 6: General Discussion

6.1 IL-33 expression in an *in vitro* model of chronic infection in CF

Results from my thesis work show that IL-33 expression is increased in a chronic model of infection using *P. aeruginosa* diffusible material (PsaDM). Interestingly this increased expression is selective for CF airway epithelial cells, since incubation of the non-CF airway epithelial cell lines N3 and NuLi with PsaDM did not increase IL-33 expression. Furthermore, 2-way ANOVA analysis correlates the increased IL-33 expression to the CF phenotype. This increased expression is followed by an increase in intracellular protein in cell lines expressing the ΔF508 mutation and the G542X mutation (CuFi and CF7, respectively). We have not addressed the mechanism by which exposure to PsaDM increases IL-33 expression only in airway epithelial cells with defective CFTR function. However, we can speculate on two potential mechanisms responsible for this effect. Our first culprit would be increased oxidative stress in CF airway epithelial cells. As mentioned in the introduction, CFTR is a transporter for reduced glutathione (GSH), an important regulator of extracellular redox state [20]. Our group has indeed shown decreased extracellular GSH levels in CFTRΔF508 airway epithelial cells. This favoured the production of reactive oxygen species (ROS) in response to PsaDM, which in turn lead to the activation of signaling pathways increasing IL-6 expression [46]. As for IL-6, increased oxidative burden could decrease the threshold required for IL-33 upregulation. Alternatively, CFTR could be involved in the control of proinflammatory signaling pathways. Evidence shows that functional CFTR dampens NFκB signaling [252]. This is potentially mediated through a negative regulatory effect of CFTR on the TNFα receptor [253] In this instance, defective CFTR function would lead to increased NFκB activation, leading to increased inflammation. Similar to what was observed for PsaDM, prolonged

exposure of CF airway epithelial cells to *P. aeruginosa* biofilm material significantly increased IL-33 expression. Taken together, these results show a preferentially increased expression of IL-33 in CF airway epithelial cells in two models of chronic infection.

6.2 IL-33 expression in a model of CF acute bacterial exacerbations

Changes in the lung microenvironment might affect the interaction of airway epithelial cells with *P aeruginosa* during CF acute exacerbations. This would cause the release of bacteria from biofilms, allowing for the direct contact of bacteria with airway epithelial cells [165]. I created an *in vitro* model of acute infection where airway epithelial cells are directly exposed to *P. aeruginosa.* This model aimed to represent the interaction of airway epithelial cells with *P. aeruginosa* during acute exacerbations. When infected with *P. aeruginosa* in this manner, CF airway epithelial cells significantly increase IL-33 mRNA. This significant increase in IL-33 expression in response to acute infection is not seen in non CF airway epithelial cell lines or in primary airway epithelial cells. We further confirmed that the increased IL-33 expression seen in CF airway epithelial cells is due to abnormal CFTR function by potentiating CFTR channel activity with VX-770 (Ivacaftor), by correcting CFTR expression to the cell membrane with VX-809 or by using a combination of both, VX-770 and VX-809. Interestingly, potentiation of CFTR function with VX-770 partially prevented the increase in IL-33 expression caused by *P. aeruginosa* in CFTRΔF508 airway epithelial cells. Although to a lesser extent, correction of CFTR expression with VX-809 also prevented the increase in IL-33 expression in response to infection. No synergism or potentiation is seen when adding both, VX-770 and VX-809. This could be explained by the fact that VX-770 decreases the stability of ΔF508 CFTR at the ER and at the cell membrane, resulting in reduced

correction efficiency of VX-809 [28]. Although these results do not directly address the mechanism through which abnormal CFTR function relates to IL-33 upregulation following infection, they implicate CFTR in the regulation of IL-33 expression in the context of an acute bacterial infection.

