The role of T-cell immunoglobulin and mucin-domain-containing molecules in inflammation in the cystic fibrosis airways

A Dissertation Submitted to the Royal College of Surgeons in Ireland for the Degree of Doctor of Philosophy

By

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Research performed under the supervision of Dr. Emer Reeves, Department of Medicine, Royal College of Surgeons in Ireland, and under the direction of Professor Prof. Noel G. McElvaney, Professor of Medicine, Royal College of Surgeons in Ireland
I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree of Doctor of Philosophy is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed

Student Number

Date
“Caminante no hay camino, se hace camino al andar”

Antonio Machado
The T-cell immunoglobulin and mucin-domain containing molecules (TIMs) have emerged as promising therapeutic targets to correct abnormal immune function in several autoimmune and chronic inflammatory conditions. It has been reported that proinflammatory cytokine dysregulation and neutrophil-dominated inflammation are the main causes of morbidity in cystic fibrosis (CF). However, the role of TIM receptors in CF has not been investigated. The aim of this study was to determine if TIM signalling mechanisms are implicated in the pathogenesis of CF lung disease.

Initially, we characterised TIMs expression in human bronchial epithelial cells. We report for the first time that TIM-1 and TIM-3 are constitutively overexpressed on the CF airway epithelial cell surface. These results were corroborated in patient bronchial brushing samples suggesting a link between CFTR function and TIM expression. Of note, blockade of CFTR function with the CFTR inhibitor-172 caused upregulation of TIM-3 and its ligand galectin-9 in normal cells. Furthermore, TIM-3 was established as a functional receptor in bronchial epithelial cells as galectin-9 induced TIM-3 phosphorylation, resulting in IL-8 and IL-17A production. We have also confirmed that the expression of TIM-3 and its ligand galectin-9 can be modulated by lipopolysaccharide (LPS) which emphasizes the role of TIM-3 under inflammatory conditions.

Additionally, TIM-3 expression was localised on resting and primed neutrophil plasma membrane. Our data suggest a novel role for TIM-3/galectin-9 in neutrophil function with potentially important consequences in neutrophil antimicrobial activity. Galectin-9 was found to modulate neutrophil activation triggering mitogen activated protein kinase (MAPK) signalling cascades and calcium mobilisation. We also provided evidence for a role of galectin-0 in neutrophil degranulation and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity, possibly via TIM-3 interactions. We have demonstrated that TIM-3 expressed on neutrophils plays a direct role in bacterial killing. Opsonisation of PA with a physiologically relevant dose of galectin-9 (50 nM) enhanced neutrophil-mediated bacterial killing by 25%, an effect abrogated by blockade of
neutrophil TIM-3 receptors. This mechanism appeared to be gram-negative bacteria specific and mediated via galectin-9/LPS binding.

Collectively our data revealed a novel role for TIM-3/galectin-9 in the modulation of the inflammatory response in the airways. Under acute inflammatory conditions in the normal lung, galectin-9 and TIM-3 expression would be upregulated in bronchial epithelial cells leading to production of chemokines and neutrophil recruitment to fight bacterial infections. In addition, gram-negative bacteria would be specifically opsonised by galectin-9 favouring interaction with neutrophils and enhancing neutrophil-mediated bacterial killing. However, in the CF lung, both TIM-3 and its ligand galectin-9 undergo rapid degradation by neutrophil derived proteases, in particular neutrophil elastase and proteinase 3, potentially contributing to the defective bacterial clearance observed within the CF lung despite the high neutrophilic presence.
ACKNOWLEDGEMENTS

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I would like to express my gratitude to the clinical staff and donors at Beaumont Hospital that so generously have provided me with samples that were critical for the completion of the thesis. I would also like to thank Dr. McNally at Our Lady’s Children’s Hospital, Crumlin for providing the pediatric samples.

The author acknowledges Prof. Kuchroo (Brigham and Women’s Hospital, USA) for providing TIM-3 blocking antibody; Prof. Hirashima (Kagawa University, Japan) and Dr. Niki (Galpharma, Japan) for providing galectin-9 recombinant protein and antibodies, and Dr. Arikawa (Kagawa University, Japan) for measurement of galectin-9 levels in BAL. I would also like to thank Dr. Thomas (Molecular Medicine, RCSI) for assistance with confocal microscopy.

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<tbody>
<tr>
<td>A1AT</td>
<td>alpha-1 antitrypsin</td>
</tr>
<tr>
<td>ABTS</td>
<td>2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td><em>B. cepacia</em></td>
<td><em>Burkholderia cepacia</em></td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>[Ca&lt;sup&gt;2+&lt;/sup&gt;]</td>
<td>Intracellular calcium</td>
</tr>
<tr>
<td>CDR</td>
<td>Carbohydrate recognition domain</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>CFBE</td>
<td>Human CF bronchial epithelial cells</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>CFTRinh-172</td>
<td>CFTR inhibitor 172; 3-[(3-trifluoromethyl)phenyl]-5-[(4-carboxyphenyl)methylene]-2-thioxo-4-thiazolidinone</td>
</tr>
<tr>
<td>CMK</td>
<td>N-(methoxysuccinyl)- Ala-Ala-Pro-Val-chloromethyl ketone</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s PBS calcium and magnesium free</td>
</tr>
<tr>
<td>DPBSG</td>
<td>5mM glucose in DPBS</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ENaC</td>
<td>Epithelial sodium channel</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>fMLP</td>
<td>N-formyl-methionine- leucine-phenylalanine</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>Gal-9</td>
<td>Galectin-9</td>
</tr>
<tr>
<td>GlyH-101</td>
<td>N-(2-naphthalenyl)-((3,5-dibromo-2,4-dihydroxyphenyl)methylene)glycine hydrazide</td>
</tr>
<tr>
<td><em>H. influenzae</em></td>
<td><em>Haemophilus influenzae</em></td>
</tr>
<tr>
<td>HAVCR</td>
<td>Hepatitis A virus receptor</td>
</tr>
<tr>
<td>HBE</td>
<td>Human bronchial epithelial cell</td>
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<tr>
<td>HMGB1</td>
<td>High mobility group box 1</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon gamma</td>
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lgV-domain  Immunoglobulin variable-domain
IL        Interleukin
IL-1β     Interleukin-1-beta
JNK       c-JUN N-terminal kinase
kDa       Kilo Dalton
KIM-1     Kidney injury molecule 1
LBB       Lamberth's Break Buffer
LSC       Laser scanning cytometry
LPS       Lipopolysaccharide
MAPK      Mitogen activated protein kinase
MEM       Minimum essential medium
MTT       3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NADPH     Nicotinamide adenine dinucleotide phosphate
NFkB      Nuclear factor kappa-light-chain-enhancer of activated B cells
NE        Neutrophil elastase
O2^-      Superoxide anion
PA        Pseudomonas aeruginosa
PBMC      Peripheral blood moncytic cells
PBS       Phosphate buffered saline
PD-1       Programmed death-1 receptor
PE        Phycoerythrin
PI        Propidium iodide
PMA       Phorbol 12-myristate 13-acetate
PMSF      Phenylmethylsulfonyl fluoride
PNS       Postnuclear supernatant
PR3       Proteinase 3
rhTIM-1   Recombinant human TIM-1
rhTIM-3   Recombinant human TIM-3 linked to human IgG1 Fc tail chimera
RIPA buffer Radioimmnoprecipitation assay buffer
ROS       Reactive oxygen species
(q)RT-PCR  (quantitative) Real-time reverse transcriptase-polymerase chain reaction
SA        Staphylococcus aureus
SBTI      Soya bean trypsin inhibitor
SD        Standard deviation
SDS       Sodium dodecyl sulphate
SDS-PAGE  Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SLPI      Serine leukoprotease inhibitor
TAPl       Tumour necrosis factor-a protease inhibitor
Tapr      T cell and airway hyperreactivity phenotype regulatory locus
TCA       Trichloroacetic acid
TEER      Transepithelial electrical resistance
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>Th</td>
<td>T-helper</td>
</tr>
<tr>
<td>TIM</td>
<td>T-cell immunoglobulin and mucin domain containing molecule</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>TLCK</td>
<td>N₆-tosyl-l-lysine chloromethyl ketone hydrochloride</td>
</tr>
<tr>
<td>TPCK</td>
<td>N-p-tosyl-l-phenylalanine chloromethyl ketone</td>
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LIST OF SELECTED PRESENTATIONS/PUBLICATIONS


(Results from Chapter 3 & 4 included in this publication)


Vega-Carrascal I., Reeves E.P., O'Neill S.J. & McElvaney N.G (2010) "The negative immune regulator TIM-3 is inactivated in the cystic fibrosis lung by proteolytic degradation" Irish Thoracic Society Annual Scientific Meeting (poster)

Vega-Carrascal I., Reeves E.P. O'Neill S.J. & McElvaney N.G. (2010) "Proteolytic degradation of T-cell immunoglobulin and mucin-domain-3 in the cystic fibrosis airways" Annual Meeting of the Royal Academy of Medicine in Ireland. Section of Biomedical Sciences (poster)


Vega-Carrascal, Reeves E.P., O'Neill S.J. & McElvaney N.G. (2010) "Altered Expression of T-Cell Immunoglobulin and Mucin-domain-Containing Molecule-1 (TIM-1) and TIM-3 in the Cystic Fibrosis Airway" 33rd European Cystic Fibrosis Conference (Oral presentation and abstract)


Vega-Carrascal, I., Reeves, E. P., O'Neill, S. J., & McElvaney, N. G (2009) "T-cell immunoglobulin and mucin-domain-containing molecule-1 (TIM-1) and TIM-3 are differentially expressed on human bronchial epithelial cells in cystic fibrosis" Annual Meeting of the Royal Academy of Medicine in Ireland. Section of Biomedical Sciences (oral presentation)

Vega-Carrascal, I., Reeves, E. P., O'Neill, S. J., & McElvaney, N. G. (2009) "Expression of T-cell immunoglobulin and mucin-domain-containing molecule-1 (TIM-1) and TIM-3 is upregulated in human bronchial epithelial cells in cystic fibrosis" Irish Thoracic Society Annual Scientific Meeting (poster)
CHAPTER 1: GENERAL INTRODUCTION

1.1 Cystic fibrosis

Cystic fibrosis is the most common lethal genetic disease in Caucasians affecting at least 60,000 individuals worldwide (Gibson et al., 2003). Ireland has the highest incidence of CF in the world, affecting 1 in 1600 people (The Cystic Fibrosis Association of Ireland, 2005). The prevalence of CF in Ireland is 2.98/10000, four times higher than in the USA or Europe (Farrell, 2008).

CF is caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR) chloride channel (Riordan et al., 1989, Rommens et al., 1989). Over 1500 CFTR mutations leading to defective chloride transport have been identified to date (Rowntree and Harris, 2003, Welsh and Smith, 1993). These mutations can be classified into five categories depending on their functional alterations within epithelial cells (Welsh and Smith, 1993). Figure 1.1 illustrates the mechanisms involved in CFTR dysfunction in each category. CFTR protein synthesis is absent in Class I mutations. Class II mutations cause the protein to misfold and be retained in the endoplasmatic reticulum. As a result the protein is targeted for degradation and the mature form is minimally expressed on the epithelial surface. Class III mutations lead to defective CFTR regulation and inactivation of chloride channel activity. Class IV mutations cause reduced chloride transport although the channel is present on the apical membrane. Class V mutations are the result of splicing defects and cause reduced production of functional CFTR protein.
<table>
<thead>
<tr>
<th>Defect Classification</th>
<th>Normal</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
</tr>
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<tbody>
<tr>
<td>Result</td>
<td>No synthesis</td>
<td>Block in Processing</td>
<td>Block in Regulation</td>
<td>Altered Conductance</td>
<td>Reduced Synthesis</td>
<td></td>
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**Figure 1.1. Functional effects of classes of CFTR mutation.** In normal epithelial cells, wild-type CFTR protein is processed in the endoplasmic reticulum, including correct glycosylation, folding and trafficking via the Golgi apparatus to the membrane where it functions as a chloride channel. Class I mutations exemplified by G542X cause lack of protein synthesis. Class II mutations (e.g. ΔF508) block CFTR processing due to misfolded protein targeted for ubiquitination and degradation. Class III mutations, including G551D, cause CFTR to reach the cell membrane but the channel is not properly activated. Class IV mutations, such as R347P or R117H, reach the apical cell surface but there is defective chloride transport due to decreased chloride conductance. Class V mutations, exemplified by incorrect splicing with the mutation 3849+10kb C→T cause reduced production of functional CFTR. Adapted from (Zielenki and Tsui, 1995)
The most common mutation is deletion of phenylalanine at position 508 (ΔF508) present in both alleles in approximately 70% of CF patients, and 90% of CF patients in at least one allele in the US (Babadilla et al., 2002, Kerem et al., 1989), making it the most studied mutation. According to the CF Registry in Ireland, 57.8% of registered patients are ΔF508 homozygous and carriers of at least one ΔF508 allele account for 90.1% of the Irish CF population (Cystic Fibrosis Registry of Ireland, 2011). This class II defect is a specific single point mutation which results in a folding defect, trafficking deficiencies and lack of CFTR activity in the plasma membrane of epithelial cells (Rowntree and Harris, 2003).

CF affects various organs, including the pancreas, liver and intestinal tract, but the most severe complications often occur in the lung (Table 1.1). CF related symptoms, although variable from patient to patient, are typically present early in life and increase in severity with age despite aggressive therapeutic intervention. CFTR absence or malfunction causes defective ion transport across the epithelium, reduction in periciliary liquid volume and persistent mucus hypersecretion. As a consequence mucus accumulates on the airway surface which leads to chronic bacterial infection, exacerbated airway inflammation, lung injury and ultimately death (Davis, 2006).
<table>
<thead>
<tr>
<th>Stage</th>
<th>Sinopulmonary disease</th>
<th>Gastrointestinal abnormalities</th>
<th>Endocrine</th>
<th>Other</th>
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</thead>
<tbody>
<tr>
<td>Infancy/ early childhood</td>
<td>Airway infection  Cough</td>
<td>Meconium ileus    Rectal prolapse</td>
<td>Acute salt depletion  Metabolic alkalosis</td>
<td>Dehydration</td>
</tr>
<tr>
<td></td>
<td>Sputum Production   Wheeze and air trapping</td>
<td>Growth retardation   Pancreatic insufficiency</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Airway obstruction</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Adolescence/ early adulthood</td>
<td>Nasal polyps  Sinusitis       ABPA</td>
<td>Reflux         DIOS   Intussusception</td>
<td>Delayed puberty     CFDR   Metabolic bone disease</td>
<td>Renal calculi  Depression  Anxiety</td>
</tr>
<tr>
<td></td>
<td>Bronchiectasis</td>
<td>Hepatic Steatosis     Biliary fibrosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mature adulthood</td>
<td>Haemoptysis          Pneumothorax      Respiratory failure</td>
<td>Biliary cirrhosis  GI adenocarcinoma</td>
<td>Osteoporotic fractures  CBAVD   Renal failure  Arthritis</td>
<td></td>
</tr>
</tbody>
</table>

Definition of abbreviations: ABPA = allergic bronchopulmonary aspergillosis; CBAVD = congenital bilateral absence of the vas deferens; CFDR = cystic fibrosis related diabetes; DIOS = distal intestinal obstruction syndrome; GI = gastrointestinal. Adapted from (Gibson et al., 2003, O’Sullivan and Freedman, 2009, Parkins et al., 2011).
1.1.1 Inflammation in the CF lung

The hallmark of CF lung disease is exacerbated inflammation accompanied by massive neutrophil infiltration that, paradoxically, cannot eradicate bacterial infections. As a result CF patients suffer from chronic bacterial infection in the lower airways. Indeed, bronchoalveolar lavage (BAL) fluid of CF patients contains elevated levels of neutrophils and proinflammatory cytokines such as interleukin (IL)-6, tumor necrosis factor alpha (TNFα) and IL-8 but decreased levels of the anti-inflammatory cytokine IL-10 (Bonfield et al., 1995).

The mechanisms linking CFTR dysfunction to chronic bacterial infection and enhanced inflammation in the lung are still not fully understood. Additionally, whether inflammation precedes infection is still a matter of debate. It has been proposed that lack of chloride transport due to defective CFTR function leads to dehydration of the airway liquid surface layer. Thus, mucus thickening impairs mucociliary clearance of bacteria. Furthermore, obstruction of the airways by mucus plugs triggers chronic bacterial colonisation (reviewed in (Boucher, 2007)). However, there is growing evidence that lack of CFTR function promotes an inflammatory status in bronchial epithelial cells even in the absence of infection (Hunter et al., 2010, Vij et al., 2009). Regardless of the initial cause, hyperinflammation in the CF lung occurs early and continues throughout life (Larson and Cohen, 2005). This inflammatory state is further amplified by bacterial infections, initially *Haemophilus influenzae* (*H. influenzae*) and *Staphylococcus aureus* (SA) in newborns (Armstrong et al., 1995), and later *Pseudomonas aeruginosa* (PA) (Hayes et al., 2010).

