Impact of the Type I Interferon mediated Antiviral Immune Response on the response to Pseudomonas *aeruginosa* infection in Bronchial Epithelial cells

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ABSTRACT

Viral infections often cause pulmonary exacerbations in cystic fibrosis (CF) patients, and this is seen in patients who are pre-colonized with pseudomonas aeruginosa, the main pathogen involved in CF lung disease. These co-infections result in significant decline of lung function over time. PA persists in the airways despite host immune response and antibiotic treatments mainly due to biofilm formation, however, our lab recently demonstrated an intracellular reservoir of P. aeruginosa inside epithelial cells. We sought to study how viral infections alter pseudomonas intracellular persistence and host antibacterial response.

To investigate this, we first transfected human bronchial epithelial cells (BEAS-2B) with Poly(I:C), a TLR3 agonist that stimulates antiviral immune responses, and co-stimulated with PA flagellin or diffusible material (PsaDM) to assess MAPK phosphorylation as part of the epithelial inflammatory response. Secondly, In the intracellular infection model, BEAS-2B cells were infected with the wild-type P. aeruginosa strain PaO1. To study PA internalization, BEAS-2B cells were treated with pIC before exposure to PaO1, and intracellular bacterial count was performed at 4 hours post-infection. To study PA persistence, BEAS-2B cells were treated with pIC post-infection, and intracellular bacterial count was performed up to 5 days. Thirdly, Flow cytometry was used to access epithelial ICAM-1 expression in response to P. aeruginosa infection, stimulation with PA diffusible material or the TLR2 agonist PAM3CSK4, in cells pretreated with pIC, as well as investigating the signaling transduction mechanisms involved. We found that, pIC stimulation prior to the TLR5 agonist flagellin and TLR2 agonist PAM treatment of BEAS-2B cells is associated with higher pro-inflammatory response characterized by increased p38α MAPK levels. Moreover, higher ICAM-1 expression is triggered by pIC pre-stimulation along with pseudomonas filtrates, the TLR2 agonist PAM, as well as pseudomonas infection by both WT and mucoid strains. In addition, pIC pre-treatment significantly decreased internalization of PA at 4h post-infection, while pIC treatment post-infection also reduced PA persistence up to 120h without apparent cytotoxicity. Lastly, ICAM-1 expression associated with pIC stimulation is almost completely abrogated by inhibiting NFκB pathway independent of MAPK activation. This study points to a role in the antiviral response in altering PA persistence and anti-bacterial response in bronchial epithelia. Ultimately, we wish to provide insights into the

pathophysiology of pulmonary exacerbations seen in CF patients in the context of viral and bacterial co-infections.

RÉSUMÉ

Les infections virales sont la cause de nombreux épisodes d'exacerbations pulmonaires chez les personnes atteintes de fibrose kystique (FK). Ces épisodes se produisent fréquemment chez des patients colonisés par la bactérie *P. aeruginosa* (PA), le pathogène le plus fréquent isolé des voies aériennes FK. Ces co-infections résultent dans la perte de fonction pulmonaire progressive. PA persiste dans les voies aériennes malgré le système immunitaire et l'utilisation d'antibiotiques, en partie grâce aux biofilmes, mais également par la présence intra-épithéliale de PA, tel que démontré récemment dans le laboratoire. Nous avons étudié comment les infections virales modulent la persistance de PA et la réponse antibactérienne de l'hôte.

Afin d'étudier ces mécanismes, le lignée cellulaire bronchique BEAS-2B a été transfectée avec le poly(I :C), un ligand du récepteur TLR3 qui stimule les réponses antivirales en absence ou présence de la flagelline ou du matériel diffusible de PA (PsaDM) pour mesurer l'activation des voies MAPK, éléments intégraux de la réponse inflammatoire. Dans le modèle d'invasion cellulaire, les BEAS-2B exposées au poly(I :C) ont été infectées avec PaO1et le dénombrement bactérien effectué à 4h post infection. À l'inverse, l'impact de poly(I :C) sur la persistance bactérienne a été mesurée en traitant les BEAS-2B après l'infection et faisant le dénombrement 5 jours post infection. L'expression de la molécule d'adhésion ICAM-1 a été mesurée par cytométrie en flux suite à l'infection par PA ou la stimulation du récepteur TLR2 en présence d'outils pharmacologiques pour déterminer les voies de signalisation impliquées. La pré-stimulation par poly(I :C) augmente l'activité de la MAPK p38 lorsqu'elle est stimulée par l'activation de TLR5 ou TLR2. De même, la pré-stimulation par poly(I :C) augmente l'expression à la surface d'ICAM-1 en réponse à PsaDM, un agoniste de TLR2 ou bien des souches PA (mucoïdes et non-mucoïdes). Les pré- et post-traitement par poly(I :C) ont diminué significativement l'internalisation et la persistance de PA. Finalement, l'expression d'ICAM-1 est dépendante de la voie NFkB et non des voies MAPK.

Cette étude démontre un rôle de la réponse antivirale dans l'altération des réponses antibactériennes des cellules bronchiques. Ces résultats ont une implication dans la pathophysiologie des co-infections respiratoires chez les personnes atteintes de FK.

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thesis. Thank you to my parents for their unconditional love, inspiration in my every single endeavor since I was born, I could not have accomplished this without your support.

PREFACE AND CONTRIBUTION OF AUTHORS

The candidate has chosen to present her study in a classical format. The thesis comprises of several sections including firstly, the rationale section along with hypotheses and objectives, followed by a detailed and comprehensive review of the literature regarding the context and background of the study. Thirdly, experimental approaches are described in the Materials and methods section; and this is followed by the results section. The discussion section then follows, which is further followed by the limitations and future directions section. Lastly, the references are listed in the end.

Dr. Rousseau, the candidate's supervisor, was the originator of the research project and has consistently guided the candidate through in all aspects of the thesis, from experimental design to troubleshooting to data analysis and interpretation. In addition, he provided comprehensive feedback on all sections of the thesis.

Dr Guy Martel, a former research associate in the laboratory, provided training for the candidate in laboratory techniques essential for this study and provided assistance in troubleshooting a few experiments.

Wanru Guo, the candidate and author of this thesis was responsible for carrying out the all of the experiments described and collecting all the data in this thesis: culturing airway epithelial cell lines, transfection experiments, agonist stimulations and P. aeruginosa infection using an airway epithelial cell line, and all analyses including determining bacterial titres, western blot and Fluorescence-activated cell sorting analyses. Moreover, the candidate performed all statistical analyses and produced all figures and tables presented in this thesis. Finally, the candidate was responsible for writing the thesis in its entirety, including original, in-depth analysis and critique that are presented in this thesis.

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LIST OF ABBREVIATIONS

CF	Cystic fibrosis
CFTR	Cystic Fibrosis transmembrane conductance regulator
Poly I:C	Polyinosinic: polycytidylic acid
HRV	Human Rhinovirus
RSV	Respiratory Syncytial Virus
ICAM-1	Intercellular adhesion molecule 1
TLR	Toll-like receptor
RLR	RIG-I like receptor
RIG-I	Retinoic-acid inducible gene I
MDA-5	Melanoma Differentiation-Associated protein-5
MAVS	Mitochondrial antiviral signaling
NLR	NOD-like receptor
Jnk	Janus Kinase
ERK	Extracellular signal–regulated kinases
TRAF	TNF receptor-associated factor
MAPK	Mitogen activated protein kinase
MKK	Mitogen activated protein kinase kinase
IKK	IkB kinase
NFκB	Nuclear factor- kappaB
TRIF	TIR-domain-containing adapter-inducing interferon-β
IL-6	Interleukin-6
IL-8	Interleukin-8
IFN-β	Interferon- β

PAMPs	Pathogen associated molecular patterns
MyD88	Myeloid Differentiation Primary Response Gene 88
TBK	TANK-binding kinase
IRF	Interferon regulatory factor
JAK	Janus kinase
STAT	Signal transducer and activator of transcription
SOCS	Suppressor of cytokine signaling
IRAK	Interleukin-1 receptor-associated kinase
ISG	IFN-stimulated genes
OAS-1	2'-5'-oligoadenylate synthetase-1
PKR	Protein kinase R
Mx proteins	Myxovirus resistance proteins
CFBE	Cystic Fibrosis Bronchial Epithelial cell line

1 INTRODUCTION

1.1 Rationale

Cystic fibrosis is an autosomal recessive disorder that is caused by over 2000 mutations in the CFTR gene, which encodes the chloride channel spanning the plasma membrane (Cystic fibrosis Canada, 2014). The decreased chloride ion conductance out of the cell results in airway epithelial cells retention of water and dehydration of the mucus. This causes diminished mucocilliary clearance of various pathogens, leading to more inflammatory response of the airways. During a CF pulmonary exacerbation, there is hyperinflammation and characterized by increased lung damage. The most common bacterial pathogen colonizing airways of adult CF patients and triggering exacerbations is P. aeruginosa (shown Fig 1), with the prevalence up to 62% in age group 25-34 (Goss & Burns 2007).

Respiratory viral infections are responsible for 65% of CF pulmonary exacerbations, and the most commonly isolated and cultured include respiratory syncytial virus (RSV) and human rhinovirus (HRV), which accounts for approximately 72.5% of the cases identified by throat swabs (Flight et al 2014). The viruses cause production of pro-inflammatory cytokines and changes the host microbial population. In CF patients in particular, it means that P. aeruginosa appear in patients previously tested negative, and changes from intermittent to chronic infection (Kiedroski et al 2018) occurs, which is why P. aeruginosa and virus infection together received widespread attention. Pseudomonal and viral infection together trigger more severe exacerbation and faster decline in lung function over time (Kiedrowski et al 2018), and since HRV comprises the majority of viral infections, thus we decided to focus on the interaction between P. aeruginosa and HRV.

Since inflammatory responses mediate immunity to bacterial infections, and type I IFN released by viral infections somewhat counteracts these pro-inflammatory responses shown in literature (next section); but on the other hand, enhances them by increasing antigen presentation and antibody secretion in vivo (Boxx and Cheng, 2016), hence our first objective is to investigate how viral infection alters the anti-bacterial inflammatory response.

Despite P. aeruginosa being seen as an extracellular pathogen, its intracellular reservoir in corneal epithelial cells (Fleiszig *et al.,* 1995) has been shown and subsequently various mechanisms have been proposed regarding its internalization into epithelial cells (Eierhoff *et al.,* 2014, Yamamoto *et al.,* 2005, Sana *et al.,* 2015). It was also shown to occupy intracellular niches formed by bleb-like structures in CF cells (Kroken *et al.,* 2018), and our group has also shown intracellular bacteria inside CF cells in immature lysosomes (Faure *et al.,* 2018). This piqued our interest to investigate its mechanism and the various factors that influence this process, including the most common viral infection that occurs in CF exacerbation.

During CF exacerbation, there is also elevated neutrophilic response. Neutrophil adhesion and migration is an important part of inflammatory response, and it is in part mediated by intercellular adhesin molecular 1 (ICAM-1). ICAM-1 is a surface glycoprotein expressed on many immune cell types (epithelial cells, dendritic cells, macrophages, lymphocytes), and is utilized by epithelial cells to bind to and activate PMN infiltration into the airway lumen and trigger an inflammatory response (Sumagin *et al.,* 2017). Most importantly, this receptor is utilized by HRV as binding and internalization receptor to epithelial cells (Bochkov *et al.,* 2016). Despite studies showing that CF cells (IB3) have higher ICAM-1 activation when co-cultured with PMNs (Tabary *et al.,* 2006), one study showed that MPA does not activate higher ICAM-1 expression and HRV binding in CF cells than their non-CF counterparts (Chattoraj *et al.,* 2011). We are therefore interested in the investigation of ICAM-1 regulation in viral and bacterial co-infections as well as sequential infections, both of which are relevant in CF exacerbations.

Since in vitro models of HRV and PA are limited, we sought to develop a sequential HRV and PA infection using the Toll-like receptor 3 (TLR3) agonist Poly I:C to mimic the IFN mediated antiviral response as an attempt to validate our model for further applications to viral-bacterial interactions. We also used a healthy bronchial epithelial cell line as certain concepts including PA intracellular persistence is a novel concept and baseline effect is unknown and yet to be established. Moreover, by looking at the effects on normal epithelial cells, it would help us better understand CF pathophysiology. Our model is also important especially due to the scarcity of literature in examining inflammation/ host immune response in CF where viral infections precede bacterial infections, which is the focus of this thesis.

Moreover, current findings on situations where bacterial infections precede viral infections in CF often yielded inconsistent findings which requires clarification. For example, Chattoraj *et al.* (2011) showed that HRV39 infection in IB3 cells that are pre-infected with mucoid PA (MPA) had decreased IFN levels whereas BEAS-2B cells do not. In contrast to this, Dauletbaev *et al.* showed neither no difference in IFNα release between healthy and CF patients, nor decreased IFN caused by P. aeruginosa infection compared with HRV alone (Daulletbaev *et al.*, 2015). Further, in the study by Das M (2014), the viral load is unaltered by MPA treatment preceding HRV infection, in stark contrast to results by Chattoraj *et al.* (2011). This could be due to the different viral strains used, and whether PA filtrates (the former study) or live PA infection is used. Despite this, both studies did agree in terms of higher IL-8 levels in CF and along with PA infection, which prompted us to investigate similar parameters but from a different angle. In another study by Chattoraj *et al.* (2011) they examined biofilm density and release of planktonic bacteria from biofilms in response to HRV infection, where the former is reduced and the latter is increased. As a model reminiscent of intracellular persistence of PA and extracellular release, we decided to investigate similar phenomenon in the context of viral and PA coinfection.

To date, none of the studies examined fully represented the in vivo scenario whereby PA is trapped with biofilms that are outside and do not contact epithelial cells, a model difficult to recapitulate in vitro. In addition, whether and how PA persistence occurs in-vivo, including any signaling pathways and molecules that regulates the process, is yet undefined. We hope that this study would provide insights into the host-pathogen interactions regarding viral and PA infection that occurs at the CF bronchial epithelia.

1.2 Hypotheses

1) Viral infection of bronchial epithelia causes higher inflammatory response along with Pseudomonas infection, which alters bacterial persistence inside airway epithelial cells.

2) Viral infection increases ICAM-1 expression in bronchial epithelial cells; The level of ICAM-1 expression is higher when pseudomonal infection occurs, resulting in a pro-neutrophilic response and decline in lung function.

1.3 Objectives

1) To determine if Poly I:C stimulation changes the levels of MAP Kinase phosphorylation as part of the antibacterial immune response;

2) To investigate if Poly I:C stimulation changes the uptake and/or persistence of pseudomonas inside airway epithelial cells.

3) To determine the level of ICAM-1 expression in the context of viral and bacterial co-infection and investigate the signal pathways involved in ICAM-1 expression (NF-κB, p38α MAPK, ERK1/2, TBK1)

2 Literature review

2.1 Genetic and physiological defects in CF

Cystic fibrosis is an autosomal recessive disorder that is caused by over 2000 mutations in the CFTR gene, which encodes the chloride channel spanning the plasma membrane (Cystic Fibrosis Canada, 2014). There are 7 main types of CFTR mutations, type I is the absence of synthesis often caused by nonsense mutations, type II mutations involve folding defect. And this includes the most common mutation – deletion of Phe508 (delF508). Type III-mutations are missense mutations and often causes channel opening defects. Type IV mutations cause reduced channel conductance and are mainly caused by missense mutations. Type V and VI mutations cause decreased protein synthesis and half-life of the protein, respectively. delF508 mutations are most common, comprising around 85% of cases (Cystic Fibrosis Canada, 2014). The decreased chloride ion conductance out of the cell results in airway epithelial cells retention of water and dehydration of the mucus. This causes diminished mucocilliary clearance of various pathogens, leading to more inflammatory response of the airways (Cystic Fibrosis Canada, 2014). This is also in part caused by the altered pH which is caused by dysregulation of bicarbonate transport, which also renders antimicrobial peptides dysfunctional (Law and Gray, 2017). Traditional therapies involve mucolytic agents such as dornase alpha that breaks down DNA. Hypertonic saline and mannitol provide osmotic gradient that draws water onto epithelium (Sermet *et al.*, 2006).

In CF epithelial cells, there is increased inflammation due to CFTR defect, mainly via the lack of extracellular export of GSH normally done by CFTR, causing reactive oxygen species (ROS) to accumulate outside of the cell (Berube *et al.,* 2010), causing a higher basal level of inflammation. CF is also a multifactorial disease with abnormalities in many other cell types that act in concert to increase inflammation and impair bacterial clearance. Neutrophils undergo defective phagocytosis due to decreased chloride concentration in phagosomes, defective degranulation by inactivation of Rab27, increased release of neutrophil extracellular traps (NETs) and undergo NETosis instead of apoptosis (Law and Gray, 2017). Macrophages are hypersensitive and release more cytokines, and have higher pH in their phagosomes causing impaired phagocytosis and clearance of neutrophils (and NETs). This results in self–perpetrating cycle of more production of NET by neutrophils and inflammation, and reduced uptake of NET by CFTR-/- macrophages and high levels of pro-inflammatory cytokines produced by epithelial cells in response to NETs (Law and Gray, 2017). This results in the dysregulated response seen in CF, explaining the higher levels of basal inflammation.

2.2 Bacterial infection in CF

Bacterial infections in CF is common, with the Gram-positive Staphylococcus aureus is most common in younger children, with up to nearly 70% in adolescence 11-17 yrs. The most common bacterial pathogen colonizing airways of adult CF patients is P. *aeruginosa* (shown Fig. 1), with the prevalence up to 62% in age group 25-34 (Cystic Fibrosis Canada, 2014). Across samples taken from patients in all ages, approximately 51.7% of sputum cultures are positive for S. aureus and 57.3% positive for P. *aeruginosa* (Goss and Burns, 2007). P. aeruginosa is an aerobic, Gram-negative bacillus bacterium, however, it is considered a facultative anaerobe since it is capable of growing in the absence of oxygen (Moradali *et al.,* 2017). The bacteria survives in harsh environments including soils, plants, water and humans. It is an opportunistic pathogen as it rarely causes infections in healthy individuals (Moradali *et al.,* 2017) and almost exclusively infect immunocompromised individuals as well as those with defects in lung function including CF and COPD (Arnason *et al.,* 2017). Over time the chronic infection causes progressive decline in lung function.

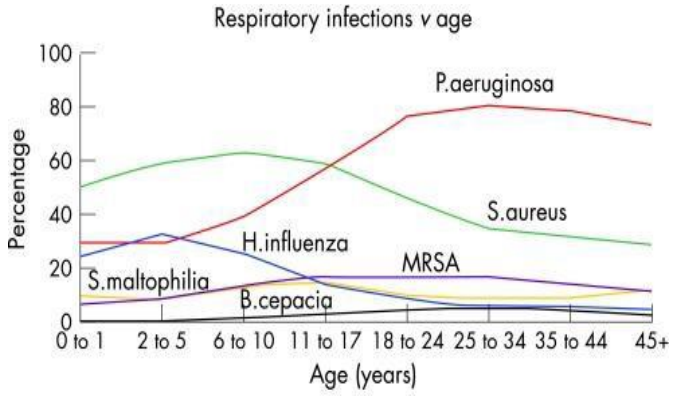


Figure 1. Age distribution of bacteria prevalence in CF patients. Adapted from: Goss, CH, Burns, JL. Thorax. 2007 Mar; 62 (4): 360-7.

In acute P. *aeruginosa* infection, they adhere to epithelium as planktonic bacteria; however, over time in the chronic infection phase, P. aeruginosa forms a biofilm structure on the surface of bronchial epithelium. Quorum sensing (QS) occurs between bacteria in response to cell population density increase, which regulate gene expression and biofilm formation by systems including LasR, Rhll, and PqsR (Rasamiravaka *et al.*, 2015). This biofilm formation is explained by Rasamiravaka *et al.* (2014). Briefly, planktonic PA firstly adheres in bronchial epithelia using flagellin and type 4 pili (and irreversible attachment occurs). In chronic PA infection, mucoid switch occurs due to MucA mutation and overproduction of polysaccharides (including alginate, Psl and Pel) occurs. These polysaccharides accumulate extracellularly (EPS) and along with the eDNA that are released by polymorphonuclear cells (PMN) form the main component of biofilm, which progresses via cycles of bacterial colony expansion and EPS accumulation (Rasamiravaka *et al.*, 2014). Lastly, equilibrium between biofilm and planktonic bacteria occurs, along with tissue damage and impaired clearance. When planktonic bacteria is released from the biofilm, this triggers a CF exacerbation.