6.3 Signaling pathways regulating IL-33 expression in the context of infection

Experiments using different TLR agonists and neutralizing antibodies show that IL-33 upregulation is mediated through TLR2, TLR3, TLR5 and TLR9 signaling pathways in CF airway epithelial cells. This is in line with previous findings that show that TLR2 and TLR3 agonists increase IL-33 expression in murine macrophages [212]. However, this is the first description of the role of TLR5 and TLR9 in the regulation of IL-33 expression by airway epithelial cells. The TLR4 agonist, LPS, increased IL-33 expression in monocytes and macrophages, but not in CF airway epithelial cells. The lack of response of airway epithelial cells to LPS might be explained by the fact that TLR4 is not present in the membrane of these cells. Experiments using inhibitors of kinases downstream TLR signaling show that the TAK1-IKKβ-TPL2-MKK1/2-ERK1/2 and the TAK1-MKK3/6-p38 MAPK signaling pathways regulate IL-33 expression in our model of acute infection in CFTRΔF508 airway epithelial cells. In contrast the TAK1-IKKβ-TPL2-MKK1/2-ERK1/2 signaling pathway seems to be the main regulator of IL-33 mRNA expression in CFTRG542X airway epithelial cells. The role of ERK1/2 in the regulation of IL-33 expression has been previously studied in colonic myofibroblasts in response to TNFα stimulation [210]. The transcriptional regulation of IL-33 by p38 MAPK has been described in the context of IFNγ stimulation of human keratinocytes [211]. However, the present results show the first description of the kinase TPL2 in the

regulation of IL-33 expression. A summary of the signaling pathways regulating IL-33 expression following an acute infection by *P. aeruginosa* is depicted in figure 4.11. The kinase TPL2 could be an interesting target for modulating IL-33 expression since blocking TPL2 would preferentially affect proinflammatory pathways triggered by infection without affecting growth, differentiation and survival pathways regulated by p38 MAPK. Interestingly, the TPL2 and p38 MAPK inhibitors do not prevent IL-33 upregulation in macrophage-like cells, suggesting that the modulation of IL-33 expression is cell-type dependent. Alternative pathways leading to ERK1/2 activation could be triggered by inflammatory stimuli in macrophage like cells. It is well known that PMA stimulation induces PKC activation, which can lead to the phosphorylation of MEK1/2 followed by ERK1/2 activation [148]. PMA was used in our *in vitro* system to differentiate THP-1 cells into macrophage-like cells, potentially activating the PKC pathway. Additionally, the activation of alternative signaling pathways could be time-dependent, with an initial activation of TLR downstream pathways and the subsequent activation of other kinases. The pitfall of using kinase inhibitors to study signaling pathways regulating gene expression is the potential for non-specific inhibition of other kinases. Our group has previously performed dose-response experiments to assess the effect of different kinase inhibitors in protein kinase phosphorylation in airway epithelial cells [150]. Additionally, the effect of the different kinase inhibitors in p38 and ERK1/2 MAPK phosphorylation was assessed in our model of infection (figures 4.4 and 4.15). There was no significant change in p38 MAPK phosphorylation upon inhibition of TPL2 and ERK1/2 with C1 and PD184352, respectively, demonstrating that these inhibitors at least do not impact those kinases. We can observe a decrease in ERK1/2 phosphorylation

upon p38 inhibition. This can be explained by the effect of p38γ and p38δ in the regulation of ERK1/2 phosphorylation by TPL2 [142]. An alternative to addressing the potential off-target effects of pharmacological inhibitors would be the use of RNA interference to knock-down specific kinase expression. Unfortunately, efforts to knock-down the kinases TPL2 and TAK1 proved unsuccessful in CF airway epithelial cells.

6.4 IL-33 subcellular localization

The increases in IL-33 mRNA seem to correlate with increased intracellular protein expression as demonstrated by immunofluorescence and immunoblotting. Interestingly, in our model of chronic infection using PsaDM, IL-33 protein seems to be mainly localized in the nuclei of CF airway epithelial cells, whereas during acute infection IL-33 is markedly increased in the cytoplasm of cells. This further underlines the differences between chronic and acute infections. Several bands can be observed when immunoblotting against IL-33 using a polyclonal antibody. This could represent different protein isoforms, post-translational modifications or non-specific binding of the antibody. The presence of several IL-33 transcripts in different transformed and non-transformed cells has been previously described [201]. Remarkably, a band of a molecular weight of approximately 45 kDa appears in the cytoplasm of CF and non CF airway epithelial cells acutely infected with *P. aeruginosa*. This could result from IL-33 mRNA upregulation or from translocation of nuclear IL-33 to the cytoplasm of cells. The fact that this band appears in non-CF airway epithelial cell lines and in primary airway epithelial cells potentially suggests that IL-33 is being shuttled from the nucleus into the cytoplasm after cells are in contact with the bacteria. Another explanation could be that, although no statistical significance is observed at the mRNA level, slight changes in IL-