The presence of bacteria promotes the airway epithelium to release proinflammatory cytokines such as IL-6 and IL-1β and the potent neutrophil chemoattractant IL-8 (Kube et al., 2001). This sustained inflammatory response recruits neutrophils in an attempt to resolve the bacterial infection, but chronic neutrophil stimulation also leads to neutrophil death by secondary necrosis rather than apoptosis (Dockrell and Whyte, 2006). As a consequence, neutrophils are not cleared effectively (Vandivier et al., 2002, Watt et al., 2005) and neutrophil contents are released into the lung perpetuating the inflammatory cycle (Griese et al., 2008). Indeed, IL-8, neutrophil elastase (NE) and neutrophil counts have been correlated with lung function (Mayer-Hamblett et al., 2007).
Defective innate immune function in the CF airways is the result of a combination of reduced mucociliary clearance, aberrant airway immune response, loss of antimicrobial activity, impaired bacterial killing, and failure to counteract the proteases and oxidants released by necrotising neutrophils.

1.1.2 Role of bronchial epithelial cells in CF airway inflammation

The defence mechanisms afforded by airway epithelium go beyond its position as a physical barrier between the host and microbial pathogens. It is now widely recognised that bronchial epithelial cells play an important role in airway defence and immune regulation (Greene and McElvaney, 2005, Proud and Leigh, 2011, Rogan et al., 2006, Ryu et al., 2010), and that these mechanisms may be defective in CF (Elizur et al., 2008, Fischer, 2009, Jacquot et al., 2008, Terheggen-Lagro et al., 2005).

Antimicrobial host defence starts in the airways by trapping pathogens in mucin-rich mucus and rapid removal by the mucociliary escalator. As mentioned above, this mechanism is severely impaired in the CF airways due to the absence of adequate chloride transport caused by CFTR mutations. Airway epithelial cells can also recognise microbes by pathogen recognition receptors binding to unique molecules in pathogens which trigger a specific innate immune response. Of these receptors, Toll-like receptors (TLR) are the most commonly studied in airway epithelium (Greene and McElvaney, 2005). Each TLR recognises a specific type of pathogen molecular pattern (Takeda and Akira, 2005). TLR2 ligands include lipoteichoic acid and lipoproteins, double-stranded viral RNA is the main ligand for TLR3, bacterial lipopolysaccharide (LPS) is a well characterised ligand of TLR4, bacterial flagellin stimulates TLR5, TLR7 and TLR8 recognise single-stranded viral RNA whereas TLR9 binds to unmethylated CpG DNA. TLR4 and TLR2 are crucial to fight bacterial infection in the airways. Bacterial wall components bind to these receptors in bronchial epithelial cells, initiating an immune response leading to expression of pro-inflammatory cytokines and recruitment of appropriate immune cells (notably, neutrophils) to tackle the infection. In order to avoid excessive neutrophil recruitment and minimise the potential harmful effects of neutrophil products to the airways, TLR4 expression and function is tightly controlled. Thus TLR4 is
only expressed on the surface during bacterial infection, however, in the constantly challenged CF bronchial epithelium, TLR4 levels are decreased compared to healthy controls (Hauber et al., 2005, John et al., 2010). Indeed, reduced TLR4 response to PA has been proposed as a cause of chronic bacterial colonisation in the CF airways (John et al., 2010). In addition to IL-8, a potent neutrophil chemoattractant, and IL-6, a master promoter of inflammation, bronchial epithelial cells produce other cytokines and growth factors to regulate the inflammation in response to bacterial infection, including TGF-β which contributes to airway remodelling and granulocyte colony stimulating factor (G-CSF) which modulates the activation and survival of neutrophils.

Airway epithelial cells also release antimicrobial peptides and proteins into the airway lining fluid. The main groups of antimicrobial peptides expressed in the airways are defensins and cathelicidins (Bals and Hiemstra, 2004). Antimicrobial peptides exhibit broad spectrum microbicidal activity against bacterial, fungal and enveloped viruses. Antimicrobial activity requires binding of the peptide to the components of the pathogen surface which is favoured by the presence of positively charged residues and surfactant properties (Rogan et al., 2006). The only known human cathelicidin in the airways is the cationic peptide LL-37. As other antimicrobial peptides, it is released as a pro-form (hCAP18) which requires proteolytic processing to release the active form, LL-37. This antimicrobial peptide is also contained in secondary neutrophil granules. In addition to small cationic antimicrobial peptides, epithelial cells also produce larger antimicrobial peptides with multiple functions including lysozyme, lactoferrin, and the antiproteases elafin and serum leukocyte protease inhibitor (SLPI). Many of these antimicrobial proteins exhibit synergistic antimicrobial properties and constitute an important part of the host defence mechanism.

Lastly, epithelial cells also produce reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂) and nitric oxide which in addition to exerting direct antimicrobial properties, also play an important role mediating intracellular signalling mechanisms to modulate the expression of cytokines, chemokines and adhesion molecules. The main sources of ROS in epithelial cells are the dual oxidases DUOX1 and DUOX2 and the NADPH oxidase system. DUOX produce H₂O₂ that is used by lactoperoxidase to produce
bactericidal hypothiocyanite anion. DUOX/lactoperoxidase system activity is impaired in CF airway epithelium due to lack of CFTR function (Fischer, 2009). CFTR mediated ion transport is necessary to provide thiocyanite anions for correct lactoperoxidase function. Additionally, defective CFTR ion transport conducts to an acidification of the airway surface layer due to diminished carbonate ion transport required for pH adjustment. Adequate proton transport has also been proposed to be required for proper DUOX/lactoperoxidase function (Fischer, 2009).

The effector antimicrobial mechanisms (mucin, antimicrobial proteins and ROS production) together with the immunomodulatory properties of the innate immune response caused by pathogen stimulation of bronchial epithelial cells, protect the airways from microbial infections. However, in the CF context all these mechanisms are impaired and cause chronic inflammation and persistent bacterial infections.

1.1.3 Bacterial infections in CF

Organisms found in CF airways are commonly opportunistic bacteria otherwise non-pathogenic in immunocompetent individuals. A key feature of CF airway infections is that bacterial pathogens evolve with age and disease status following an identified age-dependent sequence (Figure 1.2).
Figure 1.2. Age specific prevalence of airway infections in patients with CF. Organisms reported to the US CF Patient Registry, 2009. Overall percentage of patients (all ages) who had at least one respiratory tract culture (sputum, bronchoscopy, oropharyngeal, or nasal) performed in 2009 that was positive for the indicated organisms. Reproduced from (Cystic Fibrosis Foundation Patient Registry, 2010)
Infants with CF become infected early, first with gram-negative SA and non-typable *H. influenzae*. However, PA quickly infects the lower airways of young children. Initially, different PA strains, generally non-mucoid and antibiotic-susceptible, may be alternate in endobronchial infections. Later on, one strain, typically characterised by mucoid phenotype and multidrug resistant, dominates and colonises the lungs of CF children. The median age at the onset of PA colonisation is 10 years, and once PA colonisation occurs, complete bacterial eradication is frequently not possible (LiPuma, 2010).

Nevertheless, early aggressive antipseudomonal antibiotic therapy has an 80% success rate at eradicating PA infection and currently some CF centres report a shift on the median age of the onset of chronic colonisation from 10 to 18 years old (Cramer et al., 2010). Other organisms found to infect CF airways in elder children are the gram-positive bacteria *Burkholderia cepacia* (*B. cepacia*), *Stenotrophomonas maltophilia*, *Achromobacter sylosoxidans*. *B. cepacia* is composed of different species of which *B. cenocepacia* and *B. multivorans* are the most frequently isolated from CF patients in Ireland, although they constitute less than 8% of the detected organisms in sputum cultures (Cystic Fibrosis Registry of Ireland, 2011). In addition to gram-negative bacteria, non-tuberculous mycobacteria and fungi (i.e. *Aspergillus fumigatus*) are also found in the respiratory tract of CF patients. Indeed *Aspergillus fumigatus* has been reported to be present in one third of adult CF patients in Ireland (Chotirmall et al., 2008). Additionally, non-anaerobic bacteria have been frequently isolated from different types of respiratory samples from CF patients (Lambiase et al., 2010). Although the clinical significance of anaerobes remains unclear, levels of non-anaerobic bacteria detected in sputum from CF patients were similar to those of SA and PA (Zemanick et al., 2010). For instance, *Prevotella melaninogenica* was detected by PCR in 73% of sputum and oropharyngeal swab samples obtained from CF patients (Zemanick et al., 2010).

Nevertheless, PA still represents the archetypal pathogen in the CF airways and caused more than 60% of registered cardiac/pulmonary complications in CF adult patients in Ireland in 2009 (Cystic Fibrosis Registry of Ireland, 2011). Therefore, antipseudomonal therapy is still an essential component of the management of CF lung disease.
1.1.4 Role of neutrophils in CF

Neutrophil recruitment to the site of inflammation constitutes one of the first responses in the host defence against pathogen infection. Neutrophils are professional phagocytes and for this purpose, they display a range of functions that permits effective pathogen destruction (Segal, 2005). However, due to the potential tissue damage caused by neutrophil contents, such as proteases and oxidants, neutrophil activation is tightly controlled. The hallmark of CF lung disease is chronic bacterial infection despite an exuberant neutrophil response in the airways. In CF, neutrophil-dominated inflammation is believed to be responsible for the destruction of the lung architecture due to uncontrolled release of granule contents (Mayer-Hamblett et al., 2007). Whether neutrophils in CF are intrinsically abnormal or the reduced antibacterial function is a result of chronic inflammation is still a matter of debate (see (Hayes et al., 2011)). However, potential defects in CF neutrophils have been proposed for several of the main neutrophil functions: neutrophil priming and activation, neutrophil phagocytosis, oxidant production and protease degranulation.

Circulating neutrophils are considered to be in a resting state and do not express their full antimicrobial capacity. Only after exposure to certain stimuli, usually cytokines (e.g. TNFα), chemokines (e.g. IL-8) or pathogen molecules (e.g. LPS), neutrophils undergo structural changes and express cell surface receptors that facilitate transmembrane signal transduction leading to mobilisation of intracellular calcium stores and tyrosine phosphorylation signalling cascades involving MAPKs. These signalling mechanisms lead to the activation of the NADPH oxidase system and also mobilise neutrophil granules, ready to release their antimicrobial contents (Table 1.2).
### Table 1.2. Neutrophil granule contents

<table>
<thead>
<tr>
<th>Ariophil (primary)</th>
<th>Specific (secondary)</th>
<th>Gelatinase (tertiary)</th>
<th>Secretory</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloperoxidase</td>
<td>Lactoferrin</td>
<td>Gelatinase (MMP9)</td>
<td>CR1</td>
</tr>
<tr>
<td>Neutrophil Elastase</td>
<td>Cathelicidin (LL37)</td>
<td>Leukolysin</td>
<td>CR3</td>
</tr>
<tr>
<td>Cathepsin G</td>
<td>Lysozyme</td>
<td>Lysozyme</td>
<td>FPR</td>
</tr>
<tr>
<td>Proteinase 3</td>
<td>Collagenase</td>
<td>NARMP1</td>
<td>CD14</td>
</tr>
<tr>
<td>Azurocidin</td>
<td>Leukolysin</td>
<td></td>
<td>CD16</td>
</tr>
<tr>
<td>Defensins</td>
<td>Cytochrome b558</td>
<td></td>
<td>Cytochrome b558</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>NGAL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Granule contents are classified according to the timing of their biosynthesis during neutrophil differentiation. Granule contents are being degranulated in a hierarchical manner, secretory granules being the most readily exocytosed and primary granules the most resistant to exocytosis. Definition of abbreviations: CR1 = Complement 1 receptor; CR3 = Complement 3 receptor; FPR = formyl peptide receptor; NARMP1 = Natural-resistance-associated macrophage protein 1; NGAL = Neutrophil gelatinase-associated lipocalin; MMP9 = Matrix metalloproteinase 9.
Additionally, receptors required for bacterial phagocytosis are also mobilised to the neutrophil surface. The main receptors involved in neutrophil phagocytosis are Fcγ (i.e. FcγIIA, CD32 and FcγIIIIB, CD16) and complement receptors (i.e. CR1, CD35 and CR3, CD11b/CD18). Once bacteria are engulfed by the neutrophil, killing occurs within the phagocytic vacuole also known as the phagolysosome as a result of two mechanisms: NADPH oxidase dependent release of oxidants and fusion with neutrophil granules (Figure 1.3). During phagocytosis, neutrophils consume a large amount of oxygen (O₂) to produce superoxide anion (O₂⁻), this reaction [1] is catalysed by the NADPH oxidase (Babor, 1984).

\[
\text{NADPH} + \text{O}_2 \rightarrow \text{NADP}^+ + \text{H}^+ + \text{O}_2^- \quad [1]
\]

The O₂⁻ readily dismutates to form H₂O₂ and O₂ [2], a reaction that can also be catalysed by superoxide dismutase.

\[
2\text{O}_2^- \rightarrow \text{O}_2 + \text{H}_2\text{O}_2 \quad [2]
\]

Within the phagocytic vacuole H₂O₂ is used to form hypochlorous acid (HOCl) in a reaction catalysed by myeloperoxidase (MPO) in the presence of Cl⁻ [3].

\[
\text{H}_2\text{O}_2 + 2\text{Cl}^- \rightarrow 2\text{HOCl} \quad [3]
\]

HOCl is cytotoxic to bacteria due to its potent chlorinating properties. Additionally a wide range of radicals can also be produced by hydroxyl radical (·OH) produced by the iron (Fe²⁺) catalysed Fenton reaction [4].

\[
\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \cdot\text{OH} + \text{OH}^- + \text{Fe}^{3+} \quad [4]
\]
Figure 1.3. Neutrophil-mediated bacterial killing mechanisms. Neutrophils phagocytose and internalise the bacteria within the phagolysosome also known as phagocytic vacuole. Following bacterial engulfment, NADPH oxidase is activated and produces superoxide anion ($O_2^-$). NADPH oxidase activity causes a net positive charged cation entry into the phagocytic vacuole which provokes a drop in pH and change of tonicity. Potassium ($K^+$) influx triggers the release of serine proteases from their proteoglycan matrix into the phagolysosome and helps raising pH to optimal levels (Reeves et al., 2002). In addition, hydrogen peroxide ($H_2O_2$) formed by dismutation of $O_2^-$ is converted to hypochloric acid (HOCl) by myeloperoxidase which kill bacteria in combination with serine proteases. Additionally, neutrophils can also kill bacteria extracellularly by formation neutrophil extracellular traps (NETs) composed of neutrophil serine proteases and nuclear constituents. Adapted from (Pham, 2006)
Neutrophils also release a series of antimicrobial proteins and proteases into the vacuole that destroy bacteria. Among them NE is believed to be the most effective protease in degrading bacterial components (Pham, 2006). Importantly, NE can also degrade extracellular matrix components, including collagen and therefore plays an important role in CF lung destruction. Additionally, NE and other neutrophil derived proteases have also been implicated in degradation of receptors required for bacterial clearance (Griese et al., 2008) and stimulate IL-8 release by bronchial epithelium (Nakamura et al., 1992). Serine derived proteases NE, PR3 and Cath G can also cleave cytokines such as IL-8 producing more active forms (Padrines et al., 1994) and inactivate endogenous protease inhibitors disrupting the protease/antiprotease balance in the airways and contributing to lung inflammation in CF (Figure 1.4).
Figure 1.4. Role of neutrophil serine proteases in inflammation. Following activation, neutrophils release their serine proteases to the extracellular environment where they participate in the inflammatory response by activation of receptors, proteolytic degradation of chemokines and cytokines and cleavage of adhesion molecules. Adapted from (Pham, 2006).
1.1.5 Protease/antiprotease imbalance in CF

In the healthy lung, neutrophils represent approximately 1% of the inflammatory cells, however, they account for 70% of the total cell count in CF BAL (Reinhardt et al., 2003). As those neutrophils are incapable of clearing bacterial infections, they accumulate in the CF lower airways and release high amounts of proteases. Subsequently, a protease/antiprotease imbalance is established (Birrer et al., 1992) and accompanies CF patients throughout their lives.