2.3 CF exacerbation and treatments

During a CF pulmonary exacerbation, there is hyperinflammation and characterized by increased lung damage possibly by releasing planktonic bacteria from biofilm. Patients may experience fever, shortness of breath, lethargy, increased sputum volume, and decline in lung function measured by FEV1 (Goss and Burns, 2007). CF exacerbations are often treated by antibiotics, the most common being inhaled tobramycin (Schmiel *et al.*, 2014), which inhibits ribosomal 30S subunit tRNA reading of the mRNA. Other antibiotic agents such as

ceftazidime, cefepime, aztreonam, quinolones (e.g. ciprofloxacin). The combination of beta-lactam antibiotic and colistin are effective against PA, so is azithromycin as an anti-inflammatory agent whose mechanism of action is preventing protein synthesis by inhibiting the ribosomal 50S subunit (Cigana *et al.*, 2006). Recently used antibiotics include aztreonam inhalation, dry powder inhalers and Fosfomycin in combination with tobramycin (Smith *et al.*, 2017). Regular treatment of CF that target genetic defects include 3 types of drugs that affect the CFTR channel. Ataluren helps with translation readthrough of CFTR by recruiting near-cognate RNAs (Siddiqui *et al.*, 2016), however, Phase III clinical trials failed to produce beneficial effects. CFTR correctors which assist in protein folding include VX-325 and VX-809 (Lumacaftor) for type II mutations including the common delF508, and CFTR potentiators include Ivacaftor (Vx-770), which is a channel potentiator that is used in type III mutations (G551D) where there is channel gating defects as well as type IV mutations (e.g. R117H) with reduced channel conductance (Rowe and Verkman, 2013). The treatment guideline specifies that a double mutant of delF508 (which occurs 85% of the cases) requires Ivacaftor-Lumacaftor combination (Orkambi) (Rowe and Verkman, 2013). Recently, the new treatment which involves either VX-445 or VX-659, in combination with Ivacaftor/Tezacaftor (Symdeko) are assigned to patients with F508 mutation combined with another minor mutation (Mayor S, 2018). A combination of mucolytics, drugs that target the CFTR channel, as well as antibiotics for exacerbations is crucial for CF patients. By understanding host pathogen interactions in chronic infections, one can develop better treatment strategies.

2.4 Adaptations of P. aeruginosa in chronic infections

Various genetic mutations are identified in clinical strains in chronic P. aeruginosa infections. These include loss of function mutations in MucA, lasR, flagellin, Type 4 pili, type 3 secretion system, as well as increased expression of antibiotic efflux pumps, exopolysaccarides (including Psl and Pel) and type 1, 2 and 6 secretion systems (Faure *et al.*, 2018). MucA gene encodes alginate, and under normal conditions sequester AlgU to the membrane along with AlgB (Ryall *et al.*, 2014). When MucA is mutated, it no longer reacts with AlgU and it is in the cytoplasm free to activate RNA polymerase and activates transcription of AlgD, production of alginate characteristic of mucoid phenotype (Ryall *et al.*, 2014). LasR encodes proteases LasB and lasA that break down toxic proteases secreted by P. aeruginosa as well as neutrophil elastase and cytokines including IL-6 and IL-8 (Lafayette *et*

al., 2015). In chronic infections, more than a third of the clinical strains (PACF508, CF1) harbor the LasR mutation, resulting in their inability to break down inflammatory cytokine cytokines causing CF exacerbation (Moradali *et al.,* 2017). MucA mutation can also cause the downregulation of LasR and Lasl, which also in turn results in hyper-inflammation (Ryall *et al.,* 2014). There is an equilibrium between planktonic and biofilm PA, while inside the biofilm the basal levels of inflammation in biofilms is low, however, once mucoid bacteria is released it can bind to both TLR 2 and 5 (Beaudoin *et al.,* 2012), in contrast to predominantly toll-like receptor 5 in acute infections (see later sections) (Faure *et al.,* 2018). Formation of biofilm have advantages including resistance of mechanical and chemical attack as well as antibiotics; whereas lasR mutations have additional advantages including surviving in alkaline conditions and utilizing various amino acid sources and ability to act as 'social cheaters' (Lafayette *et al.,* 2015). Mutated type 4 pili and flagellin (activator of toll-like receptor 5) as well as type 3 secretion system accounts for their reduced virulence in chronic infections, which dampens host recognition and cytotoxicity (Faure *et al.,* 2018). Therefore, in chronic infection P. aeruginosa favors TLR2 over TLR5 (while activating both). Exopolysaccarides overproduction also results in increased immune system evasion. Additionally, increased expression of the type 6 secretion system increases P. aeruginosa internalization into epithelial cells (Sana *et al.,* 2015). Mutations in the bacterial lipopolysaccharide (LPS) also results in either immune evasion or enhanced TLR4 signaling (Faure *et al.,* 2018). Such mutations all have survival advantages in biofilms and chronic infections.

Another important mutation that results in increased antibiotic tolerance is the increased expression of efflux pumps by epithelial cells, which include MexXY-OprM, MaxAB-OprM/A, MexCD-OprJ and MexEF-OprN (Singh *et al.,* 2017). Loss of function mutations for down-regulators of these pumps (for example, MexR for MexA/B and MexZ for MexXY) increases their upregulation. Loss of LasR also increases expression of these efflux pumps, reducing antibiotics clearance and causing more damage (Moradali *et al.,* 2017).

2.5. P. *aeruginosa* intracellular persistence

Although P. aeruginosa had long been recognized as an extracellular pathogen, several groups have demonstrated internalization and intracellular persistence. In rat corneal

epithelial cells, Fleiszig *et al.* (1995) found PA inside vacuoles, suggesting endocytosis; subsequently, it was shown by several groups that PA internalizes epithelial cells by CFTR-caveolin in lung epithelial cells (Bajmoczi *et al.*, 2009); by lipid zipper LecA/Gb3 in MDCK cells (5) and lipid rafts in corneal epithelial cells (6). In addition, PA has also been shown to internalize by PI3K/Akt pathway (tight junction proteins) and effector injection by the T6SS which act to mobilize microtubules (7). Kroken *et al.* (2018) also showed PA persistence and replication inside corneal epithelial cells forming bleb-like niche, a process dependent on T3SS and the defective CFTR (4). Despite various findings, the mechanism and factors affecting internalization and persistence, as well as how most common viral pathogens, which often co-exist with P. aeruginosa in CF infections, alter their niche inside epithelial cells, remains understudied. Recently, our group used confocal microscopy stained with anti-pseudomonal antibody, and found that PA colocalized with LAMP-1 containing lysosomes (Faure et al 2018). These lysosomes, albeit not fully functional due to high pH, indicate an intracellular reservoir as well as potential possibility of PA utilizing the endocytosis pathway; then, using MiPACT (Microbial identification after passive CLARITY technique) that allows direct visualization of bacteria without being interfered by sputum, the presence of PA inside CF lungs is created (Faure *et al.*, 2018). Recently, The Nyugen lab also found that LasR mutation is linked to increased internalization and T3SS mutation is linked to increased persistence (unpublished data). These offers exciting perspectives to further study the mechanistic aspects of persistence, as well as how this is influenced by viral infection and inflammation, a main objective of this study.

2.6 Viral infections in CF

Respiratory viral infections are responsible for 65% of CF pulmonary exacerbations. Of these, human rhinovirus (HRV) (responsible for the common cold) is the most common isolate across multiple studies (Goffard *et al.*, 2014, Flight *et al.*, 2014). One study found that HRV comprises 72.5% of all viruses isolated (Fig. 2) using throat swab from adult CF patients (Flight *et al.*, 2014). There are a number of other viral infections in CF exacerbation, including metapneumovirus (13.2%), adenovirus (4.1%) also causing the common cold, influenza A (3.6%), and RSV (2.0%), with RSV and influenza viruses associated with the greatest decline in function (Flight *et al.*, 2014). Along with PA infections, they cause even further lung function decline and greater risk of mortality and lung transplant (Kiedrowski *et al.*, 2018). In vitro and clinical data suggested they cause significant upper and lower

respiratory tract infections. For the most common HRV virus, there are 99 strains consisting of two groups – HRV-A that contains 74 serotypes and HRV-B that contains 25 serotypes. At least 50 subtypes of HRV-C is identified using PCR techniques (Das M, 2014).

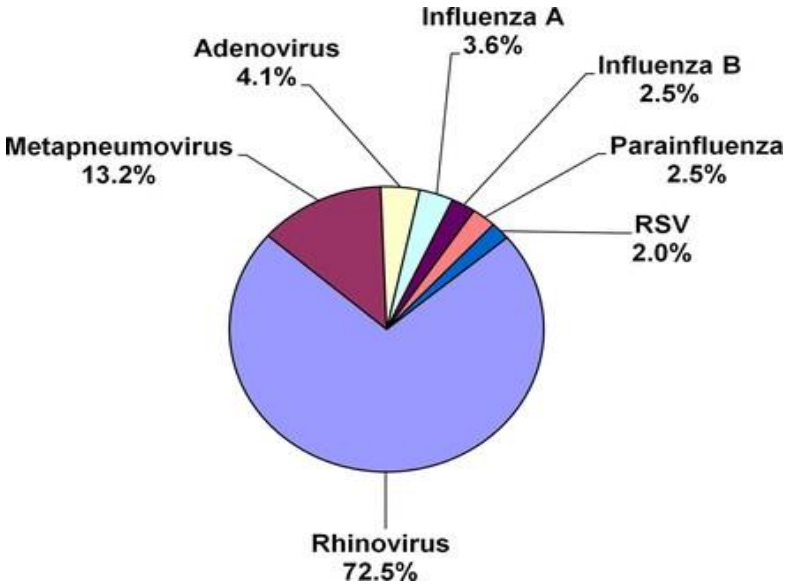


Figure 2. Percentage distribution of various viruses isolated from 100 CF patients, with rhinovirus being the most common. Adapted from Flight et al. 2013. Thorax. 2013 Oct 4. 69 (3).

It is found that CF has diminished antiviral response, especially when pre-exposed to bacterial infections. The viruses cause production of pro-inflammatory cytokines, altered immune cell recruitment, change in host microbiome composition (metabolic output), increased bacterial burden and new bacterial acquisition – meaning that P. aeruginosa appear in patients previously tested negative, and changes from intermittent to chronic infection (Kiedrowski *et al.*, 2018). Thus, viral and bacterial infections form a vicious cycle of CF exacerbation and lung damage. Additionally, reduced anti-microbial peptide secretion (Robinson *et al.*, 2013) that occur after viral infection also causes susceptibility to bacterial infections.

Despite our knowledge that viral infections predispose patients to bacterial infections in CF, how different agents and timing of infection (e.g. HRV vs. RSV, short-term vs. long-term prior to Pseudomonas infection) influence the immune response remains to be clarified, as this has been shown to be the case with different bacterial infections (Boxx and Cheng 2016).

Our study further delves into the details of the innate immune response with respect to HRV and Pseudomonas infections.

2.7 Rhinovirus internalization and replication

The human rhinovirus is a single-stranded RNA virus replicating inside the cell with a dsRNA intermediate (Bochkov *et al.,* 2016). Based on the cell surface receptors HRV binds to that allows endocytosis, HRV is grouped into the major group (e.g. HRV 16) that binds to ICAM-1, and minor group (e.g. HRV 1B) that bind to the LDL receptor (Schuler *et al.,* 2014). Binding to ICAM-1 leads to HRV internalization through PI3K/Akt and MAPK pathways similar to PA, while LDLR mainly mediates internalization via clathrin-mediated endocytosis (Bochkov *et al.,* 2016). The receptor for HRV-C has recently been identified as cadherin related family member 3 (CDHR3) (Bockhov *et al.,* 2015), although several mechanisms regarding its internalization has been proposed, including clathrin, or interacting with the cytoskeleton before involvement of tyrosine kinase (c-Src) or actin polymerization (Bockhov *et al.,* 2015). The replication of HRV consists of 6 stages (Gamarnik *et al.,* 2000): 1) binding to surface receptor for endocytosis; 2) endocytosis and conversion to hydrophobic subviral particle; 3) release from endosomes; 4) binding to ribosomes and translation to capsid (P1) and non-structural (P2, P3) proteins; 5) transcription and formation of replication complex, which involves synthesis of the negative sense strand first and use as a template to synthesize the positive strand (Gamarnik *et al.,* 2000) and 6) packaging of positive stranded RNA and proteins, releasing them from the cell. Likewise, RSV is a dsRNA virus with similar replication mechanisms (Kim & Lee, 2014). The most important innate immune receptors, Toll-like receptors (TLR), mediate most of anti-bacterial and anti-viral immunity.

2.8 Signaling mechanisms in bacterial and viral infection

2.8.1 P. *aeruginosa*, HRV and PolyI:C signaling pathways in bronchial epithelial cells

Toll-like receptors (TLR) are pattern recognition receptors that bind to pathogen associated molecular patterns (PAMP) and are integral part of the innate immune system. TLRs are present in epithelial cells and many other cell types such as dendritic cells and macrophages, acting as the link between innate and adaptive immunity (Medzhitov 2001). There are 9 subtypes, TLR1 to TLR9. The subtypes that are located on the plasma membrane are TLR2, which forms a heterodimer with either TLR 1 or 6 and binds to bacterial lipopeptides as

ligands; TLR4 which binds to bacterial lipopolysaccharides (LPS) and TLR5 binds to flagellin as the only ligand (Medzhitov 2001). The receptors located on the endosomal membrane are TLR3 that binds to dsRNA, TLR7/8 that bind to single stranded RNA as well as TLR9 that binds to CpG containing DNA (Lester & Li, 2014). All receptors except TLR3 activate MyD88 adaptor protein, leading to activation of TRAF6 which then signals to MAP Kinases (p38, ERK) via MAP kinase kinase (MKK), which then lead to transcription of inflammatory mediators (IL-6, IL-8) via AP-1 (Lester & Li, 2014). They also lead to activation of IKKα/β, degradation of IκB, and translocation to the nucleus and activation of NFκB, which also transcribe pro-inflammatory genes responsible for antibacterial response (Fig 3).

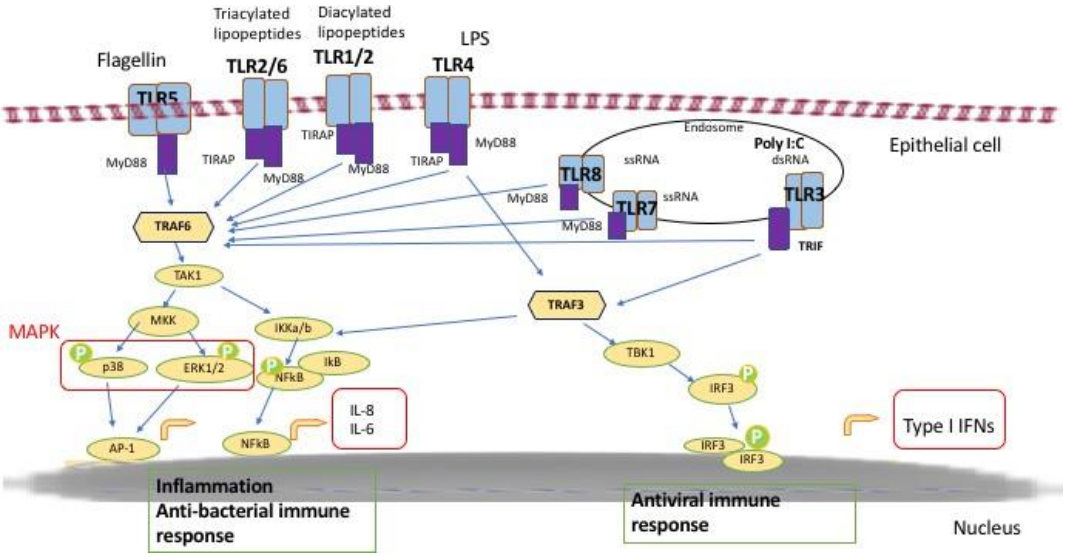


Figure 3. TLR signaling network. All receptors except TLR3 MyD88 and activate the MAPK and NF-KB pathways, while TLR3 signaling via TRIF upregulates the secretion of type I IFNs. TLR1, 2, 4, 5, 6 are located on cell surface and TLR3, 7, 8, 9 are located on endosomal membranes.

On the other hand, the TRIF dependent pathway, which is responsible for anti-viral response, is mainly downstream of TLR3 (and also RIG-I and MDA5) but also activated by TLR4

(Figueido *et al.*, 2009). Activation of the TLR3 pathway by rhinovirus, RSV and influenza virus signals the TBK1-IKKg-IRF3 pathway, where IRF3 and IRF7 act as transcription factors that form dimers and produce type I interferons (IFNα and IFNβ), which have anti-viral properties (see below) (Ivashkiv & Donlin, 2014). MyD88-dependent signaling is

classically associated with anti-bacterial response whereas TRIF-dependent signaling is associated with anti-viral responses (Triantafilou *et al.*, 2010) (Fig 4). Since two pathways co-exist in CF exacerbation, the interaction between two types of immune pathways, with P. aeruginosa predominantly utilizing the TLR5 receptor (Roussel *et al.*, 2011), and HRV using TLR3, 7, 8 and MDA5 (refer following sections) receptors (Triantafilou *et al.*, 2011), is an interesting yet understudied area.

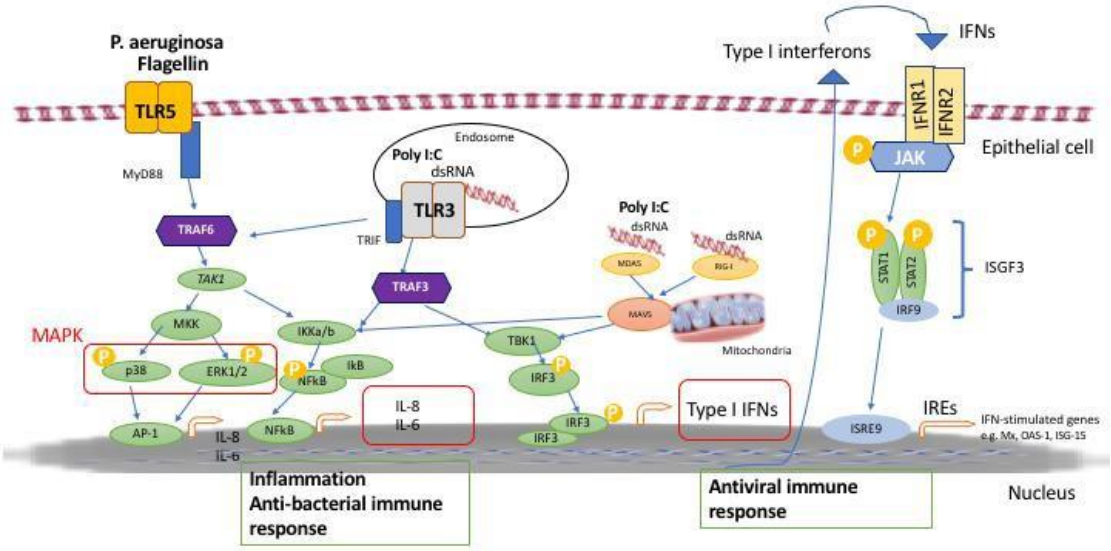


Figure 4. Anti-bacterial immune response and antiviral response signaling pathways. TLR5 mediated antibacterial response is activated by Pseudomonal flagellin, and TLR3-mediated anti-viral response is activated by poly I:C, a dsRNA mimicking viral infection.