33 mRNA could be translating into increased protein levels. It would be interesting to assess the effect of IL-33 mRNA stability in protein expression. Unfortunately, we could not confirm that the 45 kDa band observed in the immunoblotting experiments is indeed IL-33. Future experiments to address this issue could aim to knock down IL-33 by RNA interference or to identify the bands by mass spectrometry. However, experiments using a different polyclonal antibody for the detection of intracytoplasmic IL-33 by ELISA support the results observed in immunoblotting. In line with previous data, an acute infection with *P. aeruginosa* increased IL-33 in the cytoplasm of CF airway epithelial cells. This could be partially prevented by incubating cells with the IKKβ and TPL2 inhibitors, suggesting that the increase of intracytoplasmic IL-33 in acute infection is at least partially regulated through TLR signaling in CF airway epithelial cells. The presence of cytoplasmic IL-33 has been previously reported in fibroblasts and endothelial cells [208]. Furthermore, a study reported the presence of IL-33 in the cytoplasm of primary bronchial epithelial cells (NHBEs) and showed the presence of a band with an approximate molecular weight of 41 kDa when cells were exposed to the fungus *Alternaria alternata* [209]. Other reports have shown the presence of IL-33 in the cytoplasm of fibroblasts following mechanical stretch and have suggested that IL-33 is translocated to the cytoplasm of cells under stress conditions [208].

6.5 IL-33 release

Despite the increase in IL-33 expression, IL-33 is not released in our model of chronic infection. This can be explained by the fact that stimulation with neither PsaDM nor *P. aeruginosa* biofilm material induces cell death. These stimulations, however, are sufficient to induce a strong and significant release of IL-8. Due to its lack of a leader sequence for secretion through the ER-Golgi pathway, IL-33 is preferentially released

from cells following cell death [206]. Increased cell death could indeed be happening during acute CF pulmonary exacerbations, mainly as a result of the increased bacterial burden and the heavy influx and activity of neutrophils [54]. This is supported by the fact that the alarmins HMGB1 and S100A8/S100A9 are increased in sputum of CF patients suffering from these episodes [179, 188]. We demonstrated that both, apoptotic and necrotic cell death are increased in our model of acute infection with *P. aeruginosa*. However, no increase in extracellular IL-33 was observed in this scenario. This could be due to the fact that IL-33 is being degraded by bacterial proteases. Alternatively, a stronger stimulus is required to induce more than 50% necrotic cell death in order to release IL-33 from airway epithelial cells. Two previous publications have shown that the danger signal ATP prompts the release of IL-33 from airway epithelial cells via its action on the P2Y purinergic receptors [209, 216]. However, this is not the case in our acute infection model, since ATP stimulation in the context of bacterial infection did not induce IL-33 release from CF airway epithelial cells.

6.6 Potential role for IL-33 in our model

Contrary to what we initially hypothesized, IL-33 is not released in our model of acute bacterial exacerbations. The cell might be putting into play several mechanisms to prevent the release of IL-33 into the extracellular space. For instance, IL-33 might be trapped in apoptotic vesicles, preventing it from being detected in the cell supernatants. Furthermore, post-translational modifications occurring to IL-33 in response to infection might lead to its intracellular degradation thus preventing its release. These two possibilities need further investigation.

One study has reported an increase of IL-33 in bronchoalveolar lavage fluids from CF patients compared to non-CF controls [230]. According to the data obtained from my study of IL-33, it is unlikely that airway epithelial cells are the main source of extracellular IL-33. The IL-33 observed in clinical samples from CF patients might be coming from other sources such as fibroblasts or smooth muscle cells. Indeed, it has been demonstrated that fibroblasts release IL-33 in response to mechanical stretch and ATP stimulation [208]. Therefore, bronchial hyperactivity might induce IL-33 release by fibroblasts through mechanical stretch in CF patients. Another alternative is that the IL-33 observed in clinical samples comes from specific subsets of cells that are not accounted for in my model of infection. An argument that supports this notion is the fact that IL-33 is specifically produced by basal progenitor cells from COPD patients [216].

The caveat of working with a cell culture model is that it may be underrepresenting situations occurring in a physiological context. For instance, cells might not be subjected to the same pressure changes and oxygen tensions as they are in the lung. Additionally, cells in culture do not interact with other cell types as it occurs in the lung. With all this in mind, my results rather suggest that IL-33 is acting intracellularly, both at the nuclear and cytoplasmic level. We therefore determined the roles of intracellular IL-33 in our model of infection. Previous studies have shown that intracellular IL-33 suppresses the expression of NFκB target genes both, through direct interaction with NFκB itself and through the interaction of IL-33 with other transcriptional repressors [205, 215]. In our model, wild-type full-length IL-33 (FL IL-33) was localized to the nucleus of airway epithelial cells, where it repressed the NFκB-mediated transactivation of IL-8, IκBα and Groβ. The chromatin binding mutant IL-33