Neutrophils contain different classes of proteases, including serine, cysteine, aspartic and metalloproteases. Neutrophil serine proteases are considered critical mediators of the inflammatory response in the airways (Pham, 2006) and potentially destructive if not counteracted. Among them, the serine protease NE is the most harmful in the lung (Griese et al., 2008) and it has been proposed as a target for therapeutic intervention (Greene and McElvaney, 2009, Kelly et al., 2008, Quinn et al., 2010). Under normal conditions, NE degrades phagocytosed proteins but extracellularly, it can degrade virtually all structural proteins in the lung including elastin, collagen and fibronectin as well as many plasma proteins (reviewed in (Greene and McElvaney, 2009)). Other important serine proteases are cathepsin G (Cath G) and proteinase-3 (PR3). To counteract the action of these potent proteases, several anti-proteases are present in the lung. The main serine antiprotease is alpha-one antitrypsin (A1AT). Its primary role is to counteract NE activity but it can also inhibit PR3 and Cath G. The antiproteases SLPI and elafin are less abundant in the airways. SLPI has been shown to inhibit NE and Cath G among other proteases but it does not inhibit the other serine protease PR3 (Rao et al., 1993). Conversely, elafin inhibits NE and PR3 but not Cath G (Wiedow et al., 1990). In addition to the antiprotease activity, A1AT, SLPI and elafin have been shown to possess anti-inflammatory and antimicrobial activity (extensively reviewed in (Quinn et al., 2010, Rogan et al., 2006)). Table 1.3 summarises the main characteristics of serine proteases NE, PR3 and Cath. G as well as their natural inhibitors.
<table>
<thead>
<tr>
<th></th>
<th>Elastase</th>
<th>Proteinase 3</th>
<th>Cathepsin G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Mass (kDa)</td>
<td>29-33</td>
<td>29-32</td>
<td>28.5</td>
</tr>
<tr>
<td>pH</td>
<td>~10.5</td>
<td>~9.5</td>
<td>~12</td>
</tr>
<tr>
<td>No of glycosylation sites</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>No of disulfide bridges</td>
<td>4</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Optimal pH for activity</td>
<td>8.0-8.5</td>
<td>~8.0</td>
<td>~7.5</td>
</tr>
<tr>
<td>Substrate specificity</td>
<td>Small hydrophobic residues at P1 position: Val, Cys, Ala, Met, Leu, Val, Cys, Ala, Met, Ile, Leu, Ser</td>
<td>Small hydrophobic residues at P1 position: Val, Cys, Ala, Met, Leu, Val, Cys, Ala, Met, Ile, Leu, Ser</td>
<td>Aromatic or positively charged residues at P1 position: Phe, Tyr, Lys, Arg</td>
</tr>
<tr>
<td>Localization in neutrophils</td>
<td>Azurophilic granules</td>
<td>Azurophilic granules</td>
<td>Azurophilic granules</td>
</tr>
<tr>
<td>Neutrophil surface after priming</td>
<td>On unprimed neutrophil surface</td>
<td>Neutrophil surface after priming</td>
<td></td>
</tr>
<tr>
<td>Source</td>
<td>Neutrophil</td>
<td>Neutrophil</td>
<td>Neutrophil</td>
</tr>
<tr>
<td>Monocyte</td>
<td>Monocyte</td>
<td>Monocyte</td>
<td>Monocyte</td>
</tr>
<tr>
<td>Mastocyte</td>
<td>Mastocyte</td>
<td>Mastocyte</td>
<td>Mastocyte</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>Basophil</td>
<td>Basophil</td>
<td>Basophil</td>
</tr>
<tr>
<td>Endogenous inhibitor</td>
<td>A1AT</td>
<td>A1AT</td>
<td>A1AT</td>
</tr>
<tr>
<td></td>
<td>SLPI</td>
<td>Elafin/pre-elafin</td>
<td>SLPI</td>
</tr>
<tr>
<td></td>
<td>Elafin/pre-elafin</td>
<td>Elafin/pre-elafin</td>
<td>Elafin/pre-elafin</td>
</tr>
</tbody>
</table>
Proteases in the CF lung perpetuate the inflammatory status via several mechanisms. NE has been shown to promote IL-8 production by the lung mucosa (Walsh et al., 2001). NE can also activate other proteases in the airways like the matrix metalloprotease-9 (Ferry et al., 1997) and degrade antiproteases including elafin (Guyot et al., 2008), tissue inhibitor of metalloproteinase-1 (Itoh and Nagase, 1995) and A1AT. In contrast, SLPI is relatively resistant to NE and is degraded by cysteinyl cathepsins (Taggart et al., 2001). Additionally, unopposed proteolytic action can degrade different molecules important in control of inflammation including receptors required for clearance of apoptotic neutrophils (Vandivier et al., 2002) or bacterial phagocytosis (Hartl et al., 2007). As a consequence of the protease/antiprotease imbalance, lung tissue is irreversibly damaged, dramatically reducing CF patients' lung function and ultimately causing respiratory failure (Welsh and Fick, 1987). Indeed, more than 85% of CF patients die of respiratory failure (Nick et al., 2010). Ultimately, a better understanding of the mechanisms involved in inducing and controlling inflammation is required for the successful design of novel intervention strategies in CF.
1.2 T-cell Immunoglobulin and mucin-containing domain molecules (TIM)

T cell immunoglobulin and mucin domain molecules (TIM) are key regulators of immune responses (reviewed in (Wang et al., 2008, Kuchroo et al., 2008, Su et al., 2008, Rodriguez-Manzanet et al., 2009)). However, the role of TIMs in regulation of the immune response in CF remains unexplored.

TIM proteins have also been associated with several human inflammatory conditions (Recalcati et al., 2008, Su et al., 2008), including rheumatoid arthritis (Seki et al., 2008), asthma (Rennert et al., 2006), systemic lupus erythematosus (Wang et al., 2008), multiple sclerosis (Khademi et al., 2004) and diabetes (Sanchez-Fueyo et al., 2003), and more recently in tumor (Sakuishi et al., 2011) and antimicrobial immunity (Jayaraman et al., 2010).

The TIM gene family is encoded on chromosome 11B1.1 in mice (TIM1-8) and chromosome 5q33.2 in humans. There are four TIM proteins characterised in mice (TIM-1, TIM-2, TIM-3 and TIM-4). To date, only three members of the family have been identified in humans: TIM-1, TIM-3 and TIM-4. Human TIM-3 shares 63% homology with mouse TIM-3 and TIM-4 shares 49% homology with the mouse ortholog TIM-4. In contrast, human TIM-1 does not seem to have a clear ortholog in mouse as it shares 42% and 32% amino-acid sequence with murine TIM-1 and TIM-2 respectively (Meyers et al., 2005). All TIMs share a similar structure as type I membrane proteins, consisting of an N-terminal immunoglobulin variable (IgV)-like domain, a mucin-like domain, a transmembrane region and an intracellular tail (Figure 1.5).
Figure 1.5. Schematic representation of mouse and human TIM molecules. Protein structure and positions of the glycosylated sites are shown. Glycosylation sites in the mucin domain were predicted with NetOglyc and NetNglyc and are positioned approximately. Adapted from (Freeman et al., 2010)
Human TiM IgV-like domains share 40% homology (Santiago et al., 2007) and are cysteine rich which suggest a highly crosslinked structure. In contrast, the mucin domain presents in an extended conformation. The size of the mucin domain varies depending on the receptor, TIM-3 being the shortest. TIM-1 and TIM-3 are involved in intracellular signalling through tyrosine-phosphorylation motifs in the cytoplasmatic domain whereas TIM-4 does not contain any conserved motif, indicating that it exerts its function by association with other receptors. An important feature of TIMs structure is the high level of glycosylation which enables ligand binding (Santiago et al., 2007) and increases resistance to proteolytic cleavage. All TIM receptors are predicted to be N-glycosylated in the Ig-domain and at different positions close to the membrane and O-glycosylated in the mucin domain. However, the level of glycosylation varies from only one predicted site in TIM-3 to up to 56 in TIM-1 (Figure 1.6).
Figure 1.6. Schematic representation of TIM-proteins and their ligands. A) TIM-1 can bind to phosphatidylserine on apoptotic bodies through the FG-CC' binding region in the N-terminal immunoglobulin variable (IgV) domain conferring phagocytic characteristics to epithelial cells. TIM-1 can also bind to soluble TIM-4 or ligate to itself leading to T-cell activation and T-helper (Th)-2 expansion. B) TIM-3 binds to Galectin-9 through N-linked carbohydrates in the IgV domain driving a Th1-mediated inflammatory response. The ligand binding to the FG-CC' cleft on the opposite side of the IgV domain has not yet been identified. Adapted from (Freeman et al., 2010).
TIM-1 was initially identified as hepatitis A virus receptor (HAVCR) in monkey (Kaplan et al., 1996) and in humans (Feigelstock et al., 1998). TIM-1 was also cloned as kidney injury molecule-1 (KIM-1) (Ichimura et al., 1998). KIM-1 (TIM-1) is overexpressed in injured renal epithelium and can be cleaved off the cell membrane by metalloproteases (Bailly et al., 2002). Shedded TIM-1 can be detected in urine after kidney injury and it has been proposed as a urinary marker of renal injury (Han et al., 2002, Ichimura et al., 1998).

Interest in TIMs has grown dramatically after the discovery of differential TIM expression in T helper (Th) cells and their immunomodulatory properties. TIM-1 is expressed in Th2 cells, whereas TIM-3 is found in Th1 cells (Khademi et al., 2004). TIM signalling has mostly been studied in Th-cells in mice. However, the TIM-1 signalling mechanisms in Th-2 cells are poorly understood. Studies in mice suggest that in contrast to TIM-3, TIM-1 acts as a positive regulator of Th-2 cell function (Encinas et al., 2005).

TIM-3 signalling in Th-1 cells is better characterised. Galectin-9 binds to TIM-3 in a glycosylation-dependent manner (Figure 1.7). Although the downstream signalling events remain poorly characterised, TIM-3 activation causes phosphorylation of the tyrosine motif Y265 in the cytosolic domain (Van de Weyer et al., 2006). Other tyrosine residues of TIM-3 appeared to be responsible for signal transduction in TCR-dependent mechanisms (Lee et al., 2011).
Figure 1.7. Schematic representation of TIM-3 ligand binding sites. Ribbon diagram of mouse TIM-3 IgV domain. The sites of the IgV domain engaged by phosphatidyl serine (PtdSer) or galectin-9 binding are indicated. The residues at the FG and CC' loops interacting with PtdSer and coordinating the metal ion (green sphere) are shown with stick drawing. The side chains of two Asn residues which can be N-glycosylated are shown as spheres and with carbons in orange, nitrogen in blue, and oxygen in red. The complex carbohydrate modifications would extend outwards from these residues. Galectin-9 can bind to some specific motif of these N-linked carbohydrates. Adapted from (Freeman et al., 2010).
TIM-3 engagement by galectin-9 induced apoptosis of Th1 cells (Zhu et al., 2005). Blockade of galectin-TIM-3 interaction induced an exacerbation of Th-1 driven immune response and increased the number and activation of macrophages in a mouse model of autoimmune disease (Monney et al., 2002). In humans, TIM-3 blockade with monoclonal antibodies revealed that human TIM-3 signalling regulates cytokine expression at the transcriptional level rather than controlling Th1 cell expansion as in mice (Hastings et al., 2009). Collectively, these data suggest an inhibitory role for TIM-3 in Th-1-driven immunity.

TIM-3 has also been reported to play a role in the induction of peripheral tolerance. Blockade of TIM-3 function by administration of soluble TIM-3 prevented the development of tolerance in Th1 cells. Furthermore, TIM-3-deficient mice are resistant to tolerance induction by administration of high dose antigen (Sabatos et al., 2003). TIM-3 has been shown to regulate both auto- and alloimmune tolerance by modulating T regulatory cell-mediated inflammatory responses (Sanchez-Fueyo et al., 2003). This TIM-3 function has been confirmed in a murine model of graft-versus-host disease (Oikawa et al., 2006). Perhaps one of the most exciting functions of TIM-3 is its role in T cell exhaustion during chronic viral infections and in tumor immunity. Indeed blockade of TIM-3 in these settings restored normal T cell function (reviewed in (Sakuishi et al., 2011)). Figure 1.8 summarises the TIM-3 functions in the immune response described to date.
Figure 1.8. Model of TIM-3 function in the immune response. (a) In acute inflammation, TIM-3 is expressed on terminally differentiated IFN-γ-producing CD4+ and CD8+ T cells. Upon recognition of its ligand, galectin-9 (Gal-9), TIM-3-expressing T cells undergo apoptosis. (b) In chronic inflammation, TIM-3 is coexpressed with programmed death-1 receptor (PD-1) on dysfunctional or exhausted CD8+ T cells. Combined targeting of the PD-1/PD-Ligand1 and TIM-3/TIM-3 ligand pathways restores CD8+ T cell effector function and ameliorates chronic disease. (c) TIM-3 on T cells interacts with Gal-9 on myeloid derived suppressor cells (MDSC) precursors to promote MDSC expansion, which in turn suppresses T cell responses. (d) TIM-3 on CD4+ T cells can also act to facilitate killing of intracellular pathogens in macrophages through interaction with Gal-9 and a mechanism involving IL-15 and caspase-1. Overall, TIM-3 on CD4+ and CD8+ T cells control multiple mechanisms to regulate negatively effector T cell responses and terminate IFN-γ-mediated inflammation while preserving or enhancing the ability of innate cells to kill intracellular pathogens. Adapted from (Sakuishi et al., 2011).
TIM-3 is also expressed in other subsets of T cells, and cells from the innate immune system including monocytes, dendritic cells, mast cells and microglia (Su et al., 2008, Rodriguez-Manzanet et al., 2009). Thus, the role of TIM-3 is not limited to the adaptive immune response. For instance, TIM-3 is involved in macrophage phagocytic (Nakayama et al., 2009) and bactericidal activity (Jayaraman et al., 2010, Zhao et al., 2009).

In addition to the immunomodulatory properties, TIMs also play an important role in apoptotic body clearance via phosphatidyserine recognition (reviewed in (Freeman et al., 2010)). TIM-4 and TIM-1 were shown to bind to phosphatidyserine and mediate phagocytosis of apoptotic cells (Miyanishi et al., 2007, Kobayashi et al., 2007). More recently, it was demonstrated that TIM-3 presence in the phagocytic cup is required for apoptotic clearance by macrophages (Nakayama et al., 2009). The expression of TIM receptors confers phagocytic properties even in “non-professional” phagocytic cells. Indeed, TIM-1 expression in endothelial cells has been reported to confer phagocytic capacity to this cell type (Ichimura et al., 2008, Su et al., 2008).
1.3. Galectin-9

Galectin-9 was identified as the first ligand for TIM-3 (Zhu et al., 2005). In line with its lectin nature, galectin-9 binds to TIM-3 in a carbohydrate dependent manner, interacting with the N-glycosylated site in the IgV domain. Galectins have been shown to regulate immune homeostasis and inflammation (reviewed in (Liu et al., 2010)). Mammalian galectins comprise a large family of S-lectin proteins characterised by their affinity for β-galactosidase sugars and the conserved specific sequence motif in the carbohydrate recognition domain (CRD) (Paroutaud et al., 1987).

Human galectins have been classified according to their structure (Figure 1.9)

a) Prototypical galectins, which contain a single CRD and may appear associated in the form of homodimers.

b) Chimeric galectins, which contain a single CRD and a long amino-terminal domain.

c) Tandem repeat galectins, which contain two CRD domains linked by a small peptide chain.

Galectin-9 belongs to the tandem-repeat class. To date, three galectin-9 isoforms have been identified that only differ in the length of the polypeptide linker region (Figure 1.10). The short-size (s) galectin-9 has a peptide linker of 14 amino acids, the medium- (m) and the long- (l) sized forms have a linker of 26 and 58 amino acids respectively (Chabot et al., 2002). This relatively long polypeptide chain makes galectin-9 very susceptible to proteolytic cleavage (Nishi et al., 2005) which inactivates the molecule (Matsushita et al., 2000). Recently, a recombinant form of galectin-9 lacking the linker chain has been shown to be functional and resistant to proteases (Nishi et al., 2005).
Figure 1.9. Different types of galectins in humans. Adapted from (Cummings and Liu, 2009). Human galectins have been classified according to their structure into prototypical, chimeric and tandem repeat. The oval domain represents the carbohydrate recognition domain (CDR). Prototypical galectins such as galectin-1 contain only one type of CDR, chimeric galectin-3 contains a single CDR attached to a long polypeptide chain whereas tandem repeat galectins such as galectin-9 consist of two different CDR connected by an interdomain polypeptide linker.
Figure 1.10. Schematic representation of galectin-9 isoforms structure. Adapted from (Chabot et al., 2002). Sequencing of galectin-9 revealed isoforms of different sizes differing only in the linker peptide region length (small (s), medium (m) and long (l)). The structure of proteolytically resistant recombinant galectin-9 (rhgalectin-9, gal9 Null) is also shown (Nishi et al., 2005).
Galectin-9 was firstly identified in embryonic mouse kidney and found to be ubiquitously expressed in mouse and rat tissue (Wada and Kanwar, 1997). Galectin-9 is also widely distributed in human tissues and expressed in several cell types (Matsumoto et al., 1998). Galectin-9 expression has been reported in epithelial tissues such as endometrium (Shimizu et al., 2008) cervix (Liang et al., 2008) and intestine (Pielage et al., 2007). Galectin-9 expression in the oral-nasopharyngeal tract has been located to fibroblasts in nasal polyps (Park et al., 2011), periodontal ligaments in the oral cavity (Kasamatsu et al., 2005) and Epstein-Barr virus related nasopharyngeal carcinoma cells (Pioche-Durieu et al., 2005). Galectin-9 is also abundantly expressed in human endothelial cells (Ishikawa et al., 2004, Imaizumi et al., 2002) and melanocytes (Wiener et al., 2007).