Since HRV is a ssRNA virus, it mainly binds to TLR7 and 8, while the dsRNA intermediate binds to TLR3 on the endosomal membrane. Interestingly, heat inactivated RV binds to TLR2, suggesting that the capsid is responsible for the activation (Triantafilou *et al.*, 2011) (Fig 5). This is similar to RSV which has been shown to activate TLR2/6, 3 and 7 (Kim & Lee, 2014). In viral infections, Type I IFN in epithelia can act in an autocrine manner and bind to IFNA1/2 receptors, which activates major downstream signaling of Janus activated kinase 1 (Jak1) and tyrosine kinase 2 (Tyk2) protein tyrosine kinases. This activates STAT1 and STAT2 to form a heterodimer which associates with transcription factor IRF9 and forms the complex ISGF3 that translocate to the nucleus, binding to interferon response elements to activate transcription of interferon stimulated responsive genes (ISG), such as 2' 5'-oligoadenylate synthase (2' 5'-OAS), dsRNA activated protein kinase R (PKR) and Myxovirus resistance proteins (Mx proteins) (Bonjardim *et al.*, 2009). IFN receptors are also

present in other components of the innate immune system including dendritic cells, T cells and macrophages, which play roles in activating antigen presentation and effector T cell response (Ivashkiv & Donlin, 2014). The ISRE 2' 5'-OAS polymerizes to form activate tetramer pppA(2/p5'A)n, which then activate RNAseL to form a dimer and cleave viral RNA. Mx proteins form an oligomer and trap viral components. PKR forms a dimer and binds to viral RNA and phosphorylates the translation initiation factor EIF2α to inhibit translation initiation (Pindel & Sadler, 2011). Another upregulated gene is IL-10, which suppresses inflammatory responses. It is thought that type 1 IFN responses (triggered by viral infection) suppresses the anti-bacterial inflammatory response via several mechanisms (described in below section) (Bonjardam ***et al.,*** 2009), *which is the main point of this investigation.*

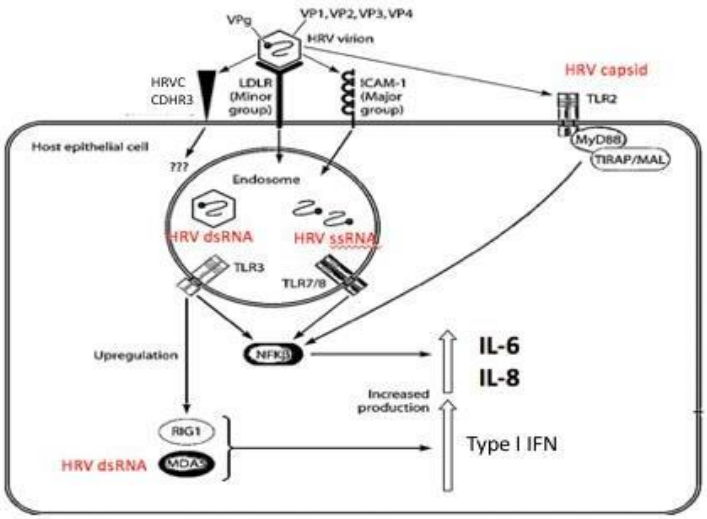


Figure 5. HRV infection signals upregulation of antiviral immunity. ICAM-1 receptor binds major group strains, while LDL binds minor group strains. HRV is known to activate TLR2, 3, 7, 8, and MDA5 receptors.

Other than TLRs, HRV also binds to Nod-like receptors NLRP3 and NLRC5 (Triantafilou *et al.,* 2013), as well as retinoic acid inducible gene-I-like receptors (RLR). These include retinoic acid inducible gene-I (RIG-I) and melanoma differentiation associated 5 (MDA5) (Reikine *et al.,* 2014). While RIG-I binds to short (<2000 bp) single or double stranded RNA with 5'-triphosphate caps, including paramyxoviruses (measles), rhabdoviruses and orthomyxoviruses (influenza A), MDA5 binds to long kilobase genomic RNA (and replicon

intermediates) such as picornavirus (including HRV) (Loo *et al.,* 2008). HRV binds to MDA-5 with its dsRNA intermediate, but not RIG-I, as their 5'-ppp is not exposed but covalently linked to viral protein g (VPg) (Bochkov *et al.,* 2016). Both RIG-I and MDA5 structures consist of an N-terminal caspase activation and recruitment domain (CARD), helicase domain and C-terminal domain. Without ligand binding RIG-I remains in inactive structure where the CARD binds to the CTD; Upon viral RNA binding the CARD is released and bind to the mitochondrial antiviral signaling protein MAVS (IPS-1) via the CARD domains, which then activates the TBK1/IKK/IRF pathway for IFN release (Reikine *et al.,* 2014). In contrast, MDA-5 does not release its CARD but uses CARD domain the nucleate and polymerize MAVS for signaling. It is found that HRV infection strongly activates MDA5 at a later stage than TLR3 (Triantafilou *et al.,* 2011), possibly because HRV increases transcription of MDA5 and it takes time to form an active receptor.

Polyinosinic: polycytidylic acid (Poly I:C) is a dsRNA TLR3 agonist, which upregulates antiviral immunity through the TLR3-TRIF-TBK1-IRF3 pathway (Torno *et al.,* 2010), in addition to a minor signal through the MAPK and NF-κB pathways. PolyI:C also binds to MDA-5 in its high-molecular weight form (1.5-8 kb). While our study utilizes HMW poly I:C, its lower MW form (0.2-1 kb) binds to TLR3 and RIG-I instead of MDA-5 (Ramezanpour *et al.,* 2018). HMW pIC is used by our study as mimicking of HRV infection as they bind to similar intracellular receptors. *Another main objective of this study th*erefore examines how TLR3 (and MDA5) mediated antiviral pathways activated by pIC affect TLR5 mediated inflammatory pathways by P. aeruginosa using the agonist flagellin, by looking at downstream signaling components MAPK and NF-κB, and potentially linking these pathways to PA intracellular persistence.

Since pIC binds exclusively to intracellular targets, it is more potent when transfected into the cell. Polyethylenimine (PEI) is a cationic polymer that facilitates its intracellular delivery by binding to negatively charged membranes. This enables pIC to be uptaken by endocytosis, leading to more sustained activation of various antiviral transcription factors as opposed to transient effects of naked pIC, and also enables activation of MDA5 receptor that non-transfected pIC cannot (Torno *et al.,* 2010). In human melanoma cells, PEI-pIC activates NOXA, leading to apoptosis induction by caspases 3, 7, 8 and 9 (Torno *et al.,* 2010).

Another promising objective of this study is to tease out the sophisticated role of viral stimulated IFN in pseudomonal infections. It is long known that type 1 IFN is a double-edged sword – can be protective or detrimental depending on the infection and time-course. In H. *pylori* infections for example, secretion of IL-10 regulated by type I IFN is protective, however, the promotion of IL-10 is detrimental in M. *tuberculosis* (Boxx & Cheng, 2016). The protective roles include 1) formation of protective epithelial barriers 2) increased bacteria clearance and 3) production of IL-10 and Setdb which reduce systemic hyper-inflammation (Boxx & Cheng 2016). In contrast, its detrimental effects include 1) triggering macrophage apoptosis and bacteria dissemination, and 2) IL-10 mediated suppression of pro-inflammatory response, in particular IL-17A mediated γδT cells, which suppresses neutrophilic response, deters secretion of antimicrobial peptides and bacterial clearance. It also suppresses many other cytokines such as IL-6 and IL-8, and 3) suppresses type II IFNs including IFNγ as well as IL-12, thus contributing to chronic infections (Boxx & Cheng, 2016). It remains unknown whether the type I IFNs are beneficial or detrimental in HRV infections prior to P. *aeruginosa* infection in terms of anti-bacterial response and bacterial clearance, which this study aims to explore.

2.8.2. Interaction between MyD88 and TRIF pathways

As mentioned above, whether the IFN signaling which is important in viral infections exerts an inhibitory effect on the pro-inflammatory pathway including IL8 in epithelial cells awaits investigation, and many evidence suggested this. It remains to see if this applies to TLR3 mediated TRIF signaling and MyD88 signaling mediated by TLR5, i.e. interaction between antibacterial and antiviral responses. There has been 3 mechanisms this inhibition occurs. Firstly, it has been shown that the IGSF3 complex, formed by STAT1/2 and IRF9, inhibits ISG (IFN responsive genes) at the CXCL8 promoter region (Laver *et al.,* 2008); IFN also reduced recruitment of cAMP response element–binding protein (CREB)–binding protein (CBP), p300 and RNA polymerase II to the promoter, as well as reducing HAT histone acetyltransferase binding (Laver *et al.,* 2008). Co-repressor proteins including HDAC1, HDAC3 and SMRT are also increased (Laver *et al.,* 2008). Secondly, there is evidence that suppressor of cytokine signaling (SOCS1) as a negative regulator of IFN signaling may also act as negative regulators of TLR signaling. SOCS1 can bind directly and inhibit JAK1

activation through its SH2 domain (Yoshimura *et al.*, 2012), and SOCS1 regulation on TLR4 mediated MAPK and NF-kB activation has also been shown (Yang & Seki, 2012). It is not yet known if this has relevance in TLR5 vs. TLR3 signaling. Additionally, SOCS1 has been reported to directly bind to IRAK4 in association with TRAF6, preventing their activation by TAK1 (Nakagawa *et al.*, 2002), however the evidence regarding this has been conflicting (Yoshimura et al, 2012). There is also reports that SOCS1 mediated ubiquitination of MYD88 associated TIRAP (MAL) also takes place in TLR4 signaling, leading to the suppression of MAL-dependent p65 NF-kB phosphorylation and activation of transcription (Mansell *et al.*, 2006).

Lastly, there is evidence that in the context of TLR4 signaling which acts through both MyD88 and TRIF pathways, that TRAF3 adaptor protein could undergo both activating and degradative ubiquitination. While K63-mediated poly-ubiquitination promotes the signal leading to the production of type I interferons, the degradation of TRAF3 by K48 mediated poly-ubiquitination mediated by Ubc13 ubiquitin conjugating enzyme is essential in activating MEKK1 and MKK4, therefore phosphorylation of MAPKs such as Jnk and p38 (Tseng *et al.*, 2011). This theory has also been supported by Hacker *et al.* (2011) in the signaling of TNFRs such as CD40. Whether this theory applies to TLR5 and TLR3 signaling in epithelial cells is yet to be determined.

Despite this, it is noted that, firstly, IFN does activate downstream signaling of MAPK as well as antigen presentation and antibody secretion (Ivashkiv & Donlin, 2014). Secondly, IFN-alpha in particular, is an activator of GAS elements which in turn increases pro-inflammatory cytokines (CXCL1, IRF9). Lastly, There is also crosstalk between the two pathways, for example, TLR3 activation could also activate TRAF6 and IKKα/β in a small percentage of the cases (Lester & Li 2014). Therefore, interaction between viral and bacterial responses by TLR3 and 5 pathways remains as a convoluted and exciting avenue.

2.9 Intercellular adhesin molecule 1 (ICAM-1)

During CF exacerbation, there is elevated neutrophilic response (Goss & Burns 2007). Neutrophil adhesion and migration is an important part of inflammatory response, and it is in part mediated by intercellular adhesin molecular 1 (ICAM-1). ICAM-1 is a surface glycoprotein expressed on many immune cell types (epithelial cells, dendritic cells,

macrophages, lymphocytes), it binds to integrins which include macrophage adhesion ligand 1 (Mac-1) and leucocyte function associated antigen-1 (LFA-1); as well as fibrinogen (Tabary *et al.,* 2006). In endothelial cells, activation of the receptor is involved in leucocyte (neutrophil) adhesion and migration to the site of injury (Lawson & Wolf, 2009). Neutrophil trans-migration across blood vessels involves three steps; 1) Rolling, where selectin on the immune cell binding to the selectin ligand on endothelium, 2) binding of chemokines to the chemokine receptor on neutrophils for integrin activation, and 3) binding of integrin (also LFA-1) to ICAM-1 enables adhesion (Lawson & Wolf, 2009). In bronchial epithelial cells, ICAM-1 binds to CD11a/18 (LFA1) and CD11b/18 (Mac1) on neutrophils and other PMN and trigger their activation and infiltration into the airway lumen through intercellular junctions (Sumagin *et al.,* 2016). Most importantly, ICAM-1 is also the entry receptor for HRV (Bockhov *et al.,* 2016), and respond to various signals that increases inflammation, thus investigating its regulation in viral and bacterial infections is of utmost importance.

ICAM-1 expression is activated by intracellular signals including protein kinase C (PKC), mitogen activated protein (MAP) kinase (JNK, ERK, and p38α), and NF-κB (Yu *et al.,* 2015). In bronchial epithelia, ICAM-1 expression is upregulated by multiple signals, one of which include tumor necrosis factor alpha (TNFα) that acts via the PLC/DAG/PKC/NF-□B pathway (Chen *et al.,* 2001). TLR agonists activating these pathways including flagellin, poly I:C all upregulate its expression. ICAM-1 gene promoter contains binding sites for many transcription factors, including AP-1, C/EBP, Ets, NF-κB, STAT, and Sp1 (Chen *et al.,* 2001). Other activators include IL-1b via the MyD88 pathway and IL-6 via STAT3 which are all upregulated in CF. IFNγ via the PKC -> cSrc -> JAK-STAT1a pathway increases ICAM1 and monocyte adhesion in uninfected human epithelial cells (Chang *et al.,* 2002), however reduce ICAM expression is infected epithelial cells (Sethi *et al.,* 1997). In CF, the high reactive oxygen species levels activates TACE to cleave EGFR pro-ligand to signal via the EGF pathway in addition to TLR (mentioned previously). Both ROS and EGF are found to increase epithelial ICAM-1 expression (Zhang *et al.,* 2008), the latter via PI3K/Akt and NF-κB pathways. Consequently, higher level of ICAM-1 expression is observed in CFTR deficient bronchial epithelial cells co-cultured with CF airway PMNs. P. aeruginosa infection upregulates ICAM-1, and this increase HRV binding and endocytosis. However, there does not appear to be difference between ICAM-1 regulation by mucoid P. *aeruginosa* in CF and

normal cells (Chattoraj *et al.,* 2011), which prompts our further investigation into how viral and pseudomonal stimuli alter ICAM-1 upregulation, *which is our third objective of the study.*

The in vitro models of HRV and PA infections are limited, especially those where viral infections precede pseudomonal infections, which is the main focus of this thesis. The mechanisms by which PA infections that precede HRV infections influence CF exacerbations have been shown but are conflicting thus requires further study. Chattoraj *et al.* (2011) showed that HRV39 infection in IB3 cells that are pre-infected with mucoid PA had decreased IFN levels whereas BEAS-2B cells do not. It points to a mechanism whereby HRV activates PI3K/Akt pathway, the increased oxidative stress ROS caused by PA infection inhibits the PI3K/Akt pathway thereby decreasing IFN secretion, causing increased viral persistence. However, the results are inconsistent, with a study by Dauletbeav *et al.* (2015) showing neither difference in IFNb release between healthy and CF patients, nor decreased IFN caused by P. aeruginosa infection compared with HRV alone. However, the study used HRV16, a major group virus compared to HRV39 in the former study (Dauletbaev *et al.,* 2015, Chattoraj *et al.,* 2011). Moreover, the later study used MPA filtrates instead of MPA infection in the former study, nor did they use cells cultured at the liquid-air interface (Dauletbaev *et al.,* 2015). Despite this, both studies did agree in terms of higher IL-8 levels in CF and along with PA infection, as well as increased viral persistence. However, it is still hard to compare different studies as the inoculation doses differ. In another study (Chattoraj *et al.,* 2011), HRV triggered reduction of biofilm density, release of planktonic bacteria, and trans-migration across membrane. All of this is triggered by increased ROS and IL8. However, none of the studies to date examined fully represented the in vivo scenario where PA is trapped with biofilms which are extracellular, whether and how PA persistence occurs in-vivo, including any signaling pathways and molecules that regulates the process, is yet undefined. Furthermore, it is yet unclear whether the increased viral persistence in CF is caused by reduced IFN response, and if not, if it is caused by bacterial co-infection, or any defects in CF including the endosomal pathway? For example, endosomal hyper-acidification in CF is corrected by Suldanefil, which then decreases IL8 production in PA infection (Poschet *et al.,* 2006). Hence, knowing the mechanism this occurs would significantly aid treatment options.

Background of study

Our study differs from previous studies in that firstly, even though the concept of PA intracellular persistence is in its naissance, it is still exciting to see how viral infection alters PA persistence. Secondly, shifting an angle and looking at the mechanism of how respiratory viral infection that predisposes a patient to secondary bacterial infection including pseudomonas, is meaningful. To date, there is only evidence that influenza virus predisposes respiratory tract to be more prone secondary bacterial infection in non-CF cells (Kiedrowski *et al.,* 2018). There is also evidence that Type I or III IFN treatment, or co-infection with RSV, RV and adenovirus promoted PA adherence and biofilm formation, but results have been conflicting. Another interesting perspective of our study is that it focuses on inflammatory signaling pathways (including MAPK, ICAM-1 etc.) other than IFN. It is not yet known whether viral priming would suppress inflammatory responses to PA, or it would potentially aggravate inflammation. Therefore, the effects of viral and bacterial infection on inflammation could be synergistic as opposed to inhibitory, and worth investigating. Even though we utilized an in vitro system due to time limitation of the study, the findings could give important insights that are applied in vivo. In all, we sought to examine the level of inflammatory mediators and ICAM-1 in the context of sequential viral and P. aeruginosa infection, as well as how viral response alter how the bacteria infect or persist inside the airways.

Table 1. Differential roles of Melanoma-Differentiation-associated gene 5 (MDA5) and Retinoic-acid-inducible protein I (RIG-I) in recognizing different virus (Kato *et al.,* 2006)

Receptor	Ligand	Examples
RIG-I	5'-ppp ssRNA Short dsRNA (<23 nucleotides)	Paramyxoviruses, influenza virus and Japanese encephalitis virus Low molecular weight poly I:C (0.2-1 kb)
MDA-5	Long ds RNA (>1000 bp)	Picornaviruses (includes HRV) High molecular weight poly I:C (1.5-8 kb)
TLR3	dsRNA >40-50 bp	Rhinovirus, respiratory syncytial virus and influenza virus

3 MATERIALS AND METHODS

3.1 Materials: The following materials are purchased from the manufacturer

Dulbecco's Modified Eagle Medium (DMEM) – Multicell
Fetal Bovine serum- Multicell
Tobramycin sulfate -GoldBio
TCEP (tris(2-carboxyethyl)phosphine) – Thermo Fisher Scientific
HEPES (1M) buffer – Gibco
Polyethylenimine – Polysciences
TC plates – Sarstedt
0.22 μm nitrocellulose membrane – Millipore
Bronchial Epithelial Growth Medium (LHC media with stock 4, trace elements and stock 11, refer to S1)
PaO1 – wild-type P. aeruginosa laboratory strain (DN276, University of Washington)
PaO1 ΔmucA – wild-type P. aeruginosa strain with mucA mutation resulting in a mucoid phenotype (DN411, M Paisck)
Phospho-p38α MAPK antibodies (Millipore), P38 MAPK antibodies (Cell signaling technologies)
Phospho-Erk1/2 antibodies (Cell signaling technologies), Erk1/2 antibodies (Cell signaling technologies)
BIRB 796 (p38 MAPK inhibitor, provided by Prof. Sir Philip Cohen, University of Dundee)
PD 18 -ERK1/2 inhibitor, US Biological, Swampscott, MA, USA
Precision Plus protein ladder (Biorad)
Bradford protein assay dye reagent (Biorad)
Ethylenediaminetetraacetic acid (EDTA) -Thermo Fisher Scientific
Sodium Dodecyl Sulfate (SDS) (Sigma Aldrich)
Tris base (Sigma Aldrich)
Tween-20 (Sigma Aldrich)
Triton x100- Thermo Fisher Scientific
ICAM-1 FITC conjugated IgG antibody (R&D systems)
Trypan Blue 0.5% solution (Multicell)
Acrylamide/Bis-acrylamide 30% solution -Biorad
Bovine serum albumin -Sigma Aldrich
Dimethyl Sulfoxide -Thermo Fisher Scientific
Ammonium persulfate - Thermo Fisher Scientific
Tetramethylethylenediamine (TEMED) - Sigma Aldrich

3.2 Cell culture

Immortalized human bronchial epithelial cells (BEAS-2B) were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 5% Fetal bovine serum (FBS) and 1%

penicillin/streptomycin. 250,000 cells/ml are seeded 1 day before experiment to reach confluence, and 125,000 cells/ml are plated 2 days before experiment for confluence.

3.3 Transfection

Linear polyethylenimine (PEI) (25kDa) is purchased from Polysciences (Warrington, PA), 1mg/ml PEI is dissolved in 20mM HEPES buffer, sterilized through 0.22um filter and stored at -80^{o}C. Cells are plated in 6 well plates with 250,000c/ml using DMEM with 5% FBS and without antibiotics to reach approximately 90% confluence. The next days, culture medium is replaced with Bronchial epithelial growth medium BEGM (without FBS, growth factors or antibiotics) for 24h. Normally BEGM consists of Bronchial epithelial basal medium (BEBM) supplemented with growth factors and antibiotics (LHC media, BSA, Bovine Pituitary extract (BPE), insulin, transferrin, hydrocortisone, triiodothyronine (T3), epinephrine, EGF, retinoic acid, ethanolamine, phosphorylethanolamine, stock 4, trace elements, stock 11, Penicillin/Streptomycin). In this case, BEGM starvation medium consists of LHC medium supplemented with Stock 4, Trace elements and stock 11 (zinc sulfate) (without antibiotics in this case since transfection). To confirm p38 activation and ascertain the timepoint, a time-course is taken with pIC transfected and non-transfected for 180 minutes. To examine the effect of sequential viral and bacterial stimulations, cells are transfected with poly I:C for 24 hours before stimulating with either flagellin or PA diffusible material for 45 mins before measuring phosphorylated p38 levels by western blot.