R48 was preferentially expressed in the cytoplasm of cells and it exerted a stronger effect in downregulating the NFκB targets IL-8, IκBα, Groα and Groβ in response to both flagellin and *P. aeruginosa*. Whether this repression is mediated directly through the interaction of IL-33 with NFκB needs further investigation. Immunoprecipitation experiments show a potential association of cytoplasmic IL-33 with the p50 subunit of NFκB in unstimulated conditions. Infection with *P. aeruginosa* led to the translocation of NFκB to the nucleus and to the association of nuclear IL-33 with the p50. This is in line with a previous study that showed the constitutive association of IL-33 with NFκB p50 in the nucleus and in the cytoplasm of cells [205]. Together, these findings suggest that intracellular IL-33, both at the nuclear and cytoplasmic levels, represses the transcription of proinflammatory cytokine genes in the context of an acute infection with *P. aeruginosa.*

6.7 IL-33 as a target for CF exacerbations

Due to the potential role of IL-33 as an anti-inflammatory mediator, it might not be beneficial to decrease its intracellular levels in the context of CF. Therefore, targeting extracellular IL-33 might be a more appropriate approach. This would decrease the excessive recruitment of inflammatory cells without affecting the intracellular transcriptional regulatory activity of IL-33. IL-33 neutralization could be achieved through the administration of a neutralizing antibody or an analog of its decoy receptor sST2. However, this is to be done with caution since it has been proposed that extracellular IL-33 plays beneficial roles in the cardiovascular system [231, 233].

Original Contribution to Knowledge

The original contribution of my thesis work to general knowledge can be summarized as follows:

1. First description of increased IL-33 expression in CFTRΔF508 and CFTRG542X airway epithelial cells in a model of chronic infection, which was published in the *Journal of Allergy and Clinical Immunology* (ref. 229).

2. First characterisation of increased IL-33 expression in CF airway epithelial cells in an *in vitro* model of acute bacterial exacerbations, which was published in *Frontiers in Cell and Developmental Biology* (ref. 244).

3. Potential role of CFTR in the modulation of IL-33 expression following an acute infection with *P. aeruginosa*, which was published in *Frontiers in Cell and Developmental Biology* (ref. 244).

4. Roles of the TLR2/TLR5-TAK1-IKKβ-TPL2-MKK1/2-ERK1/2 and the TLR2/5-TAK1-MKK3/6-p38 MAPK in the regulation of IL-33 expression in CF airway epithelial cells in a model of acute bacterial infection which has been published in *Frontiers in Cell and Developmental Biology* (ref. 244).

5. The role of ERK1/2 in the regulation of IL-33 expression in macrophage-like cells in response to bacterial derived products.

6. The presence of increased intracytoplasmic IL-33 in CF and non CF airway epithelial cells in response to *P. aeruginosa.*

7. The role of nuclear and cytoplasmic IL-33 as repressors of the NFκB target genes IL-8, IκBα and Groβ in airway epithelial cells acutely infected with *P. aeruginosa*.

Conclusions

In conclusion, infection with *P. aeruginosa* or exposure to its products increases IL-33 mRNA and protein expression in CF airway epithelial cells. TLR signaling through the TAK1-IKKβ-TPL2-MEK1/2-ERK1/2 and TAK1-MKK3/6-p38 MAPK pathways regulates IL-33 expression in response to bacterial infection. However, IL-33 is not released extracellularly in our models of acute or chronic infection. Interestingly, IL-33 is kept in the cytoplasm of cells infected with *P. aeruginosa* and potentially plays roles as a repressor of inflammation.

Future Directions

Future experiments should aim to clarify the role of intracellular IL-33 in CF airway epithelial cells. This could be achieved through knockdown of IL-33 by RNA interference and by measuring the inflammatory response of IL-33$^{-/-}$ CF airway epithelial cells in the context of infection. Additionally, it would be relevant to study the post-translational modifications occurring to IL-33 during acute infection and the mechanisms regulating IL-33 subcellular localization. Furthermore, the interaction of IL-33 with other proteins in different subcellular compartments should be assessed by mass spectrometry. Important information might also be obtained from studying the role of IL-33 in other cells types obtained from CF patients such as fibroblasts and airway smooth muscle cells. Finally, useful information could be obtained by studying the effects of knocking down IL-33 in an *in vivo* model of CFTR$^{-/-}$ or CFTR$^{\Delta F508/\ \Delta F508}$ mice.

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