Galectin-9 is broadly expressed in the immune system, including bone marrow, spleen, thymus and lymph nodes. Within immune cells, galectin-9 expression has been demonstrated in myeloid lineage cells (Spitzenberger et al., 2001) including Kupffer cells (Mengshol et al., 2010), microglia (Stancic et al., 2011), astrocytes (Yoshida et al., 2001), dendritic cells (Dietz et al., 2000) and macrophages (Jayaraman et al., 2010). Galectin-9 has also been shown to be constitutively expressed in mast cells (Wiener et al., 2007) and to a lesser extent in the Jurkat T-lymphocyte cell line (Chabot et al., 2002). Regulatory T cells also express galectin-9 (Elahi et al., 2011). Although galectin-9 was not detected in HL-60 cells, it was found to be expressed in neutrophils in the lung of ovalbumin challenged mice (Sziksz et al., 2010).

The most interesting feature of galectin-9 expression is the fact that although most immune cells exhibit constitutive expression, the extent of expression depends on cell activation and cell differentiation stage (Abedin et al., 2003). For instance, galectin-9 mRNA expression has been reported to be induced in peripheral blood monocytic cells after allergen stimulation (Matsumoto et al., 1998). Galectin-9 expression is also raised in eosinophils from hypereosinophilic patients (Saltia et al., 2002).

It has been proposed that galectin-9 may have a complex role in inflammation homeostasis by exerting pro- and anti-inflammatory events depending on concentration...
at the site of inflammation or cell type (Toscano et al., 2007). Galectin-9 binding to TIM-3 reduced interferon gamma production by inducing apoptosis of Th-1 cells (Zhu et al., 2005). Conversely, galectin-9 treatment induced TNFα production by mast cells (Anderson et al., 2007). Thus, galectin-9/TIM-3 signalling can initiate or terminate the inflammatory response by positively regulating maturation of innate cells, antigen presentation and pathogen clearance, and limiting an excessive immune response, especially T-cell mediated at a later stage (Zhao et al., 2010).

Disruption of galectin-9/TIM-3 interactions is commonly used to examine the mechanisms behind this signalling axis in several models of disease. Figure 1.11 summarises the different approaches adopted to block TIM-3 engagement by TIM-3.

In addition to TIM-3, galectin-9 has also been reported to bind to other glycoproteins including CD44, a surface receptor expressed on epithelial cells involved in cell-matrix adhesion and interaction (Katoh et al., 2007, Nobumoto et al., 2008), Glucose transporter 2 (Ohtsubo et al., 2005) and Epstein-Barr virus latent membrane protein-1 (Pioche-Durieu et al., 2005). Of interest, galectin-9 has also been reported to function as transmembrane urate transporter (Lipkowitz et al., 2004).
Figure 1.11. Schematic representations of TIM-3/galectin-9 interaction disruption strategies. A) Galectin-9 binds to TIM-3 IgV domain via carbohydrate interactions (Zhu et al., 2005). B) Blockade of TIM-3 by anti-TIM-3 antibody. C) Soluble human recombinant TIM-3-Fc chimeric protein (rhTIM-3-Fc) acts as a galectin-9 scavenger receptor preventing TIM-3-galectin-9 interaction. D) Galectin-9 preferentially binds to excess lactose in solution due to galactosidase affinity.
1.4. TIM in airway disease

The TIM gene family was located to a section of chromosome 11 in mice syntenic to human chromosome 5q23-35 (McIntire et al., 2001). This region was identified as a novel gene locus for human atopic disease (i.e. asthma, allergy and eczema) in a study comparing congenic mouse strains with different susceptibility to asthma. Despite the initial discovery linking TIM and the airway hyperreactivity regulatory locus (Tapr) in mice (McIntire et al., 2001), there is a paucity of studies on the function of TIMs in lung inflammatory disease. Most of these studies were carried out in mice models of asthma (Table 1.4). More recently, TIMs have been implicated in sarcoidosis, pneumonia, tuberculosis and CF (Table 1.4).
### Table 1.4. TIM molecules in airway disease

<table>
<thead>
<tr>
<th>Disease</th>
<th>TIM protein</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asthma</td>
<td>TIM-1(n)</td>
<td>Blocking TIM-1 antibody reduced Th-2 driven allergic response</td>
<td>(Encinas et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>TIM-1(n)</td>
<td>Antagonistic TIM-1 antibody increased Th-2 driven allergic response</td>
<td>(Umetsu et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>TIM-1(n)</td>
<td>Epitope-dependent effect of TIM-1 antibodies on Th-2 response causing</td>
<td>(Sizing et al., 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>exacerbation or inhibition of airway inflammation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TIM-3 (m)</td>
<td>Blocking antibody administration reduced Th-2 driven allergic response</td>
<td>(Kearley et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>TIM-1 (m)</td>
<td>Expression increased in T cells</td>
<td>(Xu et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>TIM-3 (m)</td>
<td>Expression increased in T cells</td>
<td>(Hu et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>TIM-3 (m,h)</td>
<td>Defective apoptotic clearance induces airway hyperresponsiveness</td>
<td>(DeKryyf et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>TIM-1 (h)</td>
<td>Antagonistic antibody reduced Th-2 response in a humanised asthma mouse</td>
<td>(Sonar et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>TIM-2 (m)</td>
<td>TIM-2 deficient mouse demonstrate exacerbated Th-2 response in asthma</td>
<td>(Rennert et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>TIM-1/3 (h)</td>
<td>TIM-3 and TIM-1 are not essential for airway hyperresponsiveness</td>
<td>(Barlow et al., 2011)</td>
</tr>
<tr>
<td>Sarcoïdosis</td>
<td>TIM-1 (h)</td>
<td>Reduced expression in T cells of non-Löfgren’s patients is in agreement</td>
<td>(Idali et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>TIM-3 (h)</td>
<td>Reduced expression in T cell in BAL and blood correlated negatively with</td>
<td>(Idali et al., 2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD4/CD8 ratio</td>
<td></td>
</tr>
<tr>
<td>Tuberculosis</td>
<td>TIM-3 (m,h)</td>
<td>TIM-3 interacts with galectin-9 on macrophages to restrict intracellular</td>
<td>(Jayaraman et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>Cystic</td>
<td>Constitutive upregulation in bronchial epithelial cells induced IL-8</td>
<td>(Vega-Carrascal et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>fibrosis</td>
<td>production</td>
<td></td>
</tr>
<tr>
<td>Pneumonia</td>
<td>TIM-3 (m)</td>
<td>Galectin-9 intraperitoneal administration reduced IL-17 production and</td>
<td>(Wang et al., 2011a)</td>
</tr>
<tr>
<td>Pulmonary</td>
<td>TIM-1 (m)</td>
<td>Costimulation on NKT cells enhances the production of IL-4 and inhibits</td>
<td>(Kim et al., 2010a)</td>
</tr>
<tr>
<td>fibrosis</td>
<td></td>
<td>production of IFN-γ</td>
<td></td>
</tr>
</tbody>
</table>

Definition of abbreviations: NKT = natural killer T cell; (m) = mouse; (h) = human.
1.4.1 TIM-1 in airway disease

Although the role of TIM-1 and TIM-3 in the development of lung allergy has recently been questioned in a study using knock-out mice (Barlow et al., 2011), TIM-1 expression has been reported to be upregulated in the lung of asthmatic mice (Xu et al., 2008). Indeed, blockade of mouse TIM-1 decreased the Th-2-type immune response and airway inflammation in a murine model of asthma in different studies (Encinas et al., 2005, Umetsu et al., 2005, Sizing et al., 2007). Blockade of TIM-1 with anti-TIM-1 antibody during initial challenge with antigen prevented airway hyperresponsiveness in a mouse model (Umetsu et al., 2005). Another group reported that administration of a different anti-TIM-1 antibody between sensitization and allergen exposure also reduced airway hyperresponsiveness (Encinas et al., 2005). Interestingly, TIM-1 ligation with monoclonal antibodies induced either positive or negative effects in a mouse allergy model, depending on the antibody. Antibodies recognising the stalk or gV domain attenuated inflammation indicating that ligation of this TIM-1 region promotes inhibitory functions. In contrast, antibodies directed against the mucin domain activated the inflammatory response (Sizing et al., 2007). Therefore, therapeutic use of anti-TIM-1 antibodies in asthma requires special attention to the TIM-1 epitope recognised by the antibody. More recently, antagonism of human TIM-1 in a humanised murine model of experimental asthma has been shown to have positive therapeutic effects (Sonar et al., 2010). In this study, blockade of TIM-1 with A6G2, an antibody against the IgV domain, ameliorated inflammation and airway hyperresponsiveness in SCID mice adoptively transferred with peripheral blood monocyctic cells from asthmatic patients. The effects of TIM-1 inhibition were exerted via suppression of Th2 cell proliferation and cytokine production.

Despite the increasing body of work in mice models of lung disease, there are virtually no studies in humans supporting the role of TIM-1 in airway inflammation (Table 1.4). Recently, T cells obtained from peripheral blood and BAL from sarcoidosis patients exhibited lower TIM-1 expression in non-Löfgren's patients (Idaii et al., 2009). This subset of patients has a marked Th-1 inflammatory response and often present with a less favourable prognosis than Löfgren's patients. Since an imbalance towards a Th-1
phenotype is believed to be the hallmark of airway inflammatory response in sarcoidosis, the study by Idali and colleagues suggested that downregulation of TIM-1 in Th cells was linked to a higher Th-1 response (Idali et al., 2009).

TIM-1 regulatory properties in airway disease are not limited to T cells. TIM-1 engagement in natural killer cells exacerbated lung injury in a bleomycin model of pulmonary fibrosis by suppressing interferon-gamma production (Kim et al., 2010a).

1.4.2 TIM-2 in airway disease

The TIM-2 knockout mouse displays an exacerbated Th2-driven response (Rennert et al., 2006). Semaphorin4-A has been recognised as a TIM-2 ligand (Kumanogoh et al., 2002). Conversely, semaphorin4-A deficient mice develop exaggerated Th-2 phenotypes (reviewed in (Takamatsu et al., 2010)) supporting TIM-2 as an inhibitor of Th-2 responses. TIM-2 was also identified as a specific heavy chain ferritin (H-ferritin) receptor leading to endocytosis of extracellular H-ferritin in liver (Chen et al., 2005) and brain (Todorich et al., 2008). H-ferritin has been reported to display immunological properties, mainly as a regulator of proliferation and differentiation of immune cells (Broxmeyer, 1992, Morikawa et al., 1995). Interestingly, elevated levels of H-ferritin were found in CF BAL fluid compared to other inflammatory respiratory conditions (Stites et al., 1999). A link between high levels of ferritin and altered TIM expression has been suggested (Recalcati et al., 2008) but not formally demonstrated. Nevertheless, none of the human TIM receptors appear to bind H-ferritin (Freeman et al., 2010). Since murine TIM-2 does not have a human ortholog, whether another human TIM receptor can adopt the Th-2 inhibitory function described in mice (Knickelbein et al., 2006, Chakravarti et al., 2005) remains to be elucidated.
1.4.3 TIM-3 in airway disease

TIM-3 has also been implicated in the development of asthma. Blockade of TIM-3 with a specific antibody reduced airway hyperreactivity and induced a switch from Th2 to Th1 type response (Kearley et al., 2007). Expression of TIM-3 in CD4+ cells was increased after ovalbumin challenge in a mouse model of asthma (Hu et al., 2009), further supporting TIM-3 as a negative regulator of Th-1 mediated immunity. In line with the described TIM-3 function, low levels of TIM-3 expression in CD4+ cells from peripheral blood and BAL were correlated with an increased Th1-type immune response in sarcoidosis (Idali et al., 2009), a prototypical Th1 inflammatory disease.

Additionally, the role of TIM-3 as a phosphatidylserine receptor has been suggested to be involved in the development of airway hyperresponsiveness as efficient clearance of apoptotic cells is crucial in preventing development of atopy (DeKruyff et al., 2010).

TIM-3 has also been suggested to be involved in the regulation of the inflammatory response to airway infection. In a mouse model of Klebsiella pneumoniae-induced pneumonia, administration of galectin-9 induced apoptosis of Th17 cells and reduction of IL-17 generation which in this model proved crucial for bacterial clearance in the lung. Decreased IL-17 production led to impaired neutrophil recruitment into the airways with subsequent reduced bacterial clearance and higher mortality (Wang et al., 2011a). These results suggested an important role for TIM-3/galectin-9 in termination of Th-17-mediated immune responses. A novel role for TIM-3 in airway infection was recently revealed (Jayaraman et al., 2010). In this study, TIM-3 was shown to act as a ligand and to stimulate galectin-9 expressed on the surface of macrophages. Through unidentified mechanisms, galectin-9 engagement on infected macrophages triggered IL-1β production and subsequent Mycobacterium tuberculosis intracellular clearance. The study by Jayaraman and colleagues suggested that TIM-3 expressed on Th-1 cells can modulate macrophage-mediated bacterial killing. This constitutes the first report on the role of TIM-3 as a ligand, in addition to the prototypical role as a Th1 receptor capable of modulating
the inflammatory response. These studies open a new area of TIM-3 research on the role of this molecule in bacterial infection.

1.5. Galectin-9 in airway disease

Galectin-9 has been shown to be involved in airway disease via TIM-3 dependent and independent mechanisms (Table 1.5). Galectin-9 expression was found to be elevated in lung tissue in animal models of asthma (Sziksz et al., 2010, Yamamoto et al., 2007). This overexpression of galectin-9 was found to be correlated with an increase in Th-2 cytokines and increased cell counts in the lungs, particularly eosinophils. A similar correlation between elevated galectin-9 and high eosinophil counts was reported in patients with acute and chronic eosinophilic pneumonia (Katoh et al., 2010). Interestingly, administration of recombinant galectin-9 attenuated lung inflammation in a mouse model of asthma (Niki et al., 2009). In this study, galectin-9 inhibited mast cell degranulation by disrupting IgE/antigen complex formation. Recombinant galectin-9 administration also ameliorated lung inflammation in a mouse model of asthma due to inhibition of CD44/ hyaluronic acid interaction which is required for recruitment of leukocytes into the airways (Katoh et al., 2007). This study also showed that galectin-9 can induce apoptosis of eosinophils thereby reducing disease severity in this asthma model.

Galectin-9 has been shown to affect antimicrobial immunity in two distinct manners. Firstly, galectin-9 stimulates immune responses via recruitment of immune cells. Secondly, galectin-9 can limit the adaptive immune response, in particular the T-cell response while promoting the expansion of regulatory cells (Kojima et al., 2011). Of interest, a recent publication reported a novel role for galectin-9 in antimicrobial immunity (Jayaraman et al., 2010). This latter study described for the first time a reversal of TIM-3/galectin-9 signalling pathway whereby galectin-9 acted as a receptor in infected macrophages and was stimulated via interactions with TIM-3 expressed on adjacent Th1 cells.
<table>
<thead>
<tr>
<th>Disease</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asthma (g)</td>
<td>Galectin-9 levels increased in an allergic AHR model and correlated with eosinophil counts</td>
<td>(Yamamoto et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>(m) Galectin-9 levels increased in an allergic AHR model and correlated with eosinophil counts</td>
<td>(Sziksza et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>(g) Galectin-9 administration attenuated allergic inflammation by binding IgE and reducing mast cell degranulation</td>
<td>(Niki et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>(m) Galectin-9 administration attenuated AHR and allergic inflammation binding to CD44 which reduced T cell migration</td>
<td>(Katoh et al., 2007)</td>
</tr>
<tr>
<td>Lung cancer (m)</td>
<td>Galectin-9 administration reduced tumor cell migration</td>
<td>(Nobumoto et al., 2008)</td>
</tr>
<tr>
<td>Acute lung injury (m)</td>
<td>Galectin-9 administration ameliorated acute lung injury expanding regulatory macrophages</td>
<td>(Kojima et al., 2011)</td>
</tr>
<tr>
<td>Tuberculosis (m/h)</td>
<td>TIM-3 interacted with galectin-9 on macrophages to restrict intracellular bacterial growth</td>
<td>(Jayaraman et al., 2010)</td>
</tr>
<tr>
<td>Cystic fibrosis (h)</td>
<td>Constitutive upregulation in bronchial epithelial cells induced IL-8 production</td>
<td>(Vega-Carrascal et al., 2011)</td>
</tr>
<tr>
<td>Pneumonia (m)</td>
<td>Galectin-9 intraperitoneal administration reduced IL-17 production and reduced bacterial clearance</td>
<td>(Weng et al., 2011a)</td>
</tr>
<tr>
<td></td>
<td>(h) Galectin-9 levels correlated with eosinophil counts in acute and chronic eosinophilic pneumonia</td>
<td>(Kato et al., 2010)</td>
</tr>
<tr>
<td>Sarcoidosis (h)</td>
<td>Galectin-9 levels not altered in Th cells</td>
<td>(Idati et al., 2009)</td>
</tr>
</tbody>
</table>

Definition of abbreviations: AHR = Airway hyperresponsiveness; (g) = guinea pig; (m) = mouse; (h) = human.
1.5 Final remarks

The data described so far emphasises a role for TIMs as modulators of the immune response. TIMs emerge as ideal candidates for therapeutic intervention in the CF lung at several levels. Firstly, TIMs may effect the inflammatory response in the airways due to their direct role in promoting generation of pro- and anti-inflammatory mediators. Secondly, TIMs may act as regulators of cellular homeostasis in the lung via induction of selective apoptosis of immune cells and preferential expansion of regulatory cells via galectin-9 interactions. Additionally, TIMs may also help fight lung infections as they have been shown to be involved in viral recognition and phagocytic cell function. Given the neutrophil-driven inflammatory component of the CF lung pathology, TIM-3 was expected to play a significant role in CF through interactions with its ligand galectin-9. Whether TIMs and in particular TIM-3 actually are involved in CF lung disease constitutes the main subject of this study.