To transfect pIC into B2B cells, 200µl FBS and antibiotics-free BEGM is used per 2ml cell culture, with added 2µg pIC and 4µl PEI per 200ml medium for pIC: PEI ratio of 1:2, as well as 2µg pIC and 2µl PEI per 200ml for a pIC: PEI ratio of 1:1. The empty cDNA vector pc-3.1 is used as a control with a concentration of 1.608 µg/µl, and 1.6µl is used for 2µg per 200ml. The mixture vortexed for 30s and incubated for 30min for PEI-pIC complex to form. After which, it is added to cells drop wise.

3.4 PA diffusible material (PsaDM)

PA filtrates, or diffusible material, contain both bacterial cell surface virulence factors (PAMP recognition ligands) and secreted virulence factors. The former include LPS (activates TLR4), Pili, flagellin (activates TLR5) and alginate (activates TLR2 and 5). The latter includes secreted enzymes, exotoxins, phospholipase C which confer tissue damage,

which are heat inactivated before use as an agonist. Hence, it is thought to activate TLR2,4 and 5, with TLR5 being the major contributor as WT PaO1 is used. PA filtrate is prepared as follows, one loop of PaO1 glycerol stock (1:1) is inoculated in 4ml Luria broth (LB). After shaking overnight, it was diluted 1:5 in LB, and the optical density at 600nm (OD 600) is measured. The filtrate is then diluted to make 5ml of OD of 0.05. The bacteria is again grown overnight at 37 ^{o}C with shaking, and is centrifuged at 3000 x g for 10min. The supernatant is transferred to a new tube, centrifuged again, sterilized through 0.22µm filter, aliquoted, and stored at -20^{o}C for a week. it is heat inactivated at 95 ^{o}C for 10 min before use.

3.5 Western blot

BEAS2B cells are plated in complete medium (DMEM with 5% FBS, 1% Pen/Strep) to 70-80% confluency and starved the following day using BEGM™ (with antibiotics). Cells are placed on ice and scraped with lysis buffer added (120µl per 2ml cells,). Lysates are transferred to tubes and centrifuged at 4^{o}C, 12,000 x g for 5 min. Afterwards the supernatant is transferred to a new series of tube, where 40ul of 4X Laemmli buffer containing TCEP is added (1ml of LB + 100µl TCEP), the mixture is then heated for 5min at 95^{o}C to denature the proteins. The Bradford assay is used to qualify protein concentrations in each sample. Briefly, standards of BCA are prepared and either 10µl of standard or 2µl of sample is added in triplicates to the reader plate. 190µl Bradford reagent is added to each well and incubated for 5 min before the OD595 is read. Different volumes of each sample are then loaded onto the SDS-PAGE gel making sure that each lane contain the same amount of protein. Gels are run using running buffer (Tris base and glycine) for 80V in stacking gel and 110V for resolving gel. Afterwards, proteins in the gel are transferred to 0.22um nitrocellulose membrane using the tank transfer method using transfer buffer (Tris base, glycine and 10% methanol) for 30 min at 100V. The membranes are then blocked with 5% milk/TBS solution for 2h at room temperature. The primary antibodies (p38α, pp38α, pERK, ERK) are diluted 1:1000 and GAPDH is dissolved 1:5000 in 1% BSA/TBST in 1ml under parafilm (1µl of each antibody added) and incubated overnight at 4^{o}C. The membranes then washed three times with TBST (mixture of TBS and Tween 20) each for 5 min. Anti-rabbit and anti-mouse secondary fluorescent conjugated IgG antibodies are added 1:15000 in 1% BSA/TBST for 1h, and they emit green and red lights respectively upon excitation by a light source. Finally,

the membranes are washed 4 times in TBST and once in PBS before scanned and the protein phosphorylation are quantified by Licor Odyssey CLx fluorescent imaging system.

3.6 Intracellular infection with P. *aeruginosa*

BEAS-2B cells were plated 250,000 cells/well in 24 well plate the night before infection. PaO1 or its mucoid counterpart PaO1 MucA is inoculated in 4ml LB and are kept on a shaker at 37ºC overnight. The overnight culture is centrifuged at 2000 x g for 10 min, washed with sterile PBS, centrifuged again and diluted in sterile PBS. The solution is calibrated until the OD reads approximately 0.9, which equals to $1x10^9$ bacteria per ml. The bacterial solution is serial diluted and the optimal volume of bacteria to inoculate the cells is determined. For example, a multiplicity of infection of 1 (MOI=1) means there is average one bacterium per cell. Once inoculated, the plate is centrifuged at 1000rpm for 3 min to allow bacteria to contact the cell. Afterwards, the plate is incubated at 37ºC for 3.5h without CO_2. After the infection is complete, 5µl of 10mg/ml tobramycin is added to each 24 well (0.5ml) for a final concentration of 100µg/ml for 30min. Tobramycin is chosen as it selectively kills extracellular bacteria without impacting the intracellular population.

At each time point, the supernatant is collected and cells are washed with PBS before lysing with 0.1% triton in PBS, 0.5ml in each well for 10 min. The lysate is then diluted according to the MOI and then 100µl is plated on each LB agar plate. The uninfected as well as the supernatant are plated on LB as controls (to confirm the activity of the antibiotic). The smallest dilutions of 10^2 and 10^3 bacteria per ml are plated on LB to check the inoculum size.

3.7 Fluorescence activated cell sorting (FACS) for ICAM-1 surface expression

BEAS-2B cells are plated 250,000 cells/ml in complete media (DMEM, 5% FBS, 1% penicillin/streptomycin) in 6-well plates and treatments are divided into unstained (US), UT and pIC groups with or without either each of the inhibitor 5Z-7-oxozeaenol (0.25µM), BI605 (7.5µM), BIRB796 (2µM), PD18 (2µM), MRT67 (2µM) in triplicates. Briefly, Cells are scraped using 100µl PBS containing 10mM EDTA to detach from plates and transferred to Eppendorf tubes, 1.4ml PBS is added to dilute EDTA. 15% Formaldehyde is added for 10 min at 4ºC to fix the cells. Cells are then centrifuged for 5 min at 500 x g. They are then

washed twice with PBS and then 1ml of 2% BSA/PBS is added is added per Eppendorf tube along with 2.5µl ICAM-1 FITC conjugated antibody for 30 min in the dark. It is centrifuged again, the supernatant is discarded, the cells are resuspended with 350µl PBS with 10% FBS. They content of each tube is then transferred into FACS tubes, filtered by centrifugation at 500 x g and samples are run by a flow cytometer. The data is then analyzed by FlowJo software. The median fluorescence intensity corresponds to ICAM-1 expression levels.

3.8 MTT assay

To assess cell viability with pIC treatment and pseudomonas infection, the yellow color 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tetrazolium compound is added to the cells, where it can be reduced by the mitochondrial enzyme, succinate dehydrogenase to a purple formazan precipitate, whose amount corresponds to the number of viable cells.

25,000 cells/ml B2B cells are plated in a 96 well plate with DMEM, 5% FBS without antibiotics. Cells are treated with pIC with or without infection by PaO1 and after treatment, the supernatant of each well is discarded and 25µl of 5mg/ml MTT is added and incubated in dark for 2h. After a purple precipitate forms (reduced formazan), 200µl isopropanol is added into each well for 30min, and the absorbance is read at 570nm. The OD is converted to the % cytotoxicity normalized to control with the formula

% viable cells = OD(sample)/ OD (control) x 100

3.9 Statistical analysis

All data are presented as the mean ± standard error (mean ± SEM) for each sample when normally distributed. All statistical analyses are performed when at least triplicate data are derived, otherwise individual data are represented as dots. All raw data are entered into GraphPad Prism 8 software (GraphPad software, CA, USA) for statistical analyses. One-way ANOVA with Bonferroni's post-hoc analysis is done with all normally distributed data. $P<0.05$ are considered statistically significant.

4 RESULTS

4.1 MAPK activation and viral and bacterial stimulants

4.1.1 Higher p38α MAPK phosphorylation is observed in pIC pre-treatment of B2B cells along with TLR2 agonist, but not in TLR5 stimulation.

BEAS2B cells were stimulated with pIC (1μg/ml) for either 24h or 48h prior to stimulation with TLR5 agonist flagellin (400ng/ml) or TLR2 agonist PAM3CSK4 (1μg/ml) for 45 min. Western blots of cell lysates were stained for phospho-p38α (1:1000) and GAPDH (1:5000) as a control. Our western blots data (**Figure 6**) showed that, pIC (1μg/ml) pretreatment for 24h and 48h did not have a significant additive effect with flagellin (TLR5 agonist, 400ng/ml)) stimulation [compare lanes 2-3 and 4-5 in (a), and lanes 2-3 and 6-7 in (b)]. This is also evident in graphs e) and f). In contrast, in c and g, although pIC pretreatment for 24h did not significantly increase p38 activation by Pam3CSK4 1μg/ml), pIC pre-treatment for 48h along with Pam3CSK4 had an additive effect of more than 20 fold that of control (comparing lanes 2-3 and 4-5 in (d), and graph h). These results indicate that TLR2 agonist PAM activates higher inflammatory response combined with pIC meaning that in chronic PA infections when TLR2 response predominates, there is a higher level of inflammation along with viral infection during CF exacerbation than the acute infection where TLR5 mediated response predominates. However, this model does not capture viral infection closely, since HRV is uptaken into the cells by the endosomal pathway, transfecting poly I:C enables better recapitulation of bacterial and viral responses.

4.1.2 pIC transfection with PEI triggers higher p38 activation at all time points.

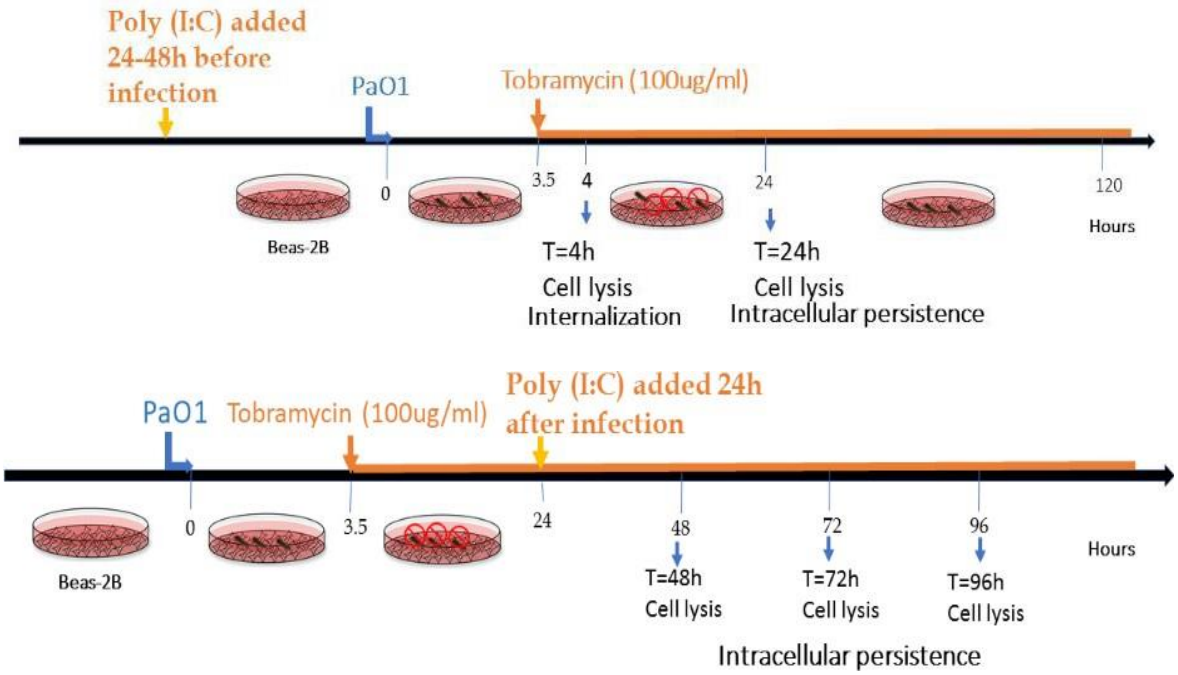
Since pIC binds exclusively to intracellular targets, it is more potent when transfected into the cell. Polyethylenimine (PEI) is a cationic polymer that binds to negatively charged cell membrane, forming a nanoparticle enabling uptake by the endosomal pathway which

involves Rab7 (small GTPase), LC3 (autophagosome marker) and lysosome sequentially. pIC to PEI ratios of 1:1 and 1:2 (1μg/ml pIC with either 1μg/ml or 2μg/ml PEI) is used to optimize the ratio for further experiments. There is higher p38 phosphorylation in the transfected pIC group (both 1:1 and 1:2) than the non-transfected (1μg/ml) at various time points of activation of 45 min, 90min, 120min and 180 min [**Figure 7**, shown by western blot in (a) and quantification p38 phosphorylation in (b)], with the peak occurring at around 120mins (Figure 7B). pIC: PEI ratios of 1:1 and 1:2 had negligible difference in activation of p38 (Fig. 7B), Hence, PEI: pIC ratio of 1:1 is used for further experiments as a mimic for HRV infection to investigate its effect along with PA infection.

4.1.3 Additive effect in p38 activation is observed in B2B cells transfected with pIC (PEI-pIC) along with stimulation with pseudomonal diffusible material (PsaDM).

BEAS2B cells are transfected with pIC in 1:1 ratio (1μg/ml pIC and 1μg/ml PEI) for 24h and stimulated with P. aeruginosa diffusible material (PsaDM) for 45 min. Non-transfected pIC (1 g/ml) is used as control. Our western blot (**Figure 8**) showed that pEI-pIC activates higher pp38 than non-transfected pIC [lane 5 vs. lane 6 in the two replicates (a) and (b)] and there is an additive effect of PEI-pIC along with PsaDM compared to either agents alone (lane 4, compared to lanes 2 and 5, also shown in (c) for quantification). In contrast, is no additive effect of PsaDM and pIC (lane 3, compared to lanes 2 and 6 in (a) and (b)). This revealed that viral infection mimicked by pIC indeed triggered higher inflammatory response along with P. aeruginosa, albeit weaker response in the acute phase than the chronic. Based upon this, we wanted to see if this heightened inflammation would alter PA internalization and persistence in epithelia, an exciting concept whose mechanism is yet unknown.

4.2 P. *aeruginosa* internalization and persistence are altered by viral stimuli



Schematic diagram of the intracellular infection protocol. To study PA internalization with pIC stimulation, pIC (1μg/ml) is either added to cells 24-48h before infection and cells are lysed 4h after infection and bacteria plated on LB agar for counting (a). To study the effect of pIC on PA persistence, pIC (1μg/ml) is added 24h after PA infection (at the persistence stage) and cells lysed at 48, 72, 96, and 120h post infection (24, 48, 72, 96h post pIC treatment) (b), as PA was previous shown to persist intracellularly up to 120h. For each PA infection, Tobramycin is added 3.5h after infection to clear extracellular bacteria while keeping intracellular bacteria.

4.2.1 pIC pre-stimulation decreases PA internalization into BEAS-2B cells

BEAS2B cells are treated with pIC (1μg/ml) for 48h before infecting with PaO1, and intracellular bacteria were counted at 4h, the time that internalization occurs (**Figure 9**). Pre-treatment with pIC significantly reduced PA internalization as early as 4h (Fig. 9a), and there is no difference between transfected and non-transfected pIC, suggesting pIC is potent in reducing PA internalization. The PEI is not toxic, as bacterial count in the PA+ pc3.1-PEI group resembles that of PA group, and data represented three experiments combined (N=12 per condition). In (b), even 24h after infection, there is only little difference in viability between pIC treated group (labelled PA+ pIC) and group without pIC (labelled PA), suggesting the difference in bacterial count is the difference in internalization. There is some toxicity of PEI after 24h, as evident by the significantly decreased viability in the PA+ pc3.1-

PEI group compared to the PA group, however, naked pIC had an effect at 4h post-infection (internalization phase) and 24h (short-term persistent phase) post-infection.

4.2.2 pIC decreases the intracellular persistence of P. *aeruginosa*

BEAS2B cells are treated with 1μg/ml pIC at 24h post-infection with PaO1 (MOI=1), which represents the short-term persistence phase (**Figure 10**). The first time-point taken for intracellular bacteria count is 24h after pIC addition, i.e. 48h post-infection (Fig 10a). Experiment was conducted for 120h (until 96h post pIC treatment) as it is the longest time shown for PA to persist inside the cell and data represents three experiments combined (N=12 per condition). The untreated group has CFU=0 and thus not shown in Figure. Shown in Fig 10a., pIC significantly decreases the number of persistent bacteria intracellularly, and there is no difference whether pIC is transfected or not. This effect is significant at 24h post pIC treatment, continuing to 96h post-treatment (120h post-infection), with lack of bacterial detection at 48h and 72h post- pIC treatment, indicating the greatest effect of pIC at this time (Fig 10a). PA+ pc3.1-PEI group has no effect on intracellular bacteria compared to PA alone meaning that the PEI is not toxic (Fig 10a). There is no significant cytotoxicity of pIC, PA or PEI in first two time points (b and c), but at the last time point (t=120h), PA and PEI-pIC groups had declined viability. However, comparing the PA+ PEI-pc3.1 group to PA group there is no difference in viability, suggesting that there is negligible cytotoxicity of transfection agent PEI but most toxicity comes from Pseudomonas infection itself. This is in contrast with Fig. 9b where the group containing PEI-pc3.1 (empty vector transfected, column 5) is the most cytotoxic. This possibly due to pIC already in the system for 48h before infection with PA for 24h compared to PA infection followed by pIC treatment for 96h. It is postulated that the former causes more damage to the cells. Whether these different sequential treatments have physiological relevance awaits further investigation in the following experiments.

Interestingly, in Fig. 10d, the group with non-transfected pIC had better viability meaning that pIC actually enhances survival, indicating a protective effect of pIC worth investigating further. This suggests that the decreased persistence shown in Fig 10a is not due to cytotoxicity, but rather through exocytosis or bacterial killing by the cell previous proposed by Fleizsig *et al.* (Fleisig *et al.*, 1995). Naked pIC (without transfection) is as potent as transfected pIC as it showed equally significant reduction of PA persistence, even though they activate different downstream mediators (which is further elaborated in the discussion

section).

In order to gain a deeper understanding of overall impact of how viral and bacterial infections interact in bronchial epithelium, we further examined regulation of intercellular adhesion molecule 1 (ICAM-1), an important mediator of neutrophilic response, inflammation, as well as adhesion and internalization receptor of HRV.

4.3 ICAM-1 upregulation by viral and bacterial stimulants

4.3.1 ICAM-1 expression is upregulated Poly I:C in BEAS-2B cells

Cells are stimulated with 1μg/ml pIC from 3h to 96h, at various time-points samples are collected and stained with ICAM1-FITC antibody for surface expression as median fluorescence intensity readout in FACS (**Figure 11**). ICAM-1 cell surface expression of B2B cells is upregulated in response to 1μg/ml pIC and gradually increases from 3 to 24h, then peaks at 48h before dropping at 72h (Fig. 11a). This decline is likely due to negative regulators of IFN signaling such as Supressor of Cytokine Signalling (SOCS). Significant expression is seen at 24h and 48h which is used for further experiments. In Fig. 11b, ICAM-1 expression showed a dose-responsive increase in expression when pIC is increased from 1μg/ml to 5μg/ml. Nevertheless, pIC of 1μg/ml is chosen for future experiments as it triggers a non-saturable level of ICAM-1 expression.

4.3.2 ICAM-1 expression is triggered by activation of NF-κB pathway dependent on TAK1 and IκB, while independent on the MAPK or TBK pathways.