1.6 Aims and goals

The aim of this study was to determine whether TIM signalling mechanisms play a role in CF lung disease, in particular if altered expression or function of TIM receptors and TIM related proteins are involved in CF lung inflammation. In order to fulfil these goals, completion of the following objectives was achieved:

1. To characterise the expression of TIM proteins in CF. Specifically in airway epithelial cells and neutrophils.
2. To establish whether TIM-3 signalling modulates the inflammatory response in CF bronchial epithelial cells in vitro.
3. To establish whether TIM-3 is involved in neutrophil function ex vivo, focusing on neutrophil-mediated bacterial killing properties.
CHAPTER 2: MATERIALS AND METHODS

2.1 Material suppliers

2.1.1 Antibodies and recombinant proteins

A list of primary antibodies employed in this study is found in Table 2.1.

Table 2.1. Primary antibodies

<table>
<thead>
<tr>
<th>Description</th>
<th>Catalogue number</th>
<th>Supplier</th>
<th>Source</th>
<th>Application</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-human TIM-1</td>
<td>MAB1750</td>
<td>R&amp;D Systems, Abington, UK</td>
<td>Mouse, Monoclonal</td>
<td>W</td>
<td>1:500</td>
</tr>
<tr>
<td>anti-human TIM-3-PE labelled</td>
<td>FAB2365P</td>
<td>R&amp;D Systems, Abington, UK</td>
<td>Rat, Monoclonal</td>
<td>F</td>
<td>10 µl/10^6 cells</td>
</tr>
<tr>
<td>anti-human MMP-9</td>
<td>AB911</td>
<td>R&amp;D Systems, Abington, UK</td>
<td>Goat, Polyclonal</td>
<td>W</td>
<td>1:1000</td>
</tr>
<tr>
<td>anti-human TIM-3</td>
<td>AF2365</td>
<td>R&amp;D Systems, Abington, UK</td>
<td>Goat, Polyclonal</td>
<td>W</td>
<td>1:1000</td>
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<tr>
<td>anti-human MPO</td>
<td>AF3174</td>
<td>R&amp;D Systems, Abington, UK</td>
<td>Goat, Polyclonal</td>
<td>W</td>
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<tr>
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<td>MAB1501</td>
<td>Millipore MA, USA</td>
<td>Mouse</td>
<td>W</td>
<td>1:1000</td>
</tr>
<tr>
<td>anti-phosphotyrosine, clone 4G10</td>
<td>05-321X</td>
<td>Millipore MA, USA</td>
<td>Mouse</td>
<td>W</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-human LL-37</td>
<td>PA-LL37-100</td>
<td>Innovagen AB Lund, Sweden</td>
<td>Rabbit, Polyclonal</td>
<td>W</td>
<td>1:1000</td>
</tr>
<tr>
<td>anti-human phospho (Thr 180/Tyr 182)-p38</td>
<td>9211S</td>
<td>Cell Signaling Technology Danvers, MA, USA</td>
<td>Rabbit, Polyclonal</td>
<td>W</td>
<td>1:1000</td>
</tr>
<tr>
<td>anti-human TIM-3</td>
<td>JM-308-100</td>
<td>MBL International Corporation Woburn, MA, USA</td>
<td>Rabbit, Polyclonal</td>
<td>W</td>
<td>1:1000</td>
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<tr>
<td>anti-human phospho (Thr 202/Tyr 204)-ERK1/ERK2</td>
<td>sc16982-R</td>
<td>Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA</td>
<td>Rabbit, Polyclonal</td>
<td>W</td>
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<tr>
<td>Anti-human ERK 2</td>
<td>sc154</td>
<td>Biotechnology Inc. Santa Cruz, CA, USA</td>
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<tr>
<td>anti p38βδ</td>
<td>sc7972</td>
<td>Biotechnology Inc. Santa Cruz, CA, USA</td>
<td>Mouse, Monoclonal</td>
<td>W</td>
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<tr>
<td>anti-human galectin-9-PE labelled</td>
<td>34690</td>
<td>Biolegend. San Diego, CA, USA</td>
<td>Mouse, Monoclonal</td>
<td>F</td>
<td>5 µl/10^6 cells</td>
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<tr>
<td>anti-human galectin-9 (clone FGB-M)</td>
<td>12-5871-81</td>
<td>Gaipharma, Kagawa, Japan</td>
<td>Mouse, Monoclonal</td>
<td>E</td>
<td>10 µg/ml</td>
</tr>
<tr>
<td>Anti-human TIM-3 derived from a hybridoma (clone 1G5)</td>
<td></td>
<td>Prof. Kuchroo, Brigham and Women's Hospital, Boston, MA, USA</td>
<td>Mouse, Monoclonal</td>
<td>B</td>
<td>10 µg/ml</td>
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<tr>
<td>(Hastings et al., 2009) Anti-mouse-TIM-3 PE labelled</td>
<td></td>
<td>e Bioscience, Ltd. Hefield, UK</td>
<td>Rat, monoclonal</td>
<td>F</td>
<td>5 µl/10^6 cells</td>
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</tbody>
</table>

Definition of abbreviations: PE= phycoerythrin; Tyr= tyrosine; Thr= threonine; Ser= serine; W= western blotting; F= Flow cytometry; I= Immunocytochemistry; IP= immunoprecipitation; B= blocking; E= enzyme linked immunosorbent assay
Secondary antibodies and isotype controls are enumerated in Table 2.2

<table>
<thead>
<tr>
<th>Description</th>
<th>Catalogue number</th>
<th>Supplier</th>
<th>Source</th>
<th>Application</th>
<th>Dilution</th>
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<tbody>
<tr>
<td>HRP-conjugated anti-goat</td>
<td>sc-2020</td>
<td>Santa Cruz Biotechnology Inc.</td>
<td>Donkey</td>
<td>E</td>
<td>1:1000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Santa Cruz, CA, USA</td>
<td></td>
<td></td>
<td>1:1000</td>
</tr>
<tr>
<td>FITC-conjugated anti-mouse</td>
<td>sc-2366</td>
<td>Santa Cruz Biotechnology Inc.</td>
<td>Bovine</td>
<td>I</td>
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<td>Santa Cruz, CA, USA</td>
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<td>FITC-conjugated anti-goat</td>
<td>sc-2024</td>
<td>Santa Cruz Biotechnology Inc.</td>
<td>Donkey</td>
<td>I</td>
<td>1:40</td>
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<td></td>
<td></td>
<td>Santa Cruz, CA, USA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HRP-conjugated anti-rabbit</td>
<td>1858415</td>
<td>Pierce Biotechnology II, USA</td>
<td>Rabbit</td>
<td>W</td>
<td>1:1000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Santa Cruz, CA, USA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HRP-conjugated anti-mouse</td>
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<td>Pierce Biotechnology II, USA</td>
<td>Mouse</td>
<td>W</td>
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<td>anti-rabbit HRP-conjugated</td>
<td>#7074</td>
<td>Cell Signaling Technology Danvers, MA, USA</td>
<td>Goat</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>PE labelled Rat IgG2A isotype control</td>
<td>IC006P</td>
<td>R&amp;D Systems, Abingdon, UK</td>
<td>Rat, IgG2A</td>
<td>F</td>
<td>10 µl/ 10⁶ cells</td>
</tr>
<tr>
<td>PE labelled mouse IgG1 isotype control</td>
<td>IC002P</td>
<td>R&amp;D Systems, Abingdon, UK</td>
<td>Mouse, IgG1</td>
<td>F</td>
<td>5 µl/ 10⁶ cells</td>
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<tr>
<td>Normal goat IgG</td>
<td>sc2028</td>
<td>Santa Cruz Biotechnology Inc.</td>
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<td>B</td>
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<tr>
<td></td>
<td></td>
<td>Santa Cruz, CA, USA</td>
<td></td>
<td></td>
<td>6 µg</td>
</tr>
<tr>
<td>normal mouse IgG</td>
<td>sc2025</td>
<td>Santa Cruz Biotechnology Inc.</td>
<td>Mouse</td>
<td>B</td>
<td>10 µg/ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Santa Cruz, CA, USA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PE labelled Rat IgG1</td>
<td>12-4301-81</td>
<td>eBioscience, Ltd Hatfield, UK</td>
<td>Rat</td>
<td>F</td>
<td>5 µl/ 10⁶ cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Definition of abbreviations: PE= phycoerythrin; FITC= fluorescein isothiocyanate; HRP= horse radish peroxidise; W= western blotting; F= Flow cytometry; I= Immunocytochemistry; IP= immunoprecipitation; B= blocking; E= enzyme linked immunosorbent assay
Human recombinant TIM-1 (rhTIM-1), TIM-3-Fc fusion protein (rhTIM-3-Fc) and human IgG1-Fc (110-HG) were purchased from R&D Systems (MN, USA). Proteolytically stable human galectin-9 was a kind gift from GalPharma (Kagawa, Japan). Expression and purification of stable human galectin-9 was performed as previously described (Seki et al., 2007, Arikawa et al., 2009). Briefly, galectins were expressed using the pET expression system (Novagen, Madison, WI) in *Escherichia coli* BL21 (DE3) and purified with a lactose–agarose column (Seikagaku Kogyo, Tokyo, Japan) and dialyzed against phosphate buffered saline (PBS). Endotoxin was eliminated with Cellufine ETclean-L, a poly-ε-lysine–conjugated resin (Chisso, Tokyo, Japan). Galectin preparations used in the present study were >95% pure as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), with <0.3 endotoxin units/ml (<0.03 ng/ml) detected by *Limulus* turbidimetric kinetic assays using a Toxnometer ET-2000 instrument (Wako, Osaka, Japan). Protein concentration was determined using a bicinchoninic acid (BCA) assay reagent (Pierce Biotechnology) with bovine serum albumin (BSA) as a standard.

### 2.1.2 Reagents

Unless stated otherwise, cell culture reagents were obtained from Gibco BRL (Karlsruhe, Germany). All other chemical reagents were purchased from Sigma-Aldrich (Dublin, Ireland) and were of the highest purity available.

### 2.2 Cell culture

Immortalised human bronchial epithelial cells 16HBE14o⁻ (HBE) (Cozens et al., 1994) and immortalised bronchial epithelial cells from a (ΔF508/ΔF508) CF patient CFBE41o⁻ (CFBE) (Bruscia et al., 2002), were kindly donated by Dr. Gruenert (University of Vermont, VT, USA).
2.2.1 Maintenance of cell cultures

Cells were grown in flasks coated with fibronectin (1 mg/ml), collagen (1 mg/ml) (BD Biosciences, Bedford, MA, USA), and BSA (1 mg/ml) in complete media (minimum essential medium [MEM] containing 10% (v/v) heat inactivated foetal calf serum (FCS) and 1% (v/v) penicillin/streptomycin) at 37°C in a humidified incubator with 5% CO₂. The media was changed every 2 to 3 days. All experiments were conducted within 10 passages. Cells were subcultured by trypsinisation. Media in flasks was removed and cells were briefly rinsed with 10 ml of pre-warmed Dulbecco's PBS, calcium and magnesium free (DPBS). After carefully removing the DPBS, cells were incubated at 37°C in 4 ml of pre-warmed PET cell dissociation solution (1% (w/v) polyvinylpyrrolidone, 0.02% (w/v) ethylene glycol tetraacetic acid, 8% (v/v) trypsin-EDTA solution [0.5 g trypsin and 0.02% EDTA] in Hank's buffered salt solution). After 5 min, PET solution was replaced by fresh PET and cell detachment was continued for another 5 min at 37°C. Trypsinisation was stopped by addition of 10 ml of complete media. Medium with suspended cells was transferred to a 15 ml conical tube and cells were pelleted by centrifugation (250 × g for 5 min). The supernatant was discarded and cells were resuspended in fresh complete medium and counted using a haemocytometer before being transferred to flasks or cell culture plates at the required cell density.

2.2.2. Cryogenic preservation of cell lines

For long-term storage, cells were trypsinised as described and resuspended in ice-cold freezing solution (90% (v/v) FCS, 10% (v/v) dimethyl sulfoxide (DMSO)) at 1 × 10⁶ cells/ml and transferred to cryovials. Cryovials were immediately placed at -80°C overnight and then stored in liquid nitrogen. Recovery of frozen cells was carried out by quickly thawing cells in cryovials in a 37°C water bath before transferring cells to 10 ml of pre-warmed complete media. Cells were pelleted by centrifugation at 250 × g for 5 min, and resuspended in 10 ml of fresh culture media before being transferred to a 75-cm² tissue culture flask.
2.2.3 Polarized cell culture.

CFBE cells were grown as polarized monolayers at a liquid-liquid interface as previously described (Chotirmall et al., 2010). Briefly, cells (7 x 10^4) were seeded onto 24-well 1.0 μm pore polyethylene hanging cell culture inserts (Millipore) and maintained in full media. Transepithelial electrical resistance (TEER) is commonly used to monitor integrity of epithelial connections (Godfrey, 1997). TEER measurements were taken every 48h using the EVOM epithelial voltohmmeater (World Precision Instruments, Stevenage, UK) according to the manufacturer's instructions. Cell monolayers were deemed confluent and used in experiments when TEER>1000Ω.cm² (Nilsson et al., 2007), this value was typically achieved after 14 days of culture. Cells were serum starved overnight before treatment with galectin-9 (50 nM) in low serum media (MEM supplemented with 1% (w/v) FCS) for 24 h. IL-8 levels in both apical (0.2 ml) and basolateral (1.2 ml) supernatants were measured by IL-8 enzyme-linked Immunosorbent Assay (ELISA).

2.3 Tissue sampling

2.3.1 Bronchial brushing sample collection

All patients (controls and CF) were undergoing diagnostic and/or therapeutic fibre-optic flexible bronchoscopy for clinical reasons. Full informed consent was obtained pre-procedure according to a protocol approved by Beaumont Hospital Ethics Committee. All procedures were carried out by respiratory specialist registrars at Beaumont Hospital. Prior to withdrawal of the bronchoscope, an area 2cm distal to the carina (medially located) in either the right or left main bronchus was selected and washed twice with 10 ml sterile 0.9% (w/v) NaCl. Subsequently, a sterile 10 mm x 1.2 mm bronchial brush (Olympus Medical Systems Corp, Japan) was inserted through the appropriate port on the bronchoscope and the chosen area sampled with a brush by gently scraping the selected area. The brush was withdrawn and immediately placed into complete media. Brushes were gently agitated to dislodge cells into the media which was centrifuged at
200 × g for 5 min and cell pellets re-suspended in 0.5 ml of TRI reagent for RNA extraction and quantitative real time polymerase chain reaction (qRT-PCR).

Ten individuals were recruited for bronchial brushing studies. Five patients (four males and one female) had CF confirmed by sweat testing or genotyping, and seven were non-CF control subjects (four males and three females). The patients characteristics are summarized as follows: CF patients (n=5; age 21.6 ± 4.6; % Forced expiratory volume in the first second (FEV1), 50.6 ± 32.1 (mean ± SD)) and the control, non-CF patients (n=7; age 50.14 ± 10.5; (mean ± SD).

2.3.2 Bronchoalveolar lavage (BAL) fluid sample collection

Bronchoscopy was performed by specialist respiratory registrars via a laryngeal mask airway and the bronchoscope was directed to the lingula and right middle lobe. BAL was performed by instilling 1 ml/kg of sterile normal saline per lobe. The characteristics of the study population are summarized in Table 2.3. All BAL samples were centrifuged at 1,000 × g for 10 min at 4°C, and cell-free supernatants were aliquoted and stored at -80 °C for subsequent analysis.