B2B cells are treated with various inhibitors for 1h before stimulating with pIC (1μg/ml or 2μg/ml) for 24h or 48h, and ICAM-1 expression level was measured by flow cytometry (**Figure 12**). Treatment of B2B cells using either 1μg/ml (Fig 12a) or 2μg/ml pIC (Fig 12b) for 24h caused upregulation of ICAM-1 compared to control (second bottom rows in a and b), manifesting as rightward shifted peak (increase in MFI). Pretreatment of cells for 1h using 7.5μl BI605, a IκB inhibitor as well as 0.25μl 5z 7-oxozeanol (TAK inhibitor) prior to pIC stimulation caused a leftward shift in MFI (reduced ICAM1 expression). In contrast,

pretreatment with (2μg/ml) MKK1/2 inhibitor (upstream of the ERK1/2 pathway) did not have an effect of ICAM-1 expression. Likewise, shown in **Fig. 13**(a), treatment with 2μg/ml pIC for 24h in the presence of TAK1 and IκB inhibitors Oxo and BI605 significantly decreased the expression of ICAM-1, whereas there is no change in expression in groups treated the p38α inhibitor BIRB796 and MKK1/2 inhibitor PD18. This indicate that pathway by which pIC upregulates ICAM-1 is mediated by NF-κB solely without involvement of MAPK. Also in Fig. 13(a), the inhibitors BI605 and Oxo decreased ICAM-1 activation by 1μg/ml pIC stimulation for 24h, but the results failed to reach significance since the overall ICAM-1 expression level is lower; nevertheless, in Fig. 13(b) at 48h post pIC (1μg/ml) stimulation, the presence of BI605 significantly reduced ICAM-1 expression but Oxo did not, possibly indicating that the inhibitor has lost its activity after 48h. Therefore, caution should be exercised in future regarding inhibitor half-lives vs. experimental duration. Future testing of these inhibitor activity for 48h and 24h using western blot is essential, which involves quantification of TAK1 protein levels in the presence of Oxo for any decrease in activity, as well as IκB protein levels in the presence of BI605 for recovery in activity (as BI605 inhibits degradation of IκB). Examining p38α and ERK activation are also useful in testing the inhibitor activity of BIRB796 and PD18.

Interestingly, in Fig. 13(b) the TBK1 inhibitor, MRT67 (2μg/ml) either alone or with pIC paradoxically increased ICAM-1 expression, partially due to its non-specificity in inhibiting TBK and off-target effects. Of note, it has been demonstrated that TLR3-TBK-IRF3 pathway is at least partially responsible for ICAM-1 expression (Matsukura *et al.*, 2006), as the knockdown of IRF3 using siRNA significantly decreased ICAM-1 expression. Future experiments investigating such pathways should consider knockdown or using a more specific inhibitor of TBK1.

4.3.3 pIC pre-treatment increases ICAM-1 expression by P. *aeruginosa* diffusible material (PAF) stimulation.

In order to investigate ICAM-1 expression regulation by viral and bacterial stimulants, we firstly treated B2B cells with pIC (1μg/ml) at the same time or 24h prior to PAF stimulation (for another 24h) to examine ICAM-1 regulation (**Figure 14**). pIC pre-stimulation for 24h

triggered significantly higher ICAM-1 expression compared to PAF alone (Fig. 14a). This is possibly due to the highest pIC signal on ICAM-1 at 48h post pIC stimulation which corresponds to 24h post PAF stimulation. In contrast, pIC and PAF co-treatment for 24h does not trigger higher ICAM-1 activation than either agonists alone (Fig. 14b). These results indicate pIC treatment short-term prior to bacterial infection could have a protective effect in terms of increased inflammation and bacterial clearance, however also potentially causing more tissue damage of the bronchial epithelia. In contrast, pIC stimulation (or viral infection) along with or after PA infection triggers a lower level of inflammatory response, which could cause less tissue damage while at the same time hinders pathogen clearance.

It is noted that PAF contains a mixture of agonists including TLR5 agonist flagellin, type 4 pilli, and considerable amount of TLR4 agonist LPS as well as lower amounts of TLR2 agonist PAM. These different receptors play distinct roles in PA acute and chronic infections, therefore further experiments are to tease out their respective regulation of ICAM-1 in combination with viral infection.

4.3.4 pIC pre-treatment for 24h increases ICAM-1 expression in response to PaO1 infection

Since we have used PA filtrates for this experiment as well as previous experiments on MAPK activation, we sought to verify our findings with PaO1 infection. B2B cells are pre-treated with pIC (1μg/ml) for 24h and 48h prior to, as well as 24h after PaO1 infection (MOI=1) for 24h, and ICAM-1 expression level is measured by FACS (**Figure 15**). Similar to PAF, there is significantly higher ICAM-1 by PaO1 infection with 1μg/ml pIC pre-stimulation for 24h (additive effect) (Fig. 15b), whereas in Fig. 15a pIC pretreatment for 48h did not significantly increase ICAM-1 along with PaO1 infection (MOI=1) compared to PaO1 alone, neither did ICAM-1 significantly increase when pIC is added 24h post infection in Fig. 15c. Also, ICAM-1 levels did not significantly change with increased dose of PaO1 infection (MOI=1 to MOI=2). The additive effect when pIC is added 24h before PaO1 infection is possibly due to the peak pIC signal on ICAM-1 at 48h post stimulation (which is the time after 24h PaO1 infection), whereas in (a) the pIC triggered response is already declining (where the stimulation had lasted for 72h when measured), and in (c) pIC is stimulated for 24h and response has not peaked. This verifies the previous result and also indicates a potentially heightened clearance of pathogens by this sequential treatment due to elevated neutrophilic response, which is achieved by the increased inflammation, albeit

causing more epithelial tissue damage.

Despite this, it is noted that we have only examined how the wild-type PA alters ICAM-1 levels in the acute phase of infection, which may not recapitulate the situation in CF airways chronically colonized with PA that harbors various mutations. To more closely mimic this, we next used the TLR2 agonist, PAM3CSK4, which is important in the mucoid switch that occurs in chronic infections, as well as the mucoid strain PaO1 *mucA* (later).

4.3.5 pIC pre-treatment for 24h increases ICAM-1 expression by PAM3CSK4 stimulation.

In acute infections, PA activates solely the TLR5 receptor, while activating both TLR2 and TLR5 during the mucoid switch accompanied by MucA (and usually LasR mutation) mutation before a decline in TLR5 activation, thus favoring TLR2 over TLR5 later during adaptation in chronic infections. Therefore it is worthwhile looking into TLR2 activation by Pam3CSK4 with or without pIC treatment and the effect on ICAM-1. B2B cells were pretreated with pIC (1μg/ml) for 24h before stimulated with PAM3CSK4 (1μg/ml) for 24h, and ICAM-1 expression is measured (**Figure 16**). There is an additive effect on ICAM-1 expression by PAM with pIC pre-stimulation for 24h (Fig. 16), indicating higher inflammation in chronic infections along with viral infection mimicked by pIC. ICAM-1 CRISPR cells are used as controls for expression of ICAM-1. Additionally, In order to better compare the respective levels of inflammation in acute and chronic states, various combination of agonists to recapitulate different states of infections are used next.

4.3.6 ICAM-1 expression by stimulation with TLR2, 3 and 5 agonists and their combination.

In order to experiment on different infection states, we used different combination of agonists, all of which are used to stimulate B2B cells for 24h and ICAM-1 expression is measured. While Flagellin (400ng/ml) and PAM (1μg/ml) are used to mimic acute and chronic infections respectively, the combination of PAM and FLAG is used to recapitulate mucoid switch but before adaptation to chronic infection and flagellin mutation, where both receptors are stimulated before favoring TLR2. The combination of PAM and pIC (1μg/ml) represent viral infection in chronic PA colonization, while PAM, Flag and pIC together represents infection with mucoid P. *aeruginosa* along with viral infection. Shown in **Fig. 17**,

TLR3 agonist pIC, TLR2 agonist PAM3CSK4 and TLR5 agonist Flagellin alone similarly upregulate ICAM-1 surface expression. An additive effect on ICAM-1 expression is observed in pIC with TLR2 agonist PAM as well as with both PAM and flagellin, whereas stimulation with PAM and flagellin together had no increase in ICAM-1 activation compared to either agent alone. Taken together, it highlights the significance of pIC as a mimic of viral infections in triggering bronchial epithelial inflammation and neutrophilic response by upregulating ICAM-1.

In addition to changes in TLR signaling that occurs during PA chronic adaptation, several dozens of other genetic mutations were documented in chronic P. *aeruginosa* isolates, we therefore tested a common mutation, *mucA*, and its effect on ICAM-1 regulation combined with pIC stimulation.

4.3.7 pIC pre-treatment for 24h increases ICAM-1 expression by PaO1 MucA infection, and no difference in ICAM-1 activation was found compared to infection by wild-type PaO1.

Dose response curves of ICAM-1 surface expression corresponding to increasing MOIs (0.5, 1, 5 and 10) of either PaO1 or PaO1 *mucA* infection (for 24h) were initially constructed to determine the optimal doses for combination of both strains. Both strains caused similar activation of ICAM-1 and plateaued at MOI of 5, representing saturable ICAM-1 activation at this multiplicity of infection (**Figure 18**). Hence, MOI of 1 is chosen for further experiments in order to observe ICAM-1 upregulation. B2B cells are stimulated with combinations of PaO1, PaO1 *mucA* (both MOI=1) and pIC (1μg/ml) for 24h and ICAM-1 expression was measured by FACS. Shown in **Figure 19**, PaO1 *mucA* (MOI=1) or PaO1 (MOI=1) along with pIC (1μg/ml) triggers significantly higher ICAM-1 activation than PaO1, PaO1 *mucA* or pIC alone (additive effect). However, there is no difference caused by the *mucA* mutation in terms of ICAM-1 activation, and neither does the co-infection by both PaO1 and PaO1 *mucA* trigger higher ICAM-1 than either alone. In addition, PaO1, PaO1 *mucA* and pIC together does not result in higher expression levels than either PA strain along with pIC, and PaO1 and PaO1 *mucA* strains did not differ significantly in terms of ICAM-1 activation. This can be due to the kinetics of infection, where the combination of MOI of 1 of both PaO1 and PaO1 *mucA* is similar to the MOI of 2 of either strains, which does not differ from MOI of 1 in ICAM-1 activation (refer to Fig. 18 dose-response curve). This could also result from factors including using a genetically engineered mutation (MucA) in wild-type

PaO1 strains as opposed to a clinical isolate (CF1), the latter differs in many traits and may harbor additional mutations including the common LasR, thereby would trigger higher inflammation compared to wild-type PaO1. This potentially explains why the ICAM-1 expression upregulated by PaO1 and PaO1 MucA are similar. ICAM-1 CRISPR cells are also included as controls for ICAM-1 expression.

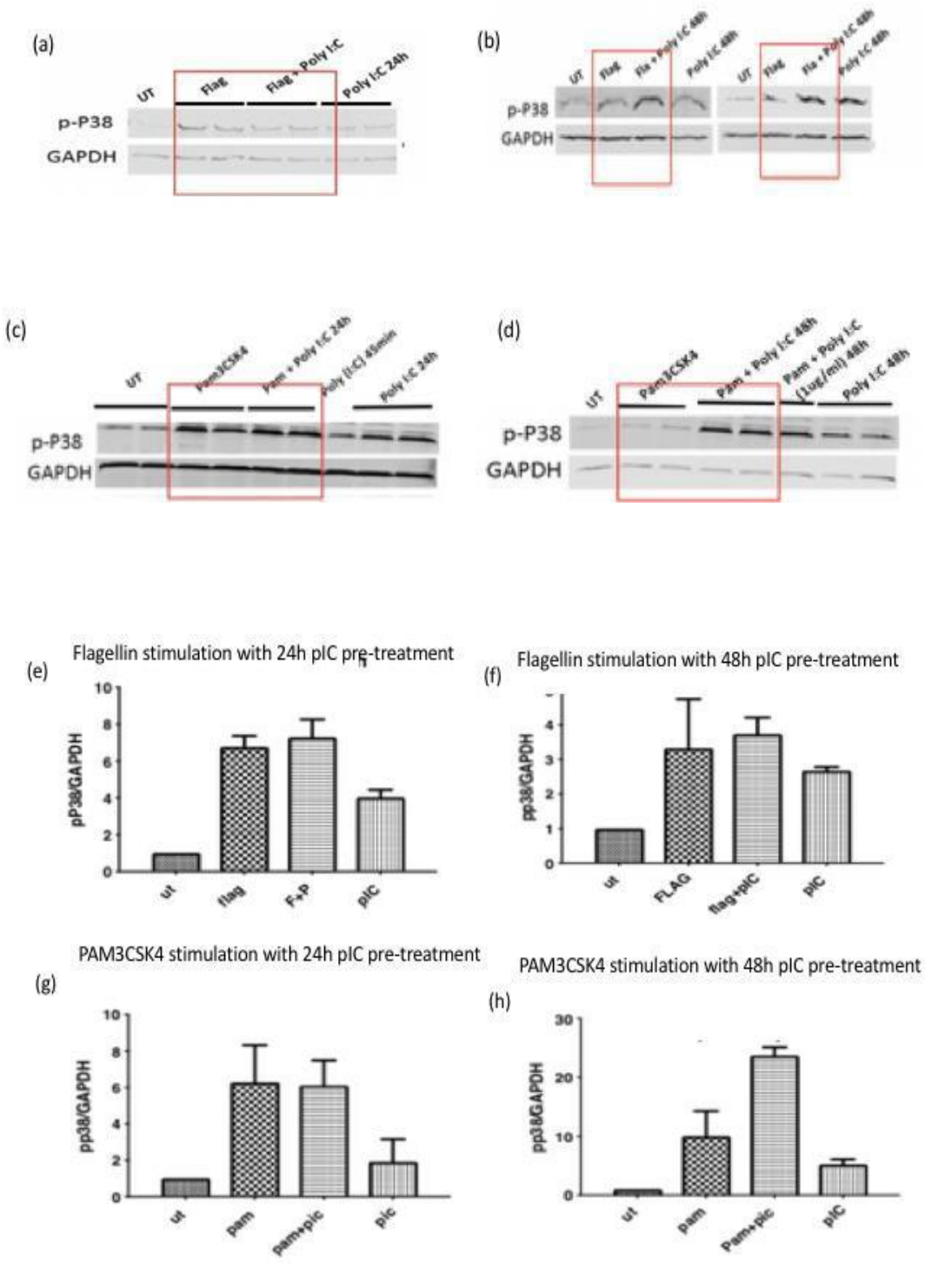

(a)
UT
Flag
Flag + Poly I:C
Poly I:C 24h
p-P38
GAPDH
(b)
UT
Flag
Fla + Poly I:C 48h
Poly I:C 48h
p-P38
GAPDH
(c)
UT
Pam3CSK4
Pam + Poly I:C 24h
Poly (I:C) 45min
Poly I:C 24h
p-P38
GAPDH
(d)
UT
Pam3CSK4
Pam + Poly I:C 48h
Pam + Poly I:C (1ug/ml) 48h
Poly I:C 48h
p-P38
GAPDH
(e)
Flagellin stimulation with 24h pIC pre-treatment
pP38/GAPDH
ut
flag
F+P
pIC
(f)
Flagellin stimulation with 48h pIC pre-treatment
pp38/GAPDH
ut
FLAG
flag+pIC
pIC
(g)
PAM3CSK4 stimulation with 24h pIC pre-treatment
pp38/GAPDH
ut
pam
pam+pic
pic
(h)
PAM3CSK4 stimulation with 48h pIC pre-treatment
pp38/GAPDH
ut
pam
Pam+pic
pIC

Figure 6. Higher p38α MAPK phosphorylation is observed in pIC pre-treatment along with TLR2 agonist, but not in TLR5 stimulation. BEAS2B cells are plated at 250,000 cells/ml in DMEM containing 5% FBS, then starved with BEGM™ overnight. (A) Cells are treated with pIC (10μg/ml) for 24h and stimulated with flagellin (400ng/ml) for 45min, The protein samples are blotted into membranes and stained with phosphor-p38α antibody (1:1000) and GAPDH as a control (1:5000). (B) cells are treated with pIC (10μg/ml) for 48h and stimulated with flagellin (400ng/ml) for 45 min. (C) cells are treated with pIC (10μg/ml) for 24h and stimulated with PAM3CSK4 (1μg/ml) for 45min (D) cells are treated with pIC (10μg/ml) for 24h and stimulated with PAM3CSK4 (1μg/ml) for 45min. Two replicates of the same samples are shown on western blots. UT, untreated. (E-H) Quantifications of the western blots A-D respectively. Y-axis represent fold change of pp38α over GAPDH normalized to control. Data represent three independent western blots with mean ± SD indicated.

In (a-d), the respective lanes are (a) Lane 1: UT. Lane 2-3: Flagellin. Lane 4-5: pIC 24h +Flagellin. Lane 6-7: pIC for 24h.

(b) Lane 1: UT, Lane 2: Flagellin. Lane 3: pIC 48h+ flagellin. Lane 4: pIC for 48h. Lanes 5-8 are a replicate of Lanes 1-4.

(c) Lanes 1-2: UT. Lanes 3-4: Pam3CSK4. Lanes 5-6: pIC 24h+ PAM. Lanes 7: pIC for 45min. Lanes 8-9: pIC for 24h.

(d) Lane 1: UT. Lane 2-3: Pam3CSK4. Lane 4-5: pIC (10ug/ml) 48h +PAM. Lane 6 pIC 48h (1ug/ml)+Pam. Lane 7-8: pIC (10 ug/ml) 48h.

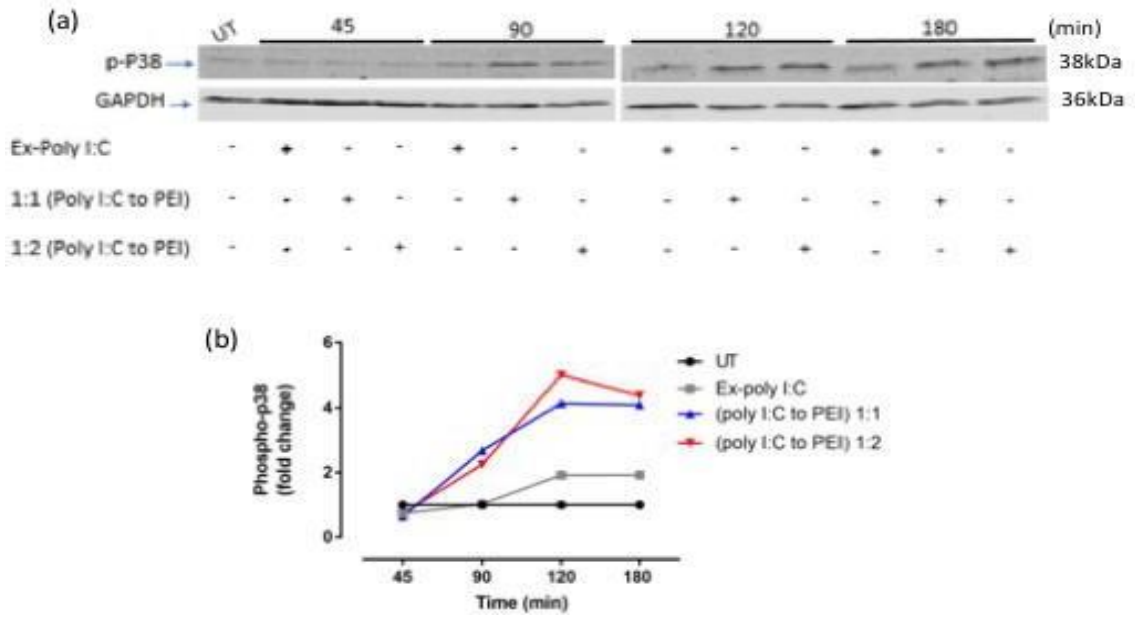


Figure 7. p38α phosphorylation by transfected and non-transfected pIC over 180 min in BEAS2B cells. B2B cells are plated 250,000 cells/ml overnight to reach 70-80% confluency. They are starved using BEGM overnight. Transfected or non-transfected pIC (pIC:PEI ratios of 1:1 or 1:2, 1μg/ml pIC with either 1μg/ml or 2μg/ml PEI) are added for 45, 90, 120 and 180min respectively, and protein samples are prepared, western blot procedure is performed using antibodies against phospho-p38α (1:1000) and GAPDH (1:5000). (a) shows p38α by different combinations at different times. Lane 1: Untreated; lane 2-4: non-transfected pIC, transfected ratio 1:1, transfected ratio 1:2 at 45 min; lane 5-7: 90 min; lane 8-10: 120 min; lane 11-13: 180 min.

(b) shows phospho-p38α activation by non-transfected pIC (grey line) or coupled with PEI (PEI-pIC) in the ratio of 1:1 (blue line) and 1:2 (red line). Fold change over pp38α/GAPDH over control is depicted. n=1 for each condition as proof of principle.