BAL fluid from adult patients with CF (n = 14), non CF-Bronchiectasis (n = 10), COPD (n=5), or healthy controls (n=5) was collected from individuals undergoing bronchoscopy for clinical reasons. Full informed consent was obtained pre-procedure according to a protocol approved by Beaumont Hospital Ethics Committee. All procedures were carried out by respiratory specialist registrars at Beaumont Hospital. BAL from CF children (n=9) was obtained from patients undergoing bronchoscopy for clinical reasons. Full informed parental consent was obtained for all procedures and ethical approval for the use of these samples was obtained from the Ethics committee of Our Lady's Children's Hospital, Crumlin.
Table 2.3. Characteristics of individuals included in BAL studies

<table>
<thead>
<tr>
<th></th>
<th>CF (adults)</th>
<th>Non-CF Bronchiectasis</th>
<th>COPD</th>
<th>control</th>
<th>CF (children)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>14</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>Age, years (± SD)</td>
<td>22.0 ± 4.5</td>
<td>52.1 ± 17.0</td>
<td>66.2 ± 7.2</td>
<td>49.8 ± 12</td>
<td>4.0 ± 2.5</td>
</tr>
<tr>
<td>Sex, % male/female</td>
<td>57/43</td>
<td>70/30</td>
<td>60/40</td>
<td>60/40</td>
<td>-</td>
</tr>
<tr>
<td>FEV$_1$, % predicted (± SD)</td>
<td>44.6 ± 21.5</td>
<td>72.7 ± 17.0</td>
<td>53.8 ± 17.5</td>
<td>&gt;80</td>
<td>-</td>
</tr>
<tr>
<td>NE activity (mU/mL BAL)</td>
<td>780 ± 291</td>
<td>64 ± 162</td>
<td>78 ± 168</td>
<td>6 ± 8</td>
<td>104 ± 194</td>
</tr>
</tbody>
</table>

Definition of abbreviations: CF = cystic fibrosis; COPD = chronic obstructive pulmonary disease; FEV$_1$ = forced expiratory volume in the first second; NE = neutrophil elastase, activity expressed in milliUnits (mU) per mL of bronchoalveolar lavage (BAL).
2.3.3. Peripheral blood neutrophil isolation

Human neutrophils were isolated from heparinized (10 U/ml; Sarstedt, Numbrecht, Germany) venous blood. Blood was immediately mixed in 50 ml Falcon tubes with 10 ml of DPBS and 10% (v/v) dextran solution (10% (w/v) dextran molecular weight 500000, D1037, Sigma) in DPBS). The mixture was gently mixed by inverting the tube three times and left to stand for 15 min at RT to allow sedimentation of erythrocytes. The supernatant containing neutrophils was transferred to a fresh 50 ml Falcon tube with a plastic Pasteur pipette and underlayered with 5 ml of Lymphoprep (Axis-Shield PoC As, Oslo, Norway). Neutrophils were pelleted by differential density gradient centrifugation at 836 × g for 10 min at RT. The supernatant was discarded and the pellet was subjected to hypotonic lysis of erythrocytes after addition of 20 ml of water for 20 sec followed by addition of an equal volume of 2 × saline solution (1.8 % (w/v) sodium chloride in ultrapure water). The resulting neutrophils were pelleted by centrifugation at 470 × g for 5 min at RT and resuspended in DPBS solution containing 5 mM glucose (DPBSG) unless specified otherwise. Cell viability was systematically monitored before each treatment by trypan blue exclusion and found to be > 98%.

2.3.4. Isolation of mouse peritoneal exudate neutrophils

Female BALB/c mice (Harlan, UK) were housed under conventional conditions in a nonbarrier facility. Mice of between 8 and 10 weeks of age were used in all experiments. Experiments were carried out according to regulations under the Home Office (UK) Animals (Scientific Procedures) Act 1986. Peritoneal elicited neutrophils were obtained as previously described (Reeves et al., 2002) with minor modifications. Briefly, mice were injected intraperitoneally with 2 ml of sterile thioglycollate broth 3% (w/v). After 6 h, mice were euthanised by CO₂ asphyxiation. Peritoneal exudate cells were harvested in 10 ml of ice-cold enzyme-free, PBS based, Cell Dissociation Buffer (Gibco). Cells were washed by centrifugation at 470 × g for 5 min at 4°C (×2) and resuspended in DPBSG.
2.4 RNA isolation and reverse transcriptase polymerase chain reaction

2.4.1 RNA Isolation

Total RNA was extracted from HBE and CFBH (1×10⁵) cells or neutrophils (1×10⁶) using TRI reagent following the manufacturer's instructions. This method permits the simultaneous isolation of DNA, protein and total RNA based on a single-step liquid-phase separation. In brief, TRI reagent (0.5-1ml) was added to cell pellets and stored at -80°C in Eppendorf tubes. Samples were allowed to stand at RT for 5 min and 200 µl of chloroform was added to each tube. Samples were vigorously shaken with vortexing for 15 sec and then allowed to stand for 2 min at RT. The resulting mixture was centrifuged at 12000 g × 15 min at 4°C. This step separated the samples into three phases: the bottom red organic phase contained protein, the white thin interphase layer contained DNA and the upper colourless aqueous phase contained RNA. The aqueous phase was carefully transferred to a fresh tube and mixed with 0.5 ml of isopropanol. Samples were allowed to stand for 5 min at RT. Subsequently, the RNA was pelleted by centrifugation at 12000 × g for 10 min at 4°C. The supernatant was discarded and the pellet was washed with 1 ml 75% (v/v) ethanol. Samples were mixed by vortexing and allowed to stand for 5 min at RT. Total RNA was pelleted by centrifugation at 12000 × g for 5 min at 4°C. The supernatant was discarded and the RNA pellet was air-dried for 5 min at RT. The pellet was resuspended in 30 µl of RNAase free water (ultrapure water treated with 0.1% (w/v) diethyl pyrocarbonate and autoclaved for 15 min to neutralise the RNAase inhibitor and avoid carboxymethylation of purine residues in RNA). The RNA content was directly quantified using a Nanodrop 8000 spectrophotometer (Thermo Scientific, Dublin, Ireland). The isolated RNA was considered free of contaminants when the A260/A280 ratio was > 1.7. RNA samples were stored at -80°C.
2.4.2 cDNA synthesis

Gene expression was analysed by two-step quantitative RT-PCR. In the first step RNA was reverse-transcribed to cDNA then amplified by polymerase chain reaction. Prior to cDNA synthesis, contaminating DNA was removed by the addition of 2 µl of DNase I (Qiagen, UK) to the RNA samples (0.2-1 µg RNA) in a total volume of 14 µl, followed by a 2 min incubation at 42°C. The RNA was then reversed-transcribed using QuantiTect Reverse Transcription Kit (Qiagen) by addition of primer mix, 1 µl reverse transcriptase and 4 µl buffer in a total 20 µl reaction volume. Samples were incubated at 42°C for 30 min, followed by a 55°C denaturing step for 5 min in a PTC-200 thermo cycler (MJ Research, MA, USA). Samples were employed directly for RT-PCR analysis or stored at -20°C.

2.4.3 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

cDNA (2 µl) was amplified with SYBR green I Master mix (Roche, Basel, Switzerland) using the LightCycler 480 PCR system (Roche). PCR was performed according to the manufacturer’s instructions using the following protocol: preincubation (95°C, 3 min); amplification, 40 cycles consisting of denaturation, annealing, elongation (10 sec 95°C, 10 sec at annealing temperature indicated in Table 2.4 and 72°C for 10 sec); melting curve analysis (95°C 5sec, 65°C 1 min and 97°C 5 continuous acquisitions°C); and final cooling step to 40°C, using previously described primers (MWG Biotech, Ebersberg, Germany). The primer sequences are provided in Table 2.4. All samples were carried out in duplicate 20 µl reactions in 96-well plates and a negative control with nc cDNA template was included in every run. Specificity of the amplicon products was confirmed by visual inspection of melting curves and products run on an agarose gel. The relative expression of the gene was determined using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008) with GAPDH as an internal control.
2.4.4. Agarose gel electrophoresis

PCR products (10 µl) were resolved on agarose gel (1.2% (w/v) agarose, 0.01% (v/v) SyBr safe DNA gel stain (Invitrogen, Biosciences, Ireland) in bionic buffer) at 150 mV for 30-40 min using bionic buffer as running buffer.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers (5'→ 3')</th>
<th>Product size (bp)</th>
<th>Annealing (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIM-1</td>
<td>Forward-GAA CAT AGT CTA CTG ACG GCC AAT AC</td>
<td>127</td>
<td>55</td>
<td>(Khademi et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>Reverse-GAA CCT CCT TTT TGA AGA ACT TTT T</td>
<td></td>
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<tr>
<td>TIM-3</td>
<td>Forward-TCC AAG GAT GCT TAC CAC CAG</td>
<td>96</td>
<td>57</td>
<td>(Khademi et al., 2004)</td>
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<tr>
<td></td>
<td>Reverse-GCC AAT GTG GAT ATT TGT GTT AGA TT</td>
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<tr>
<td>IL-8</td>
<td>Forward-TTT TGC CAA GGA GTG CTA AAG</td>
<td>194</td>
<td>56</td>
<td>(Carroll et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>Reverse-AAC CCT CAC CCA GTT TTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-17A</td>
<td>Forward-TGTCCACCATGTGGCCTAAGAG</td>
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<td></td>
<td>Reverse- GTCCGAAATGAGGTGTCTTTGA</td>
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<tr>
<td>Galectin-9</td>
<td>Forward-GATCAGAATGCTGTGGTGCG</td>
<td>260</td>
<td>59</td>
<td>(Asakura et al., 2002)</td>
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<tr>
<td></td>
<td>Reverse-GAAGCCGCTCTATGCTGCA</td>
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</tr>
<tr>
<td>GAPDH</td>
<td>Forward-CAT GAG AAG TAT GAC AAC AGC CT</td>
<td>113</td>
<td>55</td>
<td>(Carroll et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>Reverse-AGT CCT TCC ACG ATA CCA AAG T</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.5 Preparation of protein samples

2.5.1 Preparation of total cell lysate

Cells (5x10^5/well) were rinsed with DPBS and lysed in 200 μL of ice-cold radio immunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8, 0.5% (w/v) sodium deoxycholate, 1 % (w/v) Triton X-100, 0.1% (v/v) SDS) containing the protease inhibitor cocktail Complete Mini (Roche Diagnostics GmbH, Mannheim, Germany)) for 30 min on ice. Cell extracts were clarified by centrifugation at 20,000 × g for 30 min at 4°C. Where equal protein loading was required, the protein concentration of cell lysates was determined by BCA assay.

2.5.2 Subcellular fractionation by sucrose gradient ultracentrifugation

To separate subcellular fractions by sucrose gradient ultracentrifugation, HBE or CFBE cells (2 confluent 75 cm² culture flasks each) were trypsinised and resuspended in 200 μL of 10% (w/v) sucrose in ice-cold Lamberth's Break Buffer (LBB) (10 mM KCl, 3 mM NaCl, 4 mM MgCl₂, 10 mM PIPES, pH 7) containing the protease inhibitor cocktail Complete Mini. Membranes were disrupted by sonication on ice for 5 sec at 20 W output with 1 min resting intervals (×3) using a Vibra-Cell VC 130 PB ultrasonic processor (Sonic & Materials Inc., USA). Lysates were centrifuged for 5 min at 240 × g at 4°C. The membrane rich supernatant was centrifuged in a sucrose step gradient (10%, 17.5%, 34%, w/w) at 137,000 × g for 30 min at 4°C using a swing rotor RP55S (Sorvall) in an RC-M120EX ultracentrifuge (Sorvall). The membrane-protein rich fraction and the cytosolic fraction were collected separately and the membrane-protein rich fraction was diluted 1:4 in LBB containing protease inhibitors and recentrifuged at 137,000 × g for 30 min at 4°C using a fixed rotor S100AT5 (Sorvall). Pellets were resuspended in 50 μL of ice-cold RIPA buffer containing protease inhibitors. All fractions were used directly or stored at -20°C.
2.5.3 Cell surface biotinylation analysis

Outer cell surface proteins of HBE or CFBE cells were isolated using a cell surface biotinylation kit (Pierce Biotechnology) according to the manufacturer’s instructions. This kit contains a membrane-impermeable modified biotin that covalently binds to exposed primary amines on surface proteins. The biotin-labelled proteins are subsequently lysed, and affinity purified using a column containing avidin resin. The purified proteins were eluted after cleaving off the biotin label. Briefly, four 90% confluent 75-cm² flasks were quickly washed twice with 8 ml of ice-cold PBS per flask. 10 ml of ice-cold PBS containing 250 μg/ml EZ-Link sulfo-NHS-LC-biotin was added, and the cells were incubated with gentle agitation at 4°C for 30 min. Neutrophil cell surface biotinylation was carried out using 4 x 10⁷ cells washed once with ice-cold DPBS by centrifugation (500 x g for 3 min at 4°C) and resuspended in 5 ml biotin solution. The biotinylation reaction was stopped by the addition of 500 μl of quenching solution per flask, all contents were pooled and the flasks were rinsed with a single 10 ml volume of Tris-buffered saline (TBS). The cells were pelleted after centrifugation at 500 x g for 3 min at 4°C. Cells were resuspended in 500 μl of the supplied kit lysis buffer supplemented with protease inhibitor cocktail Complete Mini, incubated for 30 min on ice and sonicated using five 1-sec bursts every 10 min. To improve solubilisation efficiency cells were vortexed every 5 min for 5 sec (× 3). The cell lysate was clarified by centrifugation at 10,000 x g for 2 min at 4°C. The solubilised biotinylated proteins were isolated on immobilised NeutrAvidin agarose columns after 1 h incubation at room temperature with end-over-end rotation mixing. The column was washed with 500 μl of the kit wash buffer supplemented with protease inhibitors followed by centrifugation (1000 x g for 1 min) and the flow-through was discarded (× 4 times). The bound biotinylated proteins were eluted from the column by incubating at 95°C for 5 min with 400 μl sample buffer containing 50 mM dithiothreitol (DTT) followed by centrifugation at 2000 x g for 2 min. The isolated proteins were stored at -20°C.
2.5.4 Trichloroacetic acid (TCA) protein precipitation

Trichloroacetic acid protein precipitation has previously been shown to be an effective method to quickly preserve neutrophil proteins avoiding proteolytic degradation (Painter et al., 2006). Purified neutrophils \(2 \times 10^7\) were resuspended in 500 µl ice-cold 10% (w/v) TCA in DPBS and incubated for 1 h at 4°C. The precipitated proteins were then centrifuged at 15000 \(\times\) g for 15 min at 4°C. The supernatants were discarded and the pellets washed in 1 ml of ice-cold acetone \((\times 3)\). After washing, the pellets were air-dried at RT and resuspended in 100 µl of sample loading buffer (0.25% (w/v) bromophenol blue, 0.1 M DTT, 62.5 mM Tris, pH 6.8, 1% (w/v) sucrose, and 2% (w/v) SDS). Samples were briefly sonicated to facilitate solubilization of the pellet, boiled for 5 min and stored at -20°C. TIM-3 expression was analysed in TCA neutrophil lysates by western blotting.

2.5.5 Protein quantification by BCA assay

Protein concentration was quantified by BCA. This assay is based on the chelation of cuprous ions by BCA (Smith et al., 1985) after copper binds to aminoacids in alkaline conditions. The formed purple complex is very stable and can be monitored spectrophotometrically by measuring absorbance at 562 nm. The main advantage of this protein quantification method is the compatibility with up to 5% detergents commonly used in lysis buffers.

BSA protein standards were prepared by serial dilution in ultrapure water to a final concentration of 0, 200, 400, 600, 800, 1000, 2000, 3000 µg/ml. Standard solutions and protein samples (5 µl) were pipetted into a 96-well plate in triplicate. Fresh BCA working solution was prepared according to the manufacturer’s instructions by mixing reagent A and B (50:1) and 200 µl were pipetted into each well. The plate was covered with tin foil and incubated at 37°C for 30 min. Absorbance was read at 550 nm using a Wallac 1420 Victor2 multilabel counter (PerkinElmer, MA, USA). The protein concentration was determined from the established standard curve.
2.6. Immunoblotting

2.6.1 SDS-polyacrylamide gel electrophoresis

Proteins were separated by SDS-PAGE according to Laemmli’s method (Laemmli, 1970). Samples were denatured by adding sample loading buffer and heated for 10 min at 95°C. Samples were stored at -20°C or used immediately. Denatured protein samples (20 μl) were resolved on 10 or 12.5% (w/v) resolving gels and 4% (w/v) stacking gels. Gels (9 x 8 cm, 1 mm thickness) were cast with 8 ml of 10% resolving gels (5 ml Protogel 30% (National Diagnostics, UK), 3.75 ml 1.5M Tris-HCl pH 8.8, 150 μl 10% (w/v) SDS, 150 μl 10% (w/v) ammonium persulfate (APS), 6 μl tetramethylethylenediamine (TEMED) and 9.95 ml of deionised water) or 12.5% resolving gels (6.25 ml Protogel 30%, 3.75 ml 1.5M Tris-HCl pH 8.8, 150 μl 10% (w/v) SDS, 150 μl 10% (w/v) APS, 6 μl TEMED and 4.7 ml of deionised water), and 4 ml of stacking gels (0.85 ml Protogel, 0.85 ml 1 M Tris-HCl pH 6.8, 50 μl 10% (w/v) SDS, 50 μl 10% (w/v) APS, 5 μl TEMED and 3.4 ml deionised water). SeeBlue Plus2 Prestained molecular mass markers (4 μl; Invitrogen) were loaded on each gel for determination of molecular weight. Gels were run in an ATTO AE6450 electrophoresis tank (ATTO Corporation, Tokyo, Japan) with sufficient running buffer (25 mM Trizma base, 200 mM glycine, 1% (w/v) SDS). Electrophoresis was carried out for 60-90 min at 150 V until the loading dye had run to the bottom of the gel.

Where gradient gels were required, NuPage Bis-Tris 4-12% acrylamide precast gels (Invitrogen) were used according to the manufacturer’s instructions. Briefly, precast gels were rinsed in deionised water and mounted in a XCell SureLock Mini-cell system (Invitrogen). The electrophoresis chamber was filled with NuPage running buffer (50 ml NuPage 20X (Invitrogen), 950 ml deionised water) and samples were loaded. Electrophoresis was carried out for 35 min at 200 V.
2.6.2 Western blot analysis

Following electrophoresis, the resolving gel was equilibrated for 1 min in transfer buffer (39mM glycine, 48mM Tris pH 8.3, 20% (v/v) methanol) and placed on top of a 0.2 μm pore size nitrocellulose membrane and sandwiched between Whatmann chromatography paper (Whatmann International Ltd, Maidstone, England) pre-soaked in transfer buffer. Proteins were transferred onto membrane at 150 mA for 60 min using a semidyry blotting apparatus. Following transfer, membranes were blocked with 5% (w/v) non-fat powdered milk in PBS containing 0.1% (v/v) Tween-20 (PBST) for 1 h at RT. In phospho-protein studies PBST containing 3% (w/v) BSA was used as a blocking buffer. For immunological detection of the desired proteins, blots were incubated overnight at 4°C in blocking buffer containing antibody against TIM-1, TIM-3, LL-37, MMP-9, MPO, phospho-tyrosine, phospho-p38, phospho-ERK1/2 or anti X-actin, as a loading control marker, as required. Subsequently, nitrocellulose membranes were washed for 30 min in PBST buffer (×2), probed with corresponding HRP-conjugated secondary antibody in PBST for 1 h and then washed again. Blots were developed with Immobilon western chemiluminescent HRP substrate (Millipore, Ma, USA) and visualised on the Syngene G:Box chemi XL gel documentation system (Synoptics, Cambridge, UK) or by exposing the membrane to Kocak X-Omat LS film. Protein band size was estimated by comparison with molecular weight markers.

In phosphorylation studies, blots were stripped with 10 ml of Restore western blot stripping buffer (Pierce Biotechnology) according to the manufacturer’s instructions. Briefly, blots were incubated for 30 min at 37°C, rinsed for 1 min with deionised water and then 5 min with PBST (×3). Blots were then rebloked and probed for total p-38 or ERK 2 with the indicated antibodies (Table 2.1).
2.7 Cell imaging and cytometry methods

2.7.1 Protein detection by Laser scanning cytometry

TIM surface expression on airway epithelial cells was measured by laser scanning cytometry. Cells (1×10^5) were seeded in eight-well Lab-Tek chamber glass slides (Nalgene Nunc, Roskilde, Denmark) and incubated overnight in complete medium. The media was then aspirated and cells were fixed with 100% (v/v) methanol for 10 min at RT. Slides were washed with PBS (× 3) and blocked with 2% (w/v) BSA in PBS for 15 min at room temperature. After washing with PBS, cells were incubated at 4°C in the dark with primary antibody (1:20 in PBS) for 30 min. The slides were then washed with PBS and incubated with FITC-labelled secondary antibody (1:40 in PBS) for 30 min at 4°C in the dark. Cells in control chambers were probed with secondary antibody only. Slides were washed with PBS, then cells were permeabilised and the nuclei stained in a single step using a 1:1 ratio of permeabilising buffer (0.1 % (w/v) sodium citrate, 0.1 % (w/v) Triton X-100 in PBS) and 0.1 µg/mL solution of propidium iodide (PI) (Molecular Probes, Invitrogen) in PBS. Slides were washed with PBS and TIM expression was quantified on a CompuCyte laser scanning cytometer (CompuCyte, MA, USA). Cell nuclei were identified by PI fluorescence (588 ± 10 nm) and TIM surface expression was detected by FITC fluorescence (530 ± 20 nm) and quantified. At least 3×10^3 cells were counted in triplicate in each well. Artifacts caused by contoured debris or aggregated cells were excluded by gating out based on countour size using an algorithm.

2.7.2 Immunocytochemistry and confocal microscopy

HBE and CFB cells (1×10^5) were grown in complete media in an eight-well chamber slide or on 13 mm diameter glass coverslips (VWR International Ltd, Dublin, Ireland). After removing media and washing with PBS, cells were fixed with either methanol or 4% (w/v) paraformaldehyde at room temperature for 20 min. Cells were washed again with
PBS (×3) and blocked with 2% (w/v) BSA in PBS for 15 min at room temperature. Then cells were probed with either mouse anti-TIM-1 or goat anti-TIM-3 antibodies (1:50 in PBS) for 30 min at 4°C in the dark. After washing, cells were probed again with the corresponding FITC-labelled antibody (1:100 in PBS). Cells were washed (×3) and mounted on a glass slide using Vectashield fluorescence mounting media (Vector Laboratories LTD, UK). Controls for this experiment included secondary antibody only. Cells were visualised by confocal microscopy using a LSM510 confocal microscope (Zeiss, Welwyn Garden City, UK). Images were captured at a magnification 40× under oil immersion.

2.7.3 Flow cytometry

Samples were analysed on a FACscalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) which allows for simultaneous acquisition of fluorescence, forward light scatter and side scatter. In this study, samples were excited at 488 nm with an argon laser and the fluorescent channel FL1 (emission at 530 nm) was employed for green fluorescence such as FITC. For red fluorescence including PE, channel FL2 was employed (emission at 585 nm). At least 10000 events were acquired and analysed with CellQuest Pro Software.

2.8 Bronchial epithelial cell treatments

2.8.1 Inhibition of CFTR function

To examine the effect of CFTR function in our experimental setting, we used the CFTR inhibitor 172 (CFTRinh 172) (Calbiochem, Merck Chemicals Ltd, Nottingham, UK). This thiazolidinone channel inhibitor has been reported to be highly selective for the CFTR channel (Ma et al., 2002). CFTRinh 172 has been shown to induce CF-like inflammatory responses in HBE cells at a concentration of 10 μM without causing cytotoxicity (Perez et al., 2007) and was therefore employed at this concentration in our experiments. HBE
cells \((1 \times 10^5)\) were serum starved overnight and then treated with 10 \(\mu M\) CFTRinh 172 in low serum medium for 48 h. Control cells were treated with vehicle 0.1\% (v/v) DMSO. Cells were then collected in 0.5 ml of TRI reagent for subsequent RT-PCR analysis of TIM-3 and galectin-9 expression.

### 2.8.2 TIM expression under inflammatory conditions

HBE and CFBE cells \((1 \times 10^5)\) were grown in complete media in Lab-Tek glass chamber slides overnight. The media was aspirated and cells were washed with MEM and serum starved for 2 h. Serum free media was replaced by fresh MEM supplemented with 10 \(\mu g/ml\) of lipopolysaccharide (LPS). The LPS concentration employed has previously been reported to be within physiological levels in the CF lung and has been found to induce IL-8 production in CFBE cells (Greene et al., 2005). MEM was used as negative control. Cells were incubated for 24 h at 37\(^\circ\)C followed by washing with PBS. Subsequently, TIM-1 and TIM-3 expression was quantified by LSC.

### 2.8.3 Cell viability assay

Cell viability was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. This assay is based on the intracellular conversion of MTT (yellow) into formazan (purple) which can be monitored spectrophotometrically. MTT is a membrane permeable compound which is reduced to a water-insoluble formazan salt by mitochondrial succinate dehydrogenase (Mosmann, 1983). Since reduction of MTT only takes place in metabolically active cells, the levels of MTT can be correlated to cell viability. MTT assays were carried out in 96-well plates \((4 \times 10^4\) cells/well). An MTT stock solution of 5 mg/ml was prepared in PBS and filter-sterilised. Single-use aliquots were stored at -20 \(^\circ\)C. After treatment, 10 \(\mu l\) of MTT stock was added to each well (100 \(\mu l\) medium) and cells were incubated at 37\(^\circ\)C for 4 h. The plate was centrifuged at 700 \(\times\) g for 5 min to pellet the purple formazan crystals. The supernatant was carefully removed and 100 \(\mu l\) of acid isopropanol solution (HCl 0.1N, 10\% (v/v) Triton X-100 in anhydrous
isopropanol) was added to dissolve the formazan crystals. Plates were immediately read at 570 nm and viability was expressed as a percentage of the control cells.

2.8.4 TIM-3 immunoprecipitation

CFBE or HBE cells were grown to confluence in 10 cm dishes coated with fibronectin (1 mg/ml), collagen (2.9 mg/ml), and BSA (1 mg/ml). Cells (2 plates/treatment) were washed with PBS, and were incubated at 37°C for 15 min with 2 ml of complete media containing 500 nM galectin-9 per plate or fresh complete media as a control. Cells were washed with 5 ml ice-cold PBS (× 2) and scraped in 1 ml of LBB supplemented with protease inhibitors Complete Mini tablets and phosphatase inhibitors PhosStop Mini tablets (Roche) at 4°C. Cell lysates were sonicated on ice for 5 sec at 20 W output with 1 min resting intervals (×3) using a Vibra-Cell VC 130 PB ultrasonic processor. The lysates were clarified by centrifugation at 240 × g at 4°C for 10 min. In order to obtain a crude cell membrane fraction, clarified lysates were ultracentrifuged at 100,000 × g for 1 h at 4°C. The crude membrane pellet was resuspended in 1 ml RIPA buffer containing protease and phosphatase inhibitors and homogenized by shearing using a 1 ml syringe and a Microlance 3, 21G 1.5" needle (BD, Oxford, England). The resuspended crude membrane lysate was clarified by centrifugation and the protein concentration was determined by BCA in 5 μl aliquots. Equal amounts of crude membrane extract (500 μg) in a total volume of 1 ml were used for immunoprecipitation. Samples were precleared with 6 μg of normal goat IgG and 50 μl of protein-G Dynabeads (Invitrogen) for 1 h at 4°C with rotation. The beads were collected magnetically and washed with 1 ml PBS (× 3), and then beads were boiled in 20 μl of 2× sample buffer at 95°C for 10 min, and stored at -20°C. This sample was termed IgG immunoprecipitation control. The precleared samples or "input" were transferred to a new tube and incubated with 6 μg goat anti-TIM-3 antibody for 2 h at 4°C with rotation. The immunocomplex was captured by incubation with 50 μl of protein-G Dynabeads for 1 h at 4°C with rotation. Beads were washed with PBS and bound proteins were eluted with 20 μl of 2× sample buffer at 95°C for 10 min. The activation of TIM-3 by galectin-9 was analysed by western blot of immunoprecipitated
cell membranes probed for phospho-tyrosine. Subsequently, blots were stripped and reprobed with rabbit anti-TIM-3 antibody (1:1000) to confirm equal amounts of immunoprecipitated TIM-3.

2.8.5 Effect of galectin-9 on cytokine production

CFBE or HBE cells (1×10^5) were plated in complete media in a 24-well plate for 24 h and were then serum starved overnight to synchronise cells and eliminate effects of serum factors contained in growth media. Subsequently, cells were treated with 1 ml of low serum media containing galectin-9 (50 nM), lactose (30 mM), or rhTIM-3-Fc fusion protein (100 ng/ml). Supernatants were collected after 24 h for IL-8 content determination by ELISA. Cells were washed with 1 ml ice-cold PBS and collected in 0.5 ml TRI reagent for subsequent RT-PCR analysis. Alternatively, cells were lysed in 250 µl/well of 1 × cell lysis buffer (Raybiotech Inc, Norcross, GA, USA) supplemented with protease inhibitors Complete Mini tablets at 4°C for 20 min. Cell lysates were clarified by centrifugation at 20,000 × g for 30 min at 4°C and stored at -20°C. IL-17A levels were quantified by ELISA and protein concentration determined by BCA assay.

2.9 Proteolytic degradation of TIM-3

2.9.1 Proteolytic degradation of recombinant TIM-3

Pooled CF or pooled non-CF bronchiectasis BAL (10 µl) was incubated with rhTIM-3-Fc at 37°C. Non-CF BAL with undetectable levels of NE activity or PBS were used as a negative control. Aliquots were collected at specific time points (0-30 min, 1-24 h). The reaction was immediately stopped by the addition of sample loading buffer (2 ×) followed by 10 min incubation at 95°C. Samples were stored at -20°C for subsequent western blot analysis. In some experiments, CF BAL samples were preincubated for 1 h at 4°C with 1 µl of 1.1mg/ml aproitinin, 5mg/ml soya bean trypsin inhibitor (SBTI), 0.2 M Pefabloc SC, 5 mg/ml E-64 (Calbiochem), 10 mg/ml pepstatin A, 0.5 M ethylenediaminetetraacetic acid (EDTA), 1mg/ml alpha-1-antichymotrypsin (ACT) (Calbiochem), 2.5 mM GM6001 (Millipore), 10 mg/ml benzamidine , 20 mg/ml O-phenanthroline, 10 mg/ml tumor necrosis
factor-alpha protease inhibitor-1 (TAPI-1) (Calbiochem), 2mg/ml phosphoramidone (Calbiochem), 3 mM N-(methoxysuccinyl)-Ala-Ala-Pro-Val-chloromethyl ketone (CMK), 2mg/ml N-p-tosyl-l-phenylalanine chloromethyl ketone (TPCK), 10 mg/ml Nα-tosyl-l-lysine chloromethyl ketone hydrochloride (TLCK), 1mg/ml alpha-1-antitrypsin (A1AT) (Athens Research and Technology, Athens, GA, USA), 1mg/ml elafin (Proteo Biotech, Kiel, Germany) or 5mg/ml serine leukoprotease inhibitor (SLPI) (R&D Systems).

2.9.2 Cleavage of recombinant TIM-3 by serine proteases and determination of N-terminal sequence of proteolytic fragments

rhTIM-3-Fc (100 ng) was incubated with $10^{-7}$ M, $5\times10^{-8}$ M or $10^{-8}$ M of NE, cathepsin G (Elastin Products Co., Inc. (Owensville, MO, USA)) or PR3 (Athens Research and Technology) for 2 h at 37°C in 10 μl of 0.1 M HEPES buffer pH 7.5, containing 0.5 M NaCl, 0.05% (w/v) Brij-35. All reactions were stopped by addition of 2× loading sample buffer and boiling at 95°C for 10 min. Degradation of rhTIM-3-Fc was analysed by western blotting. For N-terminal-sequencing analysis of proteolytic fragments, samples were separated by SDS-PAGE using a 10% (w/v) polyacrylamide gel and transferred to a polyvinylidene fluoride membrane. The membrane was rinsed in methanol for 5 sec and fixed in Ponceau staining buffer (40 % (v/v) isopropanol, 10% (v/v) acetic acid, 50% (v/v) water and 0.1% (w/v) Ponceau S) for 20 min at room temperature. Proteolytic fragments were visualised after three 5 min consecutive washings in destaining buffer (45% (v/v) isopropanol, 45% (v/v) acetic acid, 10% (v/v) water). Visualised bands corresponding to TIM-3 proteolytic fragments were excised from the membrane and analysed by N-terminal-sequence Edman degradation by Altabioscience (Birmingham, UK).

2.9.3 Cleavage of native TIM-3 by NE and PR3 on CFBTE cell outer surface

CFBE cells ($1\times10^5$/well) were cultured in complete media in Lab-Tek glass chamber slides overnight. The media was then replaced by 1 ml low serum media alone or containing NE ($10^{-7}$ M), PR3 ($10^{-7}$ M), or a combination of both ($10^{-7}$ M each). Cells were
incubated for 2 h at 37°C, washed with PBS and then fixed for outer cell surface expression of TIM-3 by LSC.

2.9.4 Neutrophil elastase activity assay

NE activity was determined using the chromogenic substrate N-(Methoxysuccinyl)-Ala-Ala-Pro-Val p-nitroanilide specific for human NE. Samples (10 μl) were mixed with 90 μl of 3 mM substrate in assay buffer (0.5 M NaCl, 0.1 % (v/v) Brij-35, 0.1 M HEPES, pH 7.5). Optical density was recorded at 405 nm for 5 min at 1 min intervals at 37 °C using a Wallac 1420 Victor2 multilabel counter. NE activity in samples was calculated using an extinction coefficient of 9500 l · mole⁻¹ · cm⁻¹. NE activity was expressed as micromoles of peptide hydrolyzed/min/ml BAL. Samples were analysed in duplicate.

2.10 Enzyme linked immunosorbent assay (ELISA)

The levels of a specific protein can be easily measured using ELISA. This method is based on the formation of an antigen-antibody reaction that results in a highly specific and sensitive measurement of analytes in large numbers of biological samples (Greene, 2001).

2.10.1 Determination of cytokine levels

IL-8 protein concentration in cell supernatants and BAL was determined by sandwich ELISA (R&D Systems) according to the manufacturer’s instructions. Briefly, high-binding, Immulon 2HB flat-bottom polystyrene microtiter plates (Immulon; Thermo Scientific, Rochester, NY, USA) were coated with 100 μl of IL-8 capture antibody (MAB 208) at 2 μg/ml in Voller’s buffer (25 mM Na₂CO₃ and 15 mM NaHCO₃ in deionised water, pH 9.6) overnight at 4°C. Next, wells were rinsed with wash buffer (1% (v/v) Tween-20 in PBS), blocked with 200 μl of 1% (w/v) BSA dissolved in wash buffer and incubated for 1 h at RT. After washing wells (×3), 100 μl of samples or IL-8 standard diluted in PBS (0-2000 pg/ml) were pipetted into the wells. BAL samples were diluted 1:10 in PBS (final volume
100 μl) for IL-8 quantification by ELISA to ensure that the values were within the range of detection. Following a 2 h incubation at RT, the wells were washed again and incubated for a further 1 h with 100 μl of IL-8 detection antibody (BAF 208) diluted to 0.1 μg/ml in washing buffer. The wells were then washed and incubated with 100 μl of streptavidin-HRP (Biolegend, 405210) at 250 μg/ml in washing buffer. Following 1 h incubation at RT, wells were washed and 100 μl of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS, Invitrogen) was added for 30 min. All measurements were conducted in duplicate and recorded using a Wallac 1420 Victor2 multilabel counter set at 405 nm.