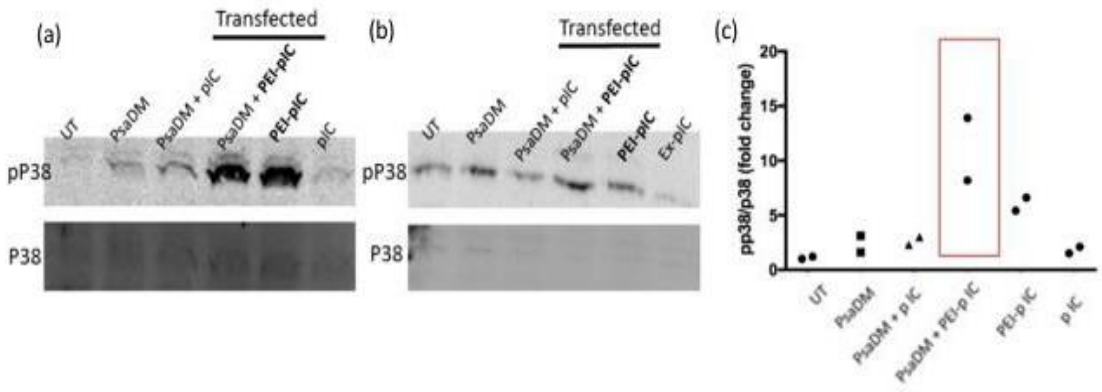


Figure 8. Additive effect of PEI-pIC and PsaDM (PAF) in p38α activation. (a) Phospho-p38 levels by western blot of BEAS2B cells stimulated with PA filtrates, pIC or both, where pIC is either transfected or not. Cells are stimulated with pIC (1μg/ml) for 24h before adding PA filtrates (45μl/ml) for 30min to activate p38. (a) and (b) show two replicates of the same experiment. Lane 1: Untreated; Lane 2: PA filtrates; lane 3: PA filtrates +pIC; lane 4: PA filtrates +PEI-pIC; lane 5:PEI-pIC. Lane 6: non-transfected pIC. Lane 4 and 5 are transfected pIC. (c) pp38α fold change over p38 in various groups. Average of control group (of pp38α/p38α) is used to calculate the fold change in each condition. N=2 and values are represented as dots.

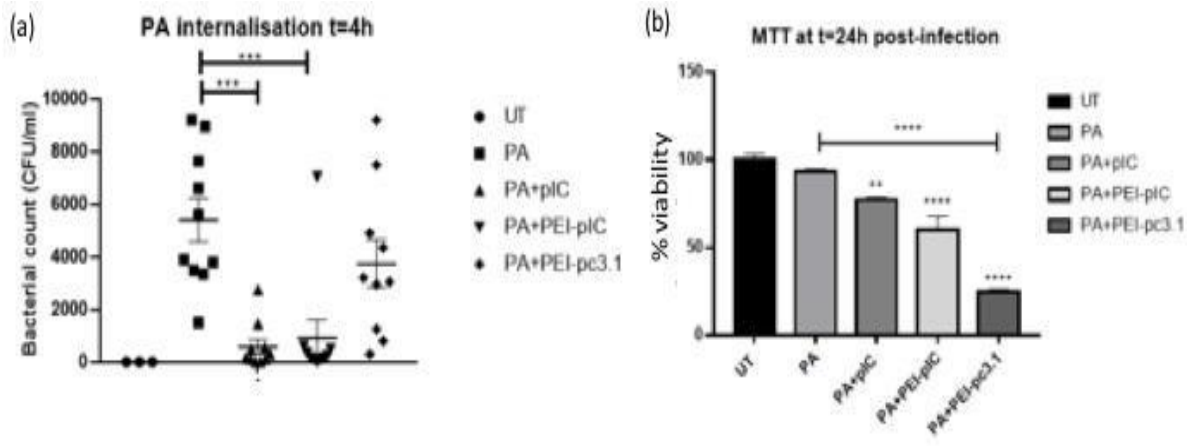


Figure 9. pIC decreases PA internalization into BEAS-2B cells. B2B cells are stimulated with pIC (1μg/ml) for 48h before infected with PA (MOI=1). (a) represents intracellular PA at 4h post infection with 48h pIC pretreatment. At 4h post infection (internalization phase), Cells are lysed and bacteria plated on LB agar and counted. PEI-pIC: transfected pIC. Pc3.1 is an empty cDNA vector as a control for PEI toxicity. Symbols include circles: PA alone, squares: PA with pIC, triangles: PA with transfected pIC, reverted triangles: PA with transfected empty vector. Data is from three independent experiments with N=4 per experiment and N=12 per condition represented. Asterisk (*) represents significant difference ($p \leq 0.05$) in comparison to PA alone with one way ANOVA with Bonferroni's post-hoc test. **($p \leq 0.01$) ***($p \leq 0.001$)

(b) MTT assay for cell viability at 24h after PA infection. % Viability is derived from OD measurements normalized to the average of uninfected cells. N=5 per condition. Asterisk (*) represents significant difference ($p \leq 0.05$) using one way ANOVA with Bonferroni's post-hoc test. **($p \leq 0.01$) ***($p \leq 0.001$)

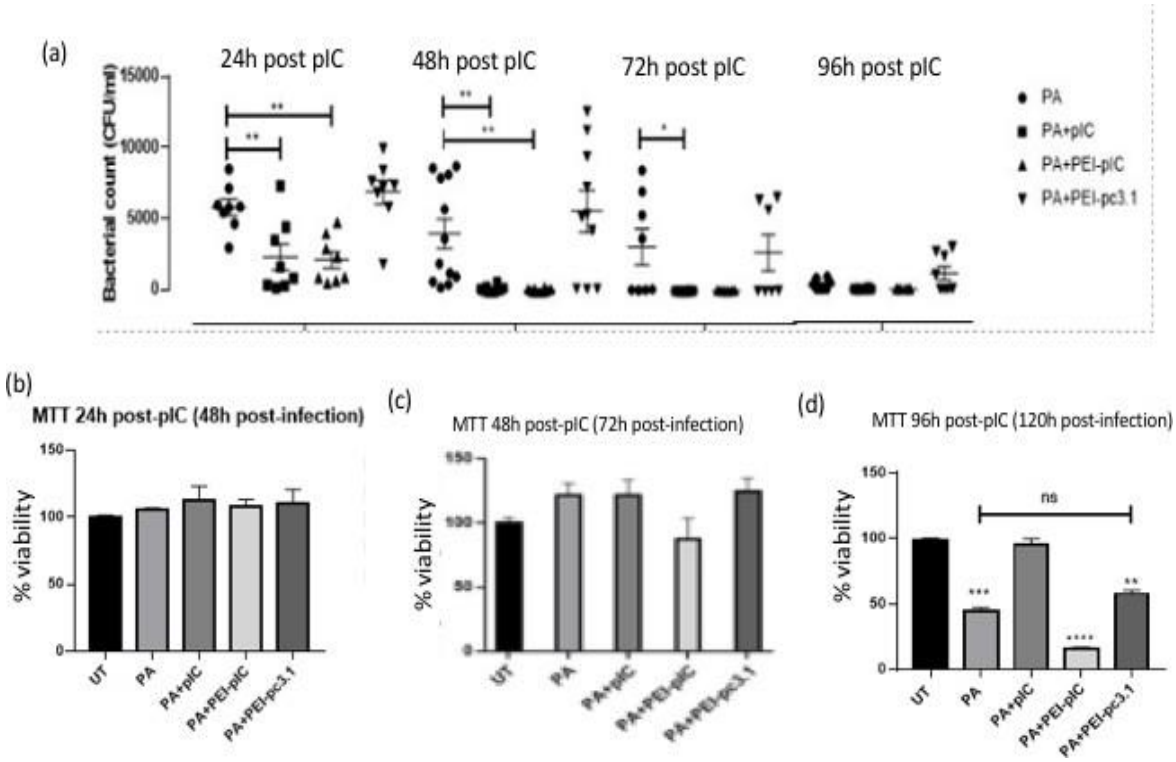


Figure 10. pIC decreases the intracellular persistence of PA. BEAS2B cells are infected with PaO1 at an MOI of 1. pIC (1μg/ml) either alone or transfected, is added 24h after the infection to mimic viral infection. At each timepoint cells are lysed with 0.1% Triton and bacteria plated on agar plates and counted. (A) represents internalized PA at the time points 24h post pIC (48h post-infection), 48h post pIC (72h post infection) and 96h post pIC (120h post infection). PEI-pIC: transfected pIC. pc3.1 is an empty cDNA vector as a control for PEI toxicity. Symbols include circles: PA alone, squares: PA with pIC, triangles: PA with transfected pIC; inverted triangles: PA with transfected empty vector. Data is from three independent experiments with N=4 per experiment and N=12 per condition represented. Asterisk (*) represents significant difference ($p \leq 0.05$) in comparison to PA alone at each timepoint with one-way ANOVA with Bonferroni's post-hoc test. **($p \leq 0.01$) ***($p \leq 0.001$) (b-d) MTT assay at 24h, 48h and 96h post-pIC treatment to assess cell viability. Y-axis is % viability derived from OD values normalized to the average of uninfected cells (control). N=5 per condition. Asterisk (*) represents significant difference ($p<0.05$) at each timepoint with one-way ANOVA with Bonferroni's post-hoc test. **($p \leq 0.01$) ***($p \leq 0.001$)

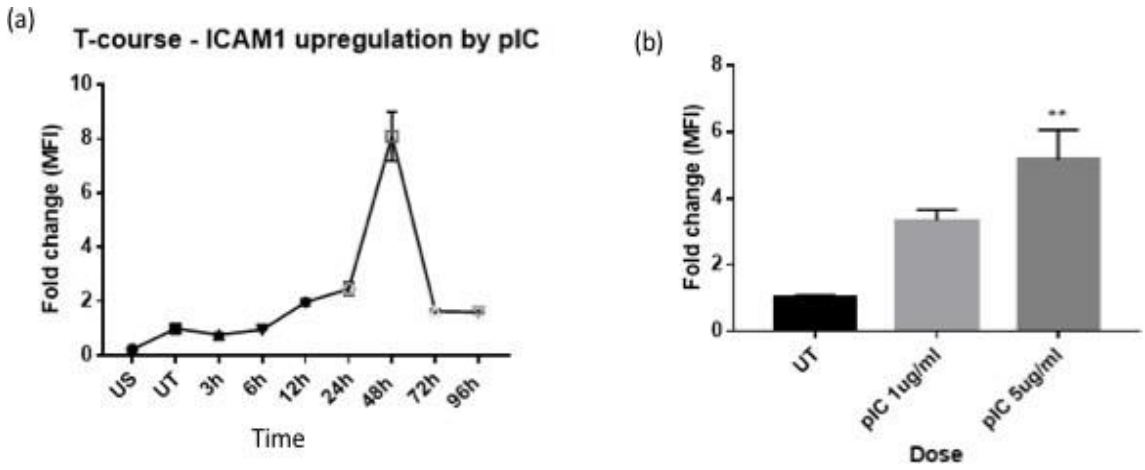


Figure 11. Time-course and dose response of pIC stimulation on surface expression of ICAM-1 measured by flow cytometry. BEAS2B cells are plated to 70-80% confluency and were stimulated with 1ug/ml pIC for varying times up to 96h (a) and at two different doses 1μg/ml and 5μg/ml (b). Y-axis represents the fold change of Median Fluorescence intensity (MFI) indicating the expression level of ICAM-1. Data is represented as mean± SEM of three replicates. In (b), One-way ANOVA with Dunnett's post hoc analysis compared to UT is used, with asterisk (*) indicating $p \leq 0.05$, **$p \leq 0.01$

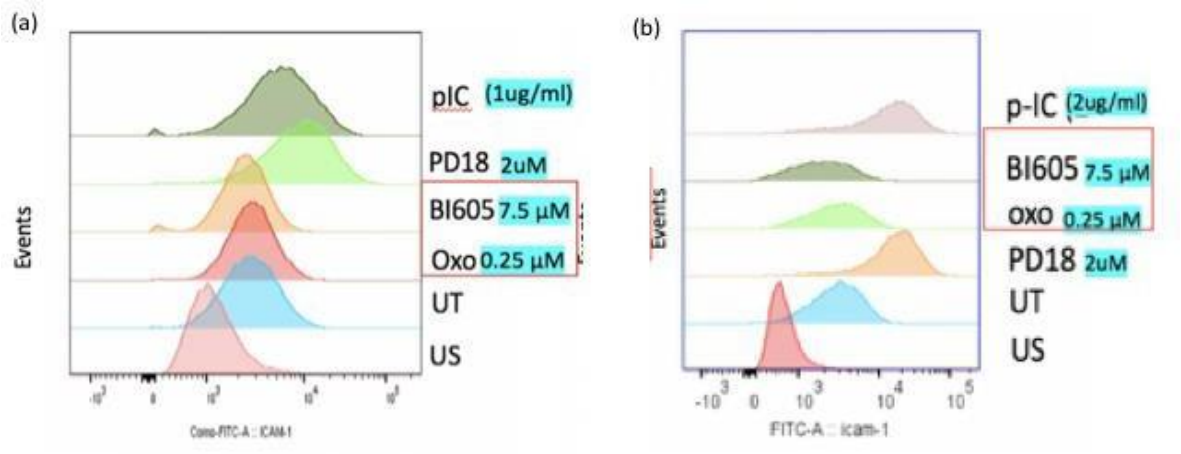


Figure 12. ICAM-1 expression is triggered by activation of NF-κB pathway dependent on TAK1 and IκB, while independent on the MAPK pathway. BEAS2B cells are treated with inhibitors for 1h before stimulated with pIC 1µg/ml in (a) and 2µg/ml in (b) for 24h. Flow cytometry diagram showing peaks of fluorescence intensity (x-axis) indicating the median level of ICAM-1 expression. Y-axis indicates number of events corresponding to each fluorescence intensity. PD18: MKK1/2 inhibitor (2µM); 5Z 7-oxozeanol: TAK1 inhibitor (0.25µM); BI605: IκB inhibitor (7.5µM). The respective concentrations are shown. US- unstained. UT- untreated.

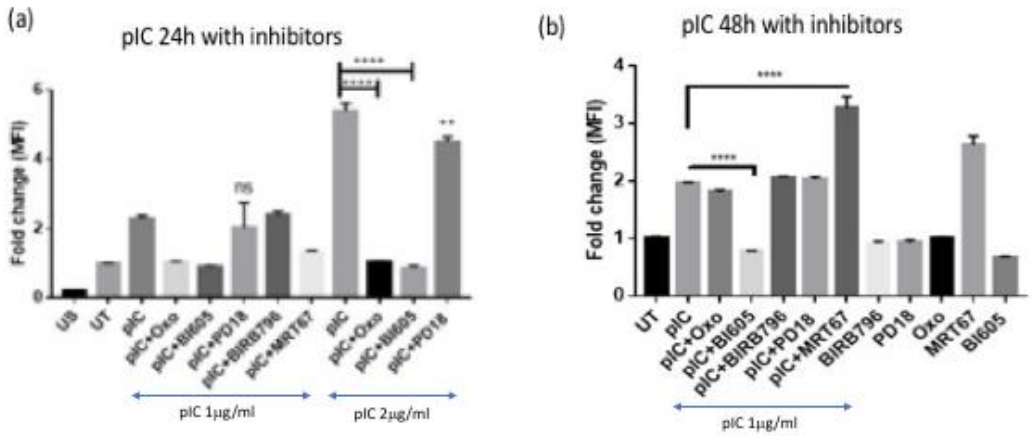


Figure 13. ICAM-1 expression is dependent on NF-κB pathway dependent on TAK1 and IkB, while independent on the p38α, ERK or TBK1 pathway. (a) BEAS2B cells are treated with inhibitors for 1h before stimulated with pIC 1μg/ml or 2μg/ml for 24h. Hence two experiments are shown. (b) BEAS2B cells are treated with inhibitors for 1h before stimulated with pIC 1μg/ml for 48 hours. It also shows the ICAM-1 expression triggered by the inhibitors alone. BRIB796: p38 inhibitor (2μM); MRT67: TBK inhibitor (2μM). The graphs all showed fold change in MFI over untreated cells. One way ANOVA is performed using Bonferroni post-hoc test with each inhibitor group compared to pIC treatment and data represents mean ± SEM of three replicates (N=3). Asterisk (*) represents significance $p \leq 0.05$, **$p \leq 0.01$ ***$p \leq 0.001$ ****$p \leq 0.0001$

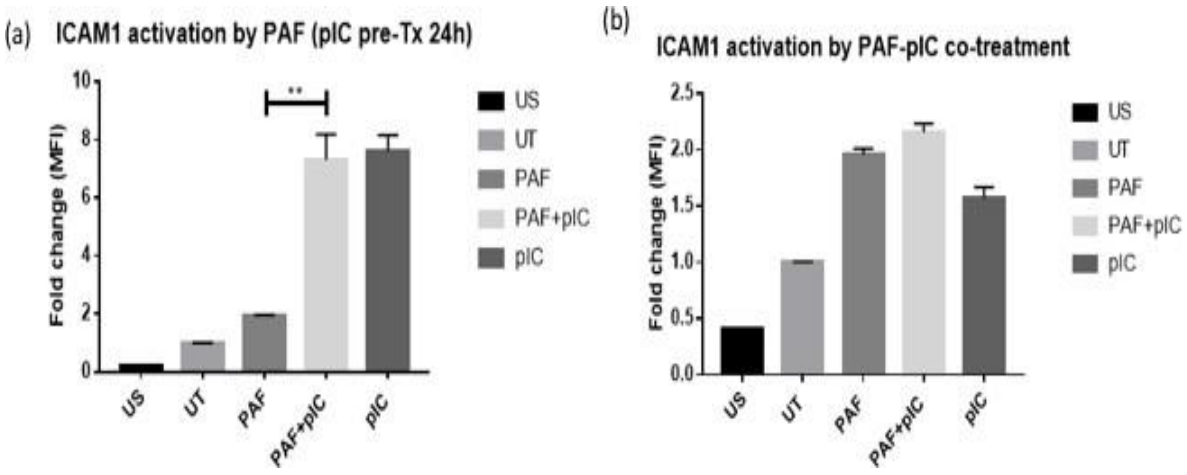


Figure 14. pIC pre-treatment increases ICAM-1 expression by PAF stimulation. BEAS2B cells are stimulated with 1μg/ml pIC for 24h before adding PA filtrates (45μg/ml of cells) for another 24h in (a), or cells are co-stimulated with both PAF and pIC for 24h in (b). Fold change in median fluorescence intensity (MFI) over UT is presented on Y-axis. One-way ANOVA with Bonferroni's test is used with both PAF and pIC groups compared to PAF+pIC group. Data are presented in triplicates with mean ± SEM represented. Asterisk (*) represents significance $p \leq 0.05$, **represent $p \leq 0.01$

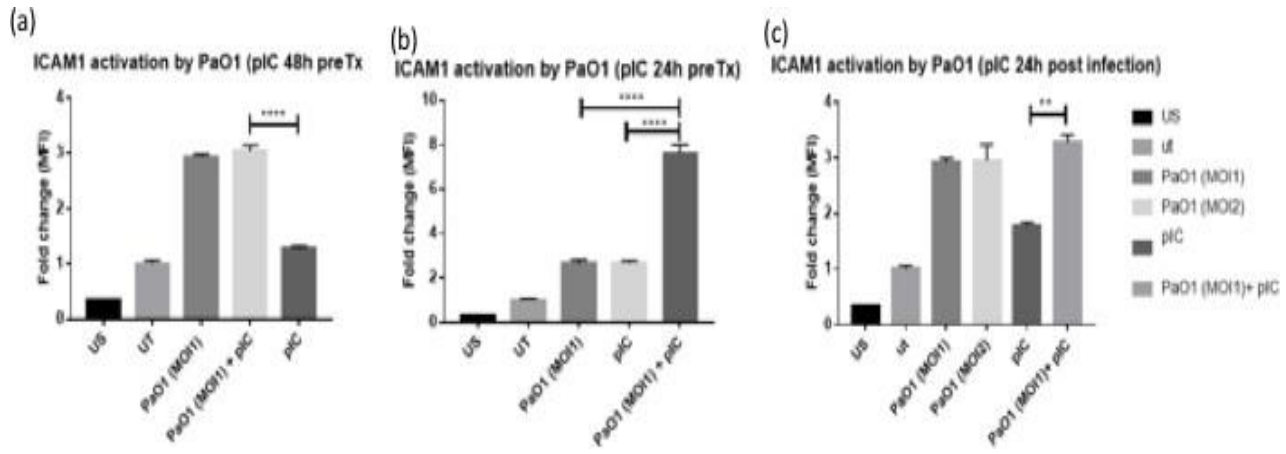


Figure 15. pIC pre-treatment for 24h increases ICAM-1 expression in response to PaO1 infection. (a) BEAS2B cells are pre-stimulated with 1μg/ml pIC for 48h are infected with PaO1 (MOI=1) for 24h. (b) Cells are pre-stimulated with pIC for 24h and infected with PaO1 (MOI=1) for 24h. (c) cells are treated with pIC 24h after PaO1 infection (thus are infected for 48h) before staining for ICAM-1. MFI fold change over UT is presented on the Y-axis. One way ANOVA with Bonferroni's post-test is used with both PAF and pIC groups compared to PAF+pIC group. Data are presented in triplicates with mean ± SEM and asterisk (*) indicating $p \leq 0.05$. ***$p \leq 0.001$ ****$p \leq 0.0001$

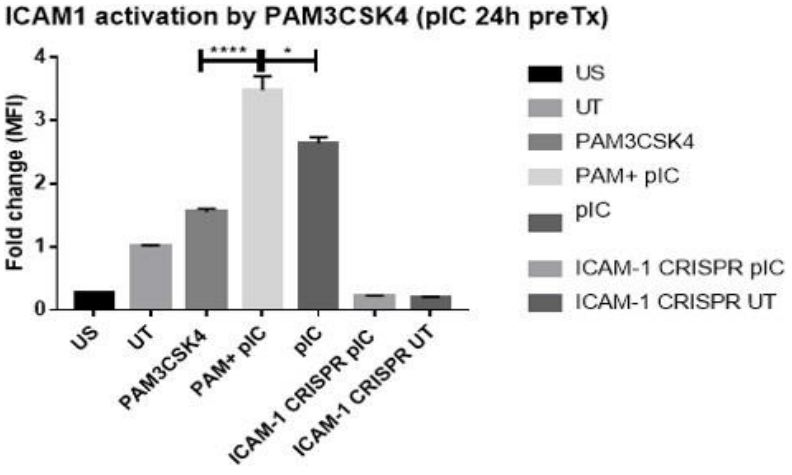


Figure 16. pIC pre-treatment for 24h increases ICAM-1 expression by PAM3CSK4 stimulation. BEAS2B cells are pre-stimulated with 1μg/ml pIC for 24h and stimulated with 1ug/ml PAM for 24h before staining for ICAM-1. ICAM-1 CRISPR cells are included as

negative controls. MFI fold change over UT is presented on Y-axis. One-way ANOVA with Bonferroni's post-hoc test is used with both PAM and pIC groups compared to PAM+ pIC group. Data are presented in triplicates with bars representing mean ± SEM, with asterisk (*) representing $p \leq 0.05$. ***$p \leq 0.001$

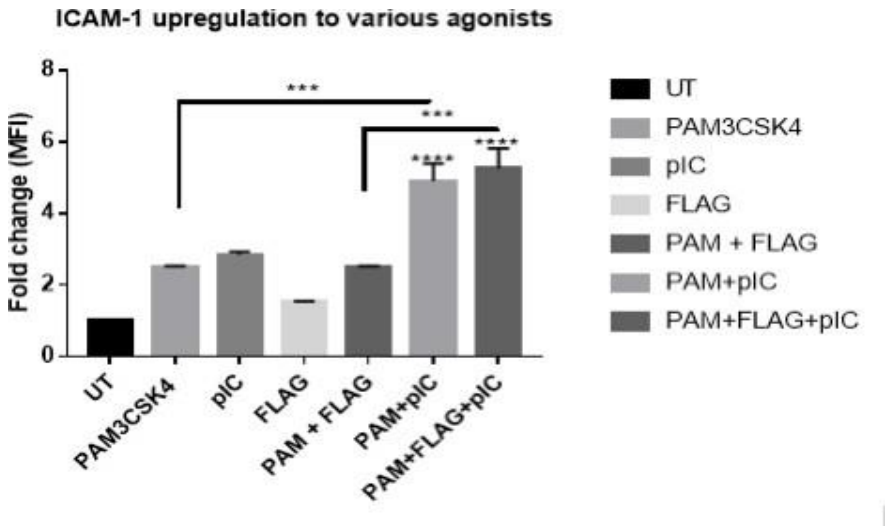


Figure 17. ICAM-1 expression by stimulation with TLR2, 3 and 5 agonists. 250,000 cells/ml BEABS-2B cells are treated with either PAM3CSK4 (1μg/ml), pIC (1μg/ml) and flagellin (400ng/ml) alone or combination using PAM+FLAG, PAM+pIC and all three agonists for 24h, after which cells are stained by ICAM-FITC antibody and fluorescence analyzed by FACS corresponding to level of expression. MFI fold change over UT is presented on Y-axis. One way ANOVA with Bonferroni post-hoc test is used, data are presented in triplicates with bars representing mean± SEM and asterisk (*) indicating $p \leq 0.05$. ***$p < 0.001$

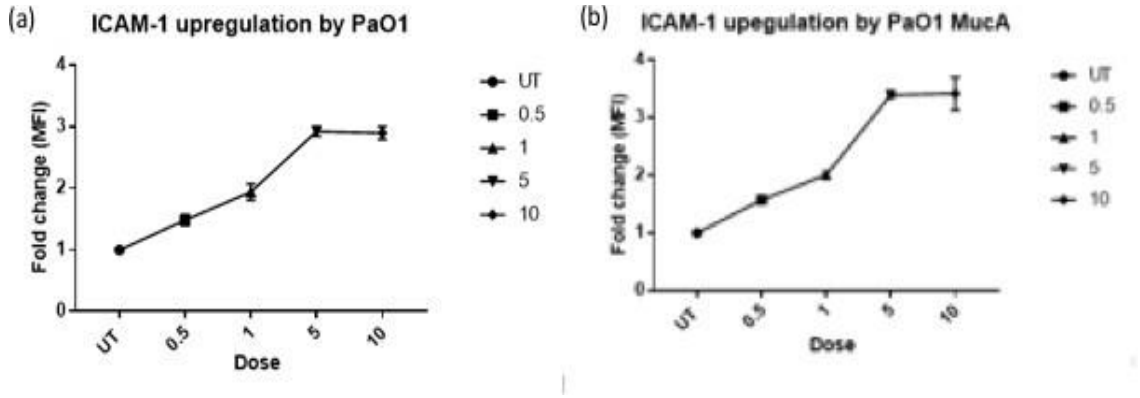


Figure 18. Dose response curves of ICAM-1 expression by PaO1 (a) and PaO1 MucA (b). Data are averages of triplicates and bars represent mean± SEM. The fold change in MFI over UT corresponds to ICAM-1 expression and increases as MOI increases from 0.5 to 10, both reaches plateau at MOI=5.

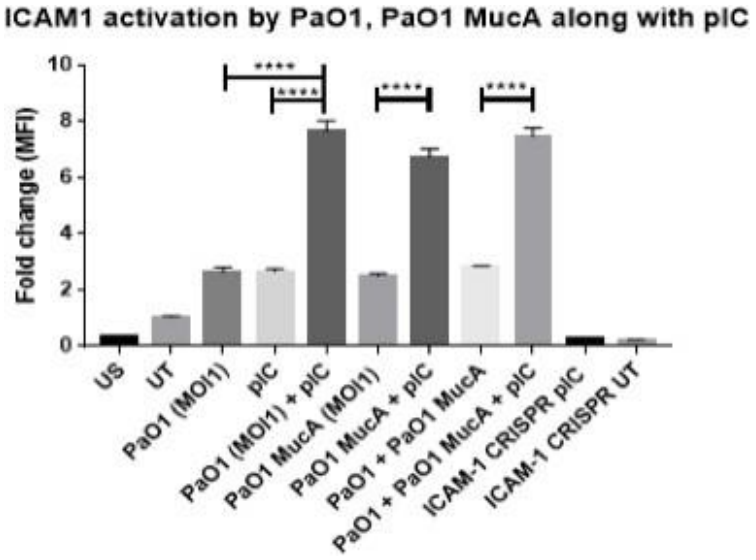


Figure 19. pIC pre-treatment for 24h increases ICAM-1 expression by both PaO1 WT and PaO1 MucA infection, and no difference was found between the two in activating ICAM-1 expression. BEAS2B cells are pre-stimulated with pIC (1ug/ml) for 24h and infected with either PaO1 or PaO1 MucA (MOI=1) as well as PaO1 together with MucA before staining for ICAM-1. ICAM-1 CRISPR cells are included as negative controls. MFI fold change over UT is presented on Y-axis. One-way ANOVA with Bonferroni post-hoc

test is used. Data are presented in triplicates with bars representing mean± SEM, with asterisk (*) representing $p \leq 0.05$. ****$p < 0.0001$

5 DISCUSSION

Overall in our study, we observed increased inflammation by poly I:C treatment prior to p. aeruginosa infection, in terms of MAPK phosphorylation, ICAM-1 surface expression, which aid in bacterial clearance albeit resulting in more tissue damage and pulmonary exacerbation. The reduction PA internalization also corroborates this finding where bacterial killing by antibiotics could occur. pIC treatment post PA infection was found to cause less overall inflammation by ICAM-1, which even though reduces the epithelial capacity to clear pathogens, also results in less tissue damage and corresponding exacerbation of pulmonary diseases including CF. This lower level of ICAM-1 mediated neutrophilic response is compensated by the reduction of epithelial PA persistence by pIC stimulation after infection. Overall, there is a role for the antiviral response to aid the clearance of P. aeruginosa mimicked by our in vitro model of sequential infections, at the same time explaining the pulmonary exacerbation potentially seen in CF and other respiratory diseases.

5.1 MAPK phosphorylation by P. *aeruginosa* and pIC

We chose to study p38α as our previous data showed highest activation at 90 min post pIC stimulation (Berube *et al.,* 2009). Transfected pIC activates p38α at a slightly later time point than non-transfected pIC (Figure 7) possibly because it has to be uptaken by the cell via autophagosomes via the Rab7- LC3 pathway, leading to the activation of the cytosolic receptor MDA5 in addition to TLR3, and causing more sustained IFN activation (Torno *et al.,* 2010). Transfected pIC triggers higher activation in pp38 as the TRIF and MyD88 pathways cross and TLR3/MDA-5 pathways often lead to activation of IKK and TRAF6, triggering NFκB and MAPK activation in a small percentage of downstream pathways. We tested both 24h and 48h after pIC stimulation, the reason is that in BEAS-2B cells IFNβ levels start to rise 24h post pIC stimulation and peaks at 48h (Wang *et al.,* 2011). We showed that p38 phosphorylation peaks when stimulating pIC for 48h (Fig. 6, b,d) probably due to the MAPK

activation property of type I IFNs, especially IFN-alpha, in addition to pIC itself (Zhang *et al.,* 2011). This paradoxical finding may be explained by the fact that far larger amounts of pro-inflammatory cytokines are released and signal through autocrine manner than the inhibition of IL-8 transcription, i.e. activation of MAPK signaling overrides downstream inhibition of transcription. This can also be explained by the different actions of IFN-beta vs. alpha, with the former having more antiviral effect and latter more inflammatory, therefore accentuating the inflammation caused by bacterial infection (Ivashkiv & Donlin, 2014).

While TLR5 agonist flagellin along with non-transfected pIC pretreatment for either 24h or 48h did not have a significant additive effect, pIC pre-treatment for 48h along with PAM had an additive effect of more than 20 fold that of control (Fig. 6; even though PAM isn't necessarily a more potent agonist than Flagellin) has ample physiological relevance. In acute infection pseudomonas mainly activate TLR5 via its flagellin (Roussel *et al.,* 2011). In CF lungs, PA adapt by formation of biofilm that is characterized by mucA and quorum sensing LasR mutation, loss in flagellin signaling and alginate production (Hoiby N, 2002). Planktonic bacteria are often released which stimulates TLR2 in addition to TLR5 (Beaudoin *et al.,* 2012). In chronic infection, in particular, overproduction of EPS coregulates increased bacterial lipopeptides (TLR2 agonist) as well as decreased flagellin (TLR5) and pili, as well as increasing LPS to stimulate TLR4, causing increased inflammation (Faure *et al.,* 2018). The upregulation of bacterial lipopeptides that bind to TLR2 (and downregulation of TLR5 agonist flagellin) is closely mimicked by the TLR2 agonist PAM3CSK4. The additive effect of pIC pre-treatment for 48h along with PAM stimulation on MAPK (Fig. 6d) closely recapitulates the switch from TLR5 to TLR2 in chronic PA infections, along with viral exacerbation causing increased inflammation. This is potentially responsible for the further decline in lung function in chronic viral and bacterial exacerbations compared to earlier stages. Similarly, the peak in pIC stimulation at 48h also has an additive effect with PAM in terms of ICAM-1 upregulation (Fig. 15b).

It is also noted that PAF (mainly containing flagellin) did have an additive effect on p38 phosphorylation with pIC when pIC is transfected using PEI 24h prior to flagellin stimulation (Fig. 8), whereas pIC without transfection 48h before flagellin stimulation did not have an additive effect (Fig. 6 b,f). It is postulated that transfection of pIC mimics viral infection more closely. Dauletbaev *et al.* (2015) demonstrated that liposome encapsulated pIC increased production of IFNβ more significantly than pure naked pIC via activation of RIG-

I/MAVS pathway, where IFNβ could in turn activate MAPK (Bonjardim *et al.*, 2009). In comparison, pure naked pIC mainly activates TLR3 and increases IL-8 through the NFkB pathway, resulting in significantly lower levels of IFN production, a finding also corroborated by Ramezanpour *et al.* (2018). Although activation of this pathway triggers MAPK activation, the extent is less pronounced than the signaling activated by transfected poly I:C.

The result in Fig. 6 (a,b,e,f) also indicates that even in the acute phase of infection (mimicked by TLR5 agonist) there are still viral exacerbation associated with increased inflammation and damage, however it is not as severe as viral exacerbation in chronic colonization of PA (mimicked by TLR2 agonist). In CF however, cells would be more sensitive and have higher basal level of phosphorylated MAPKs. Hence it is worth using CF clinically isolated PA strain to examine its effect on p38 activation in CF cell lines along with viral infection. Studies by Ramezanpour *et al.* (2018) may also explain the increased inflammation (MAPK) caused by viral infection that precedes bacterial infection observed in our study. They showed that priming of primary nasal epithelial cells (HNEC) for 24h using low molecular weight (LMW), as opposed to high molecular weight (HMW) pIC reduces the IL-6 produced in response to stimulation by agonists including TLR2, 3 and 4 agonists (HKLM, LMW pIC and LPS respectively) (Ramezanpour *et al.* 2018). It was shown LMW pIC binds to RIG-I receptor as opposed to MDA5 receptor for HMW pIC, and LMW pIC is also a less potent activator of TLR3. The reduction of inflammation by RIG-I mediated LMW pIC priming is potentially detrimental and increases susceptibility to secondary bacterial infections in the short term, which is in the context of paramyxoviruses and influenza virus as they bind to RIG-I (Kato *et al.* 2006). However, in our study we used HMW pIC which binds to MDA5 without involvement of RIG-I, and priming with HMW pIC does not trigger reduced inflammatory cytokines production when stimulated with TLR agonists, explaining our results of increased inflammation and MAPK activation. It's also worth mentioning that instead of submerged culture they used primary cells cultured at the liquid-air interface, which better recapitulates the pseudostratified mucocillary phenotype (Jiang *et al.* 2018), a model that could be attempted in the future. One main advantage of these cells is that upon pIC stimulation minimal level of IL-8 is generated compared to IFNβ, better capturing the anti-viral response.

It would also be interesting to investigate PA infection 6-7 days after viral infection with HRV, or alternatively 6-7 days post pIC treatment when the initial effect of IFNβ subsides to examine whether the effect on the inflammatory response could be reversed. TLR3 or MDA-5 KO cell lines would also be used to confirm that this effect is caused by IFN secretion.

Interestingly, another study on RV-1b exacerbation of asthma found knockout of TLR3 and MDA5 to reduce inflammation, while TLR7 and 8 knockouts impaired type I and III IFN secretion and increased inflammation, causing exacerbation (Bochkov *et al.,* 2016). Therefore, whether TLR3 and MDA5 could paradoxically increase inflammation and TLR7 and 8 could counter them is an interesting avenue to explore. If this is true, provided that HRV binds to TLR7 and 8 more readily than pIC as the former is a ssRNA, the result of HRV infection could differ from that captured by pIC as a model.

5.2 P. *aeruginosa* internalization into epithelial cells

pIC treatment in B2B cells 48h prior to PA infection significantly decreased the uptake and internalization of PA, both transfected and non-transfected, with minimal cytotoxicity of pIC even at 24h post infection (Figure 9). PEI-pIC did exhibit slight toxicity at 24h post-infection mainly due to PEI (shown by toxicity in the pc-3.1 group), as pIC had been in the system for 72h at the time (Fig. 9). In order to reduce PEI toxicity, 24h incubation with pIC prior to infection (instead of 48h) was also attempted but with inconsistent results (not shown), suggesting that the IFNβ levels at 24h was not high enough to significantly reduce PA internalization (since IFNβ levels peak at 48h in BEAS-2B cells, Wang *et al.*, 2011).

PA internalization is regulated by multiple pathways including PI3K/Akt for microtubule reorganization (Kierbel *et al.,* 2005), H6SS delivering VgrG2b effector to epithelial cells that bind to gamma-tubulin ring complex and coordinates microtubules (Sana *et al.,* 2015), actin polymerization as well as tight junction proteins (increased ZO-1 decreased internalization, increased beta-integrin increased internalization) (Lemessurier *et al.,* 2013). There are two potential explanations for decreased internalization: 1) in a study by Lemessurier *et al.* (2013) type I IFN protects against Pneumococcal disease by decreasing lung uptake and transmigration, and mechanism being increasing tight junction proteins in the epithelium.

IFN released by pIC stimulation could upregulate tight junction proteins including Cadherin 1, Cldn 4, 5, 18 and tight junction protein 1. 2) Another possibility is that ROS generated by pIC stimulation downregulates the PI3K/Akt pathway (Chattoraj *et al.*, 2011), which in turn reduces internalization as PI3K/Akt is the pathway responsible.

In CF chronic infection, PA undergoes adaptions that increases PI3K/Akt signaling by its effectors as well as increased activity of the T6SS system to promote PA internalization (Moradali *et al.*, 2017). Thus, it is speculated that they are less prone to pIC's actions on PA internalization in the chronic stage but not the acute infection. It was also found that loss of LasR in certain clinical strains in CF promotes PA internalization, the exact mechanisms are unclear, but it is most probable due to the co-regulation of other effectors or pathways along with LasR mutation. Using western blot techniques to measure the activation of PI3K/Akt pathways, one could easily verify these hypotheses in CF cells. TLR3 and MDA5 KO cell lines, as well as neutralizing antibodies for IFN, could also be used to confirm that these effects of pIC on PA internalization and persistence are caused by IFN secretion. Future attempts at HRV infection however, it is noted that as mentioned in section 5.1, HRV binds TLR7 and 8 in addition to TLR3 and MDA5, thus may interact with PA infection via production of IFN or inflammatory mediators by activating these additional receptors, which should be taken into consideration when looking for the mechanism.

5.3 P. aeruginosa intracellular persistence

pIC treatment on B2B cells 24h after PA infection significantly decreased the intracellular persistence of PA at various time points post infection up to 120h (96h post pIC) without impacting cell viability whether pIC was transfected or not. Transfected and non-transfected pIC equally decreased PA persistence, whereas the empty vector pc3.1 in complex with PEI had little effect, meaning that is negligible toxicity associated with PEI in this scenario (Fig. 10). Interestingly, pIC had a protective effect at 120h post infection, protecting the cell from damage by pseudomonas, as shown in Fig. 10d their viability is almost the same as uninfected cells and significantly higher than PA infected cells. There are several explanations for reduced PA persistence, firstly, PEI-pIC is shown to activate autophagy machinery, as they are uptaken by the cell by recruiting LC3 containing autophagosomes for clearance (Torno *et al.*, 2010). Whether autophagy that occurred while trying to clear virus induced PA clearance is worth

investigating. Secondly, it was shown by our lab that TLR5 mediates PA autophagy pathway, absence of TLR5 increases PA persistence (unpublished data, Julie Berube). Whether type I IFN released by pIC interacts with TLR5 mediated pathway is unknown and worth investigating. It is noted that the persistent bacteria in the pIC (and PEI-pIC) group was most significantly lower than PA group at 24h until 72h after pIC treatment, corresponding to the rise and peak of IFNβ (Wang *et al.*, 2011). The effect continued until 120h post-infection (96h after treatment), where there is lack of detection of PA at 72h and 96h post-infection, indicating the potency of the response.

Thirdly, it is postulated that pIC stimulation increases exocytosis of intracellular bacteria. This was initially found in CFBE WT and del508 cells, where more persistence was found in del508 cells, however, when pIC was applied both cell types released bacteria extracellularly to similar extents (unpublished data, Emmanuel Faure, Rousseau lab). The mechanism for this is unclear and could possibly involve the clathrin adaptor AP-1 and syntaxin 4, with apical exocytosis also triggered by calcium ions, actin depolarization and cholesterol depletion (Xu *et al.*, 2012). This is a novel concept worth testing, and this process could be imaged using confocal microscopy to look for syntaxin 4 colocalization with LAMP1 (marker of lysosomes), or syntaxin 4 knockout to see if intracellular persistence is increased. A similar phenomenon was found by Chattoraj *et al.* (2011), in CF cells pre-infected with MPA it was shown that HRV39 can disrupt biofilm integrity, triggering trans-migration of MPA across the biofilm as well as release of planktonic bacteria.

It is worth noting that, even though type I IFN appear to reduce PA persistence in vitro, in vivo opposite effects are shown. IFNs increase the production of SETDB2, a methyltransferase that repress transcription of neutrophil chemoattractant CXCL1 (Schliehe *et al.*, 2015). This in turn reduces inflammation promoting bacterial infection. In addition, type I IFNs inhibit also suppresses Th17 activity and increases production of IL10 (Boxx & Cheng, 2016), despite the multiple pro-inflammatory actions mediated by IFNα (mentioned in section 5.1). This warrants an in vivo CFTR-/- mice model with sequential HRV infection and intratracheal challenge of P. aeruginosa chronic isolate to investigate PA persistence and clearance further, especially those that contain *mucA* and LasR mutations which more closely captures the CF airway environment (Hoffmann *et al.*, 2015).

In CF cells, it is expected that autophagy pathway would be dysfunctional since CFTR mutations leads to endosomal dysfunction (Law & Gray 2017), resulting in higher P. *aeruginosa* persistence. Daeulethaev *et al.* (2015) similarly reasoned that increased HRV persistence with PA was the result of dysfunctional endosomal pathway. Another mechanism of increased persistence in CF is the loss of T3SS activity, forming osmotically active membrane blebs (Kroken *et al.*, 2018). Despite previous results showing similar extent of PA exocytosis between WT and del508 CFBE cells, healthy cells including B2B should be used to compare with CF cell line in the future. Whether CF cells have reduced type I IFN secretion is still a controversy, therefore it is harder to predict the effect of pIC in CF cells if the second hypothesis applies. Future experiments done in CF cells, as well as measuring various parameters and mediators to verify the potential mechanism of exocytosis is highly useful.

5.4 ICAM-1 surface expression

Treatment with pIC at 2μg/ml for 24h in the presence of TAK1 and IκB inhibitor Oxo and BI605 significantly decreased the expression of ICAM-1, whereas there is no change in expression in groups treated with the p38 inhibitor BIRB and the MKK1/2 inhibitor PD18. This indicates that the pathway is mediated by NFκB solely without involvement of the MAPK orTBK pathway (Fig. 12 and 13). In particular, the TBK inhibitor MRT67 paradoxically increased ICAM-1 expression possibly due to its off-target effects. It is also possible, though, that shutting off the TBK pathway actually increases the inflammatory pathway by NF-κB thus ICAM-1 expression, potentially explained by downregulation of SOCS and causing upregulation in the MyD88 pathway, since SOCS directly inhibits many MyD88 pathway components including TIRAP and IRAK (Yang & Seki 2012).

It is noted that this model only tested pIC stimulation short-term (2-3 days) prior to PAF stimulation (Fig. 14), where there is higher level of ICAM-1 and the inflammation peaks, which could be considered protective against secondary infection whilst causing more damage. Whether the opposite response occurs when treated with pIC longer term before PA infection should be examined, which was shown in literature to occur in the model of influenza infection (Rynda-Apple *et al.*, 2015). To test this, cells could be cocultured with PMNs and pretreated with pIC for longer time (e.g. 6-7 days), then stimulated with PAF or

infected with PaO1 and ICAM-1 expression could be measured.

PAF also contains a mixture and agonists (PAMPs), thus it is unknown which receptors are responsible for their additive effect, although in WT PaO1 filtrates the agonist that is mainly responsible is flagellin (Roussel *et al.,* 2011). In the following experiments we tested flagellin (which is known to activate TLR5 receptor primarily), Pam3CSK4 (which is TLR2 receptor agonist) and PaO1 infection by activating mainly the TLR5 receptor (PaO1 is mainly uptaken by TLR5 via autophagy as absence of TLR5 increases persistence).

There is significantly higher ICAM-1 by PaO1 infection with pIC pre-stimulation for 24h (additive effect) (Fig. 15). Similar to above, this has physiological relevance in that there is higher inflammation and causes more tissue damage when pIC is exposed short term (24h) before PA infection, and both longer term (48h), when the inflammatory response by pIC decreases and when the PA infection precedes viral infection reduces ICAM-1 mediated inflammatory response, resulting in less tissue damage but at the same time increasing the susceptibility to secondary infections. Although in different systems, this is also in line with the finding by Rynda-Apple *et al.* where longer periods after influenza virus infection causes exacerbation because it is when the level of type I IFN declines. When pIC is added along with or 24h after PA infection the inflammatory response is also lower, in line with the findings by Chattoraj *et al.* (2011) that there is a decline in IFN caused by MPA infection (caused by increased oxidative stress) prior to HRV infection. To confirm these findings, IFNα/β levels could be quantified using HEK-blue cells calorimetric assay and IL-6 and IL-8 quantified by ELISA.

It is also worthwhile looking into TLR2 activation by Pam3CSK4, a receptor important in mucoid PA stimulating epithelial cells. There is significantly higher ICAM-1 by PAM with pIC pre-stimulation for 24h (additive effect) (Fig. 16). This indicates an even higher inflammation in chronic infections along with viral infection. Even though the combination of pIC and PAM did not trigger a significantly higher ICAM-1 upregulation compared to that of PAF or PaO1, unlike p38 upregulation in the western blot results (Fig. 6), the viral infection is sufficient to trigger a CF exacerbation along with mucoid PA.

Higher ICAM-1 is observed in pIC with PAM as well as with both PAM and Flag together,

whereas stimulation with PAM and Flagellin together had no increase compared to either alone (Fig. 17). In both conditions, stimulation with pIC (viral stimulation) increases more ICAM-1 than both Pam and Flagellin (PA stimulants alone), highlighting the significance of viral infections in triggering a CF exacerbation. This experiment closely mimics the stimulants in CF airways in acute and chronic infections. As addressed earlier, the mucoid switch in PA activates TLR2 in addition to TLR5, before switching to favor TLR2 and reducing TLR5 stimulation at a later stage. PAM and Flag co-stimulation is no more inflammatory than either alone, indicating the fact that the initial stage of mucoid switch before adaptation could be little different than the acute infection stage where TLR5 is dominant (Fig. 17). however, In chronic infection, CF cells are often co-infected with viruses such as HRV. It is seen that in both the initial mucoid stage (represented by Pam+ Flag+ pIC) as well as the adapted stage (Pam+ pIC) ICAM- 1 expression are significantly higher along with viral infection (pIC). Nevertheless, it is hard to draw conclusion from healthy B2B cells with low basal inflammation therefore re- examining this in CF cells is important.

The dose response curves of mucoid PA and its WT counterpart for ICAM-1 expression plateaued at MOI=5. Hence, we selected MOI=1 for combination with pIC to investigate ICAM-1. The two curves showed similar ICAM-1 activation at the same doses, which is not within expectation (Fig. 18), since mucoid strains activate TLR5 in addition to TLR2 (Beaudoin *et al.,* 2012). In addition, *mucA* mutations downregulates lasR (Ryall *et al.,* 2014), and during this process it is speculated that it reduces the breakdown of hyper-inflammatory cytokines such as IL-8 and IL-6, they could in turn stimulate ICAM-1 expression, therefore it is hypothesized that *mucA* mutations would have higher ICAM1 levels. However, the real situation is more complex. First of all, PaO1 is a laboratory strain with genetically engineered *mucA* mutant, which lacks many properties of clinical strains. Clinical mucoid strains have gone through various adaptations and carry dozens of genetic mutations other than *mucA*, including the more important LasR mutation that consists of over one third of the bacterial population, increased EPS that coregulates with TLR2, mutated flagellin (resulting in reduced stimulation of TLR5) as well as Lipid A of LPS whose mutation stimulates more TLR4 activity (Faure *et al.,* 2018). These are also mutations including AmpR and RetS (regulation of cyclic-di-GMP pathway) which reduce virulence and T3SS expression (Moradali *et al.,* 2017). Another important mutation that results in increased antibiotic tolerance is the efflux pumps MexXY-OprM, and MaxAB-OprM/A. The former responsible for resistance to

tobramycin and quinolones, the latter to quinolones and β-lactams (Singh *et al.*, 2017). Loss of function mutation for down-regulators of these pumps include MexR for MexA/B and MexZ for MexXY (and MexA/B). Loss of LasR also increases expression of these efflux pumps, reducing antibiotics clearance and causing more damage (Moradali *et al.*, 2017). All of these explains why clinical strains are more valuable in studying viral and bacterial interactions in CF and ICAM-1 regulation. In an unpublished study by the Nyugen lab, CF1 LasR mutant increases internalization of PA into CFBE cells whereas the mutant of the WT PaO1 did not. Secondly, the unpublished study also found that only the late filtrate of CF1 (isolated from patients later in life) increased ICAM-1, while early LasR competent filtrates had no impact. Another study by Ruffin *et al*. (2016) concluded that a single LasB mutation encoded by LasR is responsible for most hyper-inflammation, much more than other genes such as LasA and AprA. Thus one hardly thinks a single *mucA* mutation without accompanying defects would result in increased inflammation or ICAM-1 regulation. It would be reasonable to say PaO1 *mucA* would not differ from their WT counterparts in terms of MAPK phosphorylation either.

It is worth mentioning the studies mentioned above are all performed in CF cell lines. In healthy cells such as B2B, the hyperinflammation is much harder to observe. There is intact anti-oxidant mechanism like GSH, functional autophagy mechanisms, and other mechanisms to counter inflammation (Berube *et al*., 2010). The immune cells are functional unlike CF where macrophages and neutrophils are defective in clearing pathogens, and the inflammation uncontrolled as neutrophil elastase traps (NET) overwhelms the capacity of anti-protease activities (Law & Gray 2017). This also explains why PaO1 *mucA* infection did not increase ICAM-1 expression level in BEAS-2B cells compared to WT PaO1.

Nevertheless, as shown in Fig 17, PAM and Flagellin together did not increase ICAM-1 compared to either agent alone. Similarly, PaO1 and MucA together did not increase ICAM-1 expression compared to either alone, possibly due to inability of PaO1 MucA (MOI=1) to trigger higher ICAM-1 response than WT PaO1 (MOI=1) in this scenario, which is equivalent to doubling the PaO1 dose (MOI=2), which does not trigger much higher ICAM-1 activation (refer to dose response curve Fig. 18). Hence, future attempts should focus on the ICAM-1 expression caused by infection with a clinical strain with mucA mutation in CF cells with or without viral infection. Future experiments should also look at whether simply lasR

downregulation (without mutation) caused by *mucA* mutation affects PA internalization and persistence, as we already know that LasR and T3SS mutations promote internalization and persistence respectively.

In addition, one study showed that MPA increases ICAM-1 to the same extent in CF IB3 and non-CF cells (Chatteroraj *et al*., 2011), whereas another study shows that IB3 cells co-culture with PMNs significantly upregulated ICAM-1 compared with control (Tabary *et al*., 2006). The difference is that co-culturing mimics the in vivo situation more closely, whereby PMNs secrete paracrine acting cytokines, that act in concert with PA on ICAM-1. This could be used in future studies for a better representation of CF airways, in addition to culturing at air liquid interface.

How do our results translate into the scenario in CF? Other than the higher basal level of inflammation, there is more persistence and internalized P. aeruginosa (by T6SS) therefore would dampen the protective response of pIC – causing more internalization and more persistence, which acts to evade antibiotics and immune cell clearance. Indeed, bacteria trapped in biofilms have less virulence, but the already high basal inflammatory response makes any small infections prone to exacerbations and tissue damage, accentuating the detrimental effects of HRV infection (pIC).

CF exacerbations triggered by both viral and bacterial infections are extremely common and detrimental. They cause accelerated decline in lung function and increased risk of lung transplant (Wark *et al*., 2012; Kiedrowski *et al*., 2018). To date, evidences on interaction of viral and bacterial pathogen are scarce, with only studies showing alteration in IFN levels and increased HRV persistence in CF patients when bacterial infection precedes viral infection (Moradali *et al*., 2017). Chattoraj *et al*. concluded that the decreased IFN was due to ROS mediated inhibition of PI3K/AKt pathway, which is triggered by PA infection, resulting in viral persistence. Dauletbeav *et al*. (2015) also found viral persistence despite the normal IFN response, which does not differ between normal cells and CF. They reasoned that their results differ from that of Chattoraj *et al.* as they used HRV16, a major group HRV which binds to different extracellular receptors, and that there could be other interferons, such as IFNλ that are reduced prior to the IFNβ peak, which does not occur until 48h post-infection (Dauletbaev *et al*. 2015). Most importantly, it remains unclear whether the increased viral

persistence is due to defect in IFN or the CFTR causing endocytosis defects. This is why we used healthy epithelial cells B2B first, as well as WT PaO1 removing the influence of any mutations in PA adaptation and natural defects in CF such as the endosomal pathway (causing increased internalization and persistence). Hence, the mechanism underlying these infections and the roles type I IFNs play is still unclear.

Two studies on viral infection that precedes bacterial infections found that HRV and RSV increased PA biofilm growth (Van Ewijk *et al*., 2007), and that influenza virus A infection caused reduced lipocalin 2, an antimicrobial peptide which predisposes patients to secondary pneumonia (Robinson *et al*. 2013). The increased inflammation of early viral infections is also documented. However, none of the studies point to detailed mechanisms (except for Chattoraj *et al*. but only where bacterial colonization precedes viral infection not the opposite process), especially for the most common PA and HRV confections where viral infection precedes bacterial infection in many occasions. Our study not only established that there is increased MAPK and ICAM-1 mediated inflammation using poly I:C as a model for HRV and PA co-infection, but also uncovered a role of the antiviral response in altering PA persistence, a novel concept. The effect is double-sided - viral infection reduces PA persistence which facilitates clearance using antibiotics like tobramycin that fails to penetrate membranes as well as attack by immune cells, however this in turn triggers more inflammation during the attack and the bacteria released could re-infect epithelial cells triggering more tissue damage. This is our reasoning, that together with hyper-inflammation, causes CF exacerbation. This is also line with previous findings that late CF isolates increase ICAM-1 compared to early isolates (unpublished data, Nyugen lab) and that IL-8 is elevated in MPA and HRV coinfections, a finding that agrees across different studies (Chattoraj *et al.* 2011, Dauletbaev *et al.* 2015). Our findings that PA in chronic infection is more inflammatory, also agrees with previous studies that mutated LasR in late PA isolates increases IL-6 and IL-8 due to their inability of the proteases produced to degrade these cytokines (mediated by LasB) (Lafayette *et al*., 2015). However, there are contradictory findings, one study found PA exoproducts produced by QS sensing causes more damage and impaired wound healing in both CF and non-CF hECs but the effect is reversed in late isolates with LasR mutations and QS inhibitors (Ruffin *et al*., 2016). Another study found PA exoproducts reduce synthesis and degrade CFTR in WT CFBE and del508, which reduces the efficacy of CFTR correctors (e.g VX-325), and are also reversed by QS inhibitors and LasR

mutation in chronic colonization (Maille *et al*., 2017). In both studies the increase in tissue damage and CFTR degradation cause higher inflammation in acute infection than in the chronic. Admittedly these studies do not involve viral infections, but it is worth clarifying the inflammatory findings using clinical PA strain in CF cells along with viral infection and directly measure cytokines IL-8 and IL-6, perhaps in co-culture with PMNs and even an in vivo model of HRV infection with PA, where CF mice is intratracheally challenged with a chronic clinical isolate of P. aeruginosa with or without HRV co-infection, since it is long sought that IFN and IL-8 responses are mutually inhibitory (Boxx & Cheng 2016), it is extremely useful for those in vivo models to verify these results where both the innate and adaptive systems come into play.

6 LIMITATIONS AND FUTURE DIRECTIONS

One of most important area to improve is using clinical PA isolates in CF cultures with the human rhinovirus. Developing PA biofilms in vitro is essential, as they differ from planktonic bacteria in many ways, including loss of virulence and many inflammatory responses. Using an in vivo model of HRV and pseudomonas infection where CF mice is intratracheally challenged with a chronic clinical isolate of P. *aeruginosa* is essential to recapitulate CF lung disease, especially since Type I IFN actions are versatile and in vitro models are difficult to fully capture its various actions. For example, the model proposed by Hoffmann *et al.* (2005) where the Pseudomonas strain contained both MucA and LasR mutations. Moreover, The type I IFN secreted is most important part of antiviral response. We learned that IFNβ levels peak at 48h post HRV from literature, but it is worth measuring IFNλ levels using a time course to determine peak levels, thus eliminating confounding of other mediators. Directly measuring cytokine levels provides more accuracy of the inflammatory response compared to using MAPK as an indirect measure. Since HRV binds to TLR7 and 8, in addition to TLR3 and MDA5, CRISPR cell lines each individual receptor, or multiple knockouts should be generated to confirm their respective contributions to increased MAPK, ICAM-1 and any alterations in PA internalization and persistence, by looking at whether these effects could be reversed.

Of note, this study did not include the fluorescence isotype control for ICAM-1 (mouse

IgG2- FITC) to exclude the non-specific binding of ICAM-1 antibody to other Fc receptors present on the cell membrane. However, we included ICAM-1 CRISPR cells stained with ICAM-1 antibody as a control in most experiments and the fluorescence is similar to unstained controls, which indicates that there is negligible non-specific binding of the ICAM-1 antibody. Nevertheless, future experiments would take this into account, especially when examining multiple markers beyond ICAM-1. In addition, the intracellular delivery of pIC (e.g. via transfection using PEI) could be used in the future to study ICAM-1 expression in combination with bacterial stimulants, as transfected pIC signals via the RIG-I/MAVS pathway resulted in a more potent IFN antiviral response than TLR3 (Dauletbaev *et al.*, 2015), serving as a better model to study the antiviral response mediated by type I IFN.

Understanding how common viruses in addition to HRV, such as RSV, contribute to CF exacerbation is crucial, as they utilize different immune pathways and produce different outcomes in terms of susceptibility to secondary infection. RSV normally only causes pediatric infections (bronchiectasis), but in CF it causes exacerbation in both adults and children, while in children associated with severe mortality therefore understanding its mechanism is crucial (Van Ewijk *et al.*, 2007). RSV is a dsRNA virus binding to the RIG-I receptor (MDA5 contribution is only auxiliary) (Kim & Lee 2014). According to the theory of Ramezanpour *et al.*, we postulate that, unlike HRV which signals through MDA5 and induces a potent IFN response, RIG-I signaling by RSV produces a weak IFN response. Since we suspected that the IFN response triggered by pIC had a protective role (against secondary infections) short term after, the weaker IFN response triggered by RSV increases the susceptibility to secondary bacterial infections *in the short term* thus explaining the severe pulmonary exacerbation. We speculate that RV infections confer protection to bacterial infections in the short term, but not long term after the infection. Therefore, comparing the infections by RSV and HRV and they impact bronchial epithelial response to P. *aeruginosa* is useful.

It is noted that other important mediators including IL-17 and IL-12, other than type I IFN, are all important in PA clearance. The former is secreted by Th17 (cell-mediated) and clears extracellular pathogen, the latter is secreted by Th1 cells and participates in Type I immunity together with IFNγ, which clears intracellular bacteria (Leung *et al.*, 2010). It is known that type I IFN downregulates both cytokines, thus it would be a matter of which one dominates

and examining such in vivo is extremely useful in understanding host pathogen interactions. Therefore, thorough analysis of complete physiology would yield a more powerful conclusion with respect to understanding the pathophysiology of CF exacerbations.

7 CONCLUSIONS

This study presented a model of sequential viral and bacterial infections in bronchial epithelial cells. The results show that the increased MAPK and ICAM-1 mediated inflammatory and neutrophilic response in pIC and PA infections helps clear pathogens, but is exacerbated by increased damage caused by inflammation; pIC restricts PA from entering the cells reducing intracellular persistence, increasing their chance of clearance (by antibiotics and immune cells), but the result is more extracellular and planktonic bacteria and more inflammation either through signaling pathways or reinfection, again causing higher pulmonary exacerbation. Therefore, future or existing treatments that target one pathway without considering the other can lead to serious consequences, highlighting the importance of keeping a balance between targeting inflammation and interfering with pathogen clearance.

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