IL-17A levels in cell supernatants or cell lysates (100 μl) were determined by sandwich ELISA according to the manufacturer’s instructions (eBioscience, Hatfield, UK) as follows. Plates were coated overnight with capture antibody diluted 1:250 in the provided coating buffer, incubated overnight at 4°C, washed and blocked for 1 h at RT with assay diluent. After five washes with PBS containing 0.05 % (v/v) Tween-20, samples or standards (0-500 pg/ml) were added and incubated overnight at 4°C. The wells were then washed and incubated with detection antibody (1:250) for 2 h at RT, following by a washing step and incubation with avidin-HRP (1:1000) for 1 h at RT. After a final wash, ABTS solution was added and the plate was read at 405 after 1 h incubation at RT. The IL-8 and IL-17A lower limits of detection were 3.5 pg/ml and 4pg/ml respectively.

2.10.2 Determination of galectin-9 levels in BAL fluid

Levels of galectin-9 in BAL were determined by Prof. Arikawa and Prof. Niki (Kagawa University, Japan) using a custom made sandwich ELISA as previously described (Seki et al., 2007, Arikawa et al., 2009). In brief, 96-well plates (Nunc, Naperville, IL) were coated with an anti-human galectin-9 monoclonal antibody (9S2-3, GalPharma, Japan), blocked with 3% (w/v) BSA containing 0.05% (v/v) Tween 20 in PBS, then incubated for 1 h at 37°C with BAL (100 μl) or known concentrations of recombinant human galectin-9. After several washings, bound galectin-9 was recognized by polyclonal anti-human galectin-9 antibody conjugated with biotin using EZ-Link Sulfo-NHS-Biotin reagent (Pierce-
Biotechnology). Quantification was performed using HRP-streptavidin (Invitrogen, Tokyo, Japan) and the colorimetric substrate tetramethylbenzidine (KPL, Gaithersburg, MD). The optical density was recorded using a microplate spectrophotometer (Bio-Rad). The galectin-9 ELISA limit of detection was 15.6 pg/ml.

2.10.3 Determination of TIM-3 in BAL fluid

Levels of TIM-3 in BAL were determined by direct ELISA according to the manufacturer's instructions as follows. Samples were diluted 1:8 in Vollers buffer and 100 μl were plated in a 96-well plate along with rhTIM-3-Fc standards (0-2000 pg/ml) and incubated overnight at 4°C. Following washing, wells were blocked with 300 μl of 3% (w/v) BSA in PBS and incubated for 1.5 h at RT. Next, plates were washed with PBS containing 0.05% Tween-20 and incubated for 2 h at RT with 100 μl TIM-3 antibody (R&D systems, AF2365) at 1 μg/ml in diluent buffer (1% (w/v) BSA in PBS). The plates were then washed, incubated for 1 h at RT with 100 μl of HRP-conjugated anti-goat antibody (sc-2020) diluted 1:1000 in diluent buffer, washed again and incubated 30 min with ABTS. The OD was measured at 405 nm. The limit of detection was 0.6 ng/well.

2.11 Determination of TIM-3 expression in human and murine neutrophils

Freshly isolated human neutrophils were counted using a haemocytometer and pelleted by centrifugation at 250 x g for 3 min at 4°C and immediately resuspended in Tri reagent for RT-PCR analysis or TCA for whole protein isolation. For flow cytometry quantification of TIM-3 surface expression, neutrophils (1 x 10⁷) were fixed in ice-cold paraformaldehyde solution (2% (w/v) paraformaldehyde in DPBS, pH 7.4) for 15 min at 4°C. Cells were then pelleted and washed in 1 ml of blocking buffer (1% (w/v) BSA in DPBS) at 4°C (x 3) and blocked for 15 min in 500 μl of blocking buffer at 4°C. Subsequently, 2 x 10⁶ neutrophils (50 μl) were transferred to a fresh Eppendorf tube and incubated for 30 min at RT in the dark with 20 μl of PE-TIM-3 antibody or PE isotype
control followed by 3 washes in 1 ml DPBS. Cells were immediately analysed by flow cytometry.

Thioglycollate-elicited murine neutrophils (1 × 10⁶) were fixed in 100 μl ice-cold paraformaldehyde solution (2% (w/v) paraformaldehyde in DPBS, pH 7.4) for 15 min at 4°C, washed in 300 μl DPBS by centrifugation and blocked in 100 μl blocking buffer for at least 15 min at 4°C. Subsequently, 5 μl of either PE-antimouse-TIM-3 antibody or PE-isotype control were added and incubated for 30 min at 4°C in the dark. Neutrophils were washed twice in DPBS, resuspended in 200 μl blocking buffer and kept at 4°C until analysed the following day by flow cytometry.

2.12 Determination of role of galectin-9 in neutrophil function

2.12.1 Intracellular calcium mobilisation measurement

Intracellular calcium mobilisation was monitored using a Fluo-4 NW kit (Invitrogen) as previously described (Bergin et al., 2010). Fluo-4 is a fluorescein ester derivative that is easily loaded into cells and once inside the cell, esterases cleave off the lipophilic groups resulting in a negatively charged fluorescent molecule. The fluorescence is highly enhanced by calcium binding which facilitates intracellular calcium measurements.

According to the manufacturer’s instructions, neutrophils were resuspended in sterile polypropylene tubes (Sarstedt) at 2.5 × 10⁶ cells/ml in assay buffer containing 30 mM lactose, 30 mM sucrose or buffer alone and incubated for 30 min at 37°C, 5% CO₂. An equal volume of 2 × loading solution was next added (final dye concentration 5 mM) and cells were further incubated for 30 min. 100 μl of neutrophil suspension (1.25 × 10⁵ neutrophils) were pipetted into a black 96-well plate in duplicate and fluorescence was recorded at RT every 5 sec in a Victor X3 2030 Multilabel reader (PerkinElmer) using excitation and emission wavelengths of 485 and 535 nm respectively. After 60-90 sec, galectin-9 was added (final assay concentration 0, 50, 100, 500 nM) and intracellular calcium fluorescence was monitored for up to 10 min.
2.12.2 Oxidative burst

Neutrophil oxidative burst was analysed as production of $O_2^-$ (Dalhlgren et al., 2007). Extracellular $O_2^-$ production upon galectin-9 treatment was monitored by the cytochrome-C reduction assay (Babior et al., 1973). Cytochrome C is reduced by $O_2^-$ following equimolar stoichiometry, therefore, the formation of $O_2^-$ can be directly detected by measuring the formation of reduced cytochrome C at 550 nm. Neutrophils were resuspended at $2.5 \times 10^8$ cells/ml in cytochrome C assay buffer (0.5 mM MgCl$_2$, 0.5 mM CaCl$_2$, 5 mM glucose, 100 $\mu$M cytochrome C from bovine heart in DPBS) pre-warmed at 37°C. Neutrophil suspensions (200 $\mu$l equivalent to $5 \times 10^5$ cells) were pipetted into a 96-well plate in duplicate in the presence of buffer, galectin-9 at concentrations 0, 50, 100, 250 and 500 nM, or 500 ng of phorbol myristate acetate (PMA) as a positive control. Reduction of cytochrome C was measured at 550 nm every min. After 10 min, N-formyl-methionine- leucine-phenylalanine (fMLP, final concentration 1 $\mu$M) was added to cells to assess whether galectin-9 is capable of neutrophil priming. $O_2^-$ production was monitored every min for an additional 30 min and the concentration of $O_2^-$ produced was calculated using the Beer-Lambert law with an extinction coefficient ($\varepsilon$) of 21.1 nM$^{-1}$cm$^{-1}$. The amount of $O_2^-$ produced was calculated using the following equation:

$$\Delta\text{OD}_{550} \times 25.28 = \text{nmoles } O_2^- /10^8 \text{ cells/ min}$$

Taking in consideration a 200 $\mu$l reaction volume contained $5 \times 10^5$ neutrophils per well over a 0.75 cm path length.

2.12.3 Neutrophil degranulation assays

The effect of galectin-9 on neutrophil degranulation was investigated by western blot analysis of supernatants after stimulation. Freshly isolated neutrophils ($1 \times 10^7$) were transferred to an Eppendorf tube, pelleted by centrifugation at 250 x g for 3 min at RT and resuspended in 440 $\mu$l of DPBSG. Neutrophils were incubated at 37 °C and 100 $\mu$l aliquots were taken at 0, 15, 30 and 60 min, immediately placed on ice to stop the
reaction and centrifuged at 250 × g for 3 min at 4 °C. Supernatants were carefully collected to avoid disturbing the pellets and immediately stored at -80 °C for NE activity analysis or 54 µl were mixed with 6 µl of 10 × sample loading buffer, immediately heated at 95 °C for 10 min and stored at -20 °C for western blot analysis. Reduced samples (5 µl, equivalent to 1 × 10⁵ neutrophils) were resolved in 4-12% gradient gels. Membranes were probed for MPO, LL37 and MMP-9 and as markers of primary, secondary and tertiary granules respectively.

In order to determine if galectin-9 induced degranulation via TIM-3 interaction, neutrophils were preincubated in the presence or absence of 10 µg/ml TIM-3 blocking antibody (Golden-Mason et al., 2009) or 10 µg/ml mouse IgG control or DPBSG for 10 min at RT. Next, neutrophils were pelleted, resuspended in 440 µl of DPBSG and incubated at 37 °C in the presence of buffer alone or TNF-α (30 ng/10⁷ neutrophils), 1 µM fMLP or galectin-9 (0, 50, 500 nM). Aliquots were taken at 0 and 15 min for NE activity determination and western blot analysis of granule markers.

2.12.4 Galectin-9 induced intracellular signalling

The effect of galectin-9 in neutrophil intracellular signalling indicated by protein phosphorylation was analysed by western blotting as previously described (Marois et al., 2011) with minor modifications. Briefly, freshly isolated neutrophils (1 × 10⁵) were resuspended in 300 µl DPBSG in the presence of 0, 50, 100 or 500 nM galectin-9 and incubated at 37 °C. Aliquots (50 µl; 1.6 × 10⁶ neutrophils) were taken at 0, 1, 2, 5 and 10 min, transferred into an equal volume of 2 × sample loading buffer containing phosStop phosphatase inhibitor and complete mini protease inhibitor (Roche) and boiled for 10 min. Samples (20 µl equivalent to 3 × 10⁵ cells) were directly loaded onto 4-12% gradient gels and tyrosine phosphorylation was analysed by immunoblotting with the anti-phosphotyrosine antibody 4G10. Phosphorylation of p38 and ERK 1/2 was also investigated by immunoblotting after samples were resolved on 10% SDS-PAGE gels.
2.13 Determination of bactericidal activity

2.13.1 Bacterial culture

*Pseudomonas aeruginosa* (*P. aeruginosa*, PA) strain (PAO1) and *Staphylococcus aureus* (*S. aureus*, SA) strain (8325–4) were used in this study. Bacteria were stored at -80°C as glycerol stocks (50% (v/v) glycerol in tryptic soy broth) and streaked onto tryptic soy agar plates to obtain single colonies after an overnight incubation at 37°C. A single colony was then suspended in BBL trypticase soy broth (Becton Dickinson) and grown overnight to stationary phase at 37°C and 200 rpm in a shaker incubator (New Brunswick Scientific, Eppendorf). Bacterial colony quantification was achieved by measuring absorbance at 600 nm in 1.5 ml disposable cuvettes (Plastibrand, Postfach, Germany) using a Biophotometer (Eppendorf, Cambridge, UK) according to the following conversion values:

\[
\text{PAO1 (OD}_{600}\text{) 0.185} = 10^8 \text{ cells/ml}
\]

\[
\text{SA (OD}_{600}\text{) 0.170} = 10^8 \text{ cells/ml}
\]

2.13.2 Killing assays

The role of TIM-3 and galectin-9 in bacterial killing was assessed *in vitro* following a previously described method (Reeves et al., 2002) which mimics *in vivo* killing and involves rapid stirring of the neutrophil/bacterial mixture (1:1). In brief, overnight bacterial cultures (5 × 10⁷) were pelleted by centrifugation at 20000 × g for 10 min at RT, resuspended in 500 µl of 50% (v/v) autologous plasma in DPBS and opsonised at 200 rpm for 30 min at 37°C. In a subset of experiments, galectin-9 in DPBS (0, 50, 100 and 500 nM) was used as the opsonisation agent. Freshly isolated neutrophils (1 × 10⁷) were resuspended in Eppendorf tubes in a total volume of 500 µl of DPBSG, in the presence or absence of rhTIM-3-Fc (5 µg/ml) or control rhIgG1Fc (5 µg/ml). Neutrophils were placed in a Thermomixer compact apparatus (Eppendorf) and shaken at 1400 rpm at 37°C. Opsonised bacteria (100 µl, 1 × 10⁷) were immediately added to the neutrophils and after a brief mix by pipetting, 100 µl aliquots were transferred to 10ml of ice-cold
trypticase soy broth to stop the reaction. Aliquots were also taken at 2, 4, 8 and 16 min. Serial dilutions of the bacterial suspensions were plated on triplicate tryptic soy agar plates and incubated at 37°C. Viable bacterial colony forming units were counted the following day. Bacterial viability was expressed as a percentage of bacterial counts at time= 0 min representing 100% viability.

In order to determine if TIM-3 was involved in galectin-9 mediated bacterial killing, neutrophils were preincubated with TIM-3 blocking antibody or mouse IgG control (final concentration of 10 μg/ml) for 10 min at RT prior to the addition of bacteria. The blocking antibody was present throughout the entire reaction.

2.13.3 Galectin-9/LPS binding assays

Interaction between PA LPS and galectin-9 was determined by an in-house developed solid-phase binding assay based on previously published galectin-3/LPS binding studies (Gupta et al., 1997, Li et al., 2008, Mey et al., 1996). High-binding, Immulon 2HB flat-bottom polystyrene microtiter plates were coated overnight at 4°C with 100 μl/well of commercial LPS purified from PA, serotype 10 (50 μg/ml in Vollers buffer). Following three washes with PBS containing 1% (v/v) Tween-20, 100 μl of PBS containing galectin-9 (0-1.5 μM, 0-50 μg/ml) was added to each well and incubated for 2 h at 37 °C. The plates were then washed (×5), incubated for 2 h with 100 μl of 10 μg/ml galectin-9 antibody (clone FG9-M, Galpharma) in PBS, washed once more and then incubated for 1 h with anti-mouse-HRP antibody (1:1000 in PBS). After a final wash, plates were incubated for 1 h with ABTS and galectin-9/LPS binding was confirmed by measuring absorbance at 405 nm. Controls included uncoated LPS wells, wells without galectin-9 and wells treated with antibodies only.

Galectin-9 binding to bacteria was measured by flow cytometry. PA and SA (5 × 10^7) were pelleted by centrifugation, fixed in 3.7 % (w/v) paraformaldehyde in PBS for 15 min at RT, washed in 1 ml PBS (×3) and resuspended in 1 ml ice-cold PBS. Bacteria (1 × 10^6) were incubated overnight at 4°C with end-to-end rotation in 500 μl of PBS containing 500
nM galectin-9 or PBS alone. Following centrifugation, bacteria were washed in PBS (×3) and incubated with 20 μl PE-labelled galectin-9 antibody or PE-labelled mouse IgG control for 30 min at RT in the dark. After a final wash, the bacteria were re-suspended in PBS and galectin-9 binding assessed by flow cytometry.

2.14 Statistical analysis

Data were analysed with GraphPad Prism 4.0 software package (GraphPad Software, CA, USA). Unless specified otherwise, data are expressed as mean ± standard deviation (mean ± SD) of at least three independent experiments in triplicate. Results were considered significant (*) when p<0.05.

Data were tested for normality (one sample Kolmogorov–Smirnoff test) and where normal, differences were calculated by two-tailed unpaired t-test. For non-normal data, differences were calculated by two-tailed Mann-Whitney test (2 data sets) or Kruskal-Wallis test with post-hoc Dunn’s multiple comparison test (>2 data sets).

The correlation between galectin-9 and IL-8 concentration or galectin-9 and NE activity in BAL was determined by one-tailed Spearman correlation analysis.

Results of cytochrome C assays, intracellular calcium measurements and killing assays were analysed by 2-way ANOVA.
CHAPTER 3: EXPRESSION OF TIMS IN THE CF AIRWAYS

3.1 Introduction

Despite the initial discovery of the TIM gene family in a chromosomal region linked to airway hyperreactivity (McIntire et al., 2001), the role of TIM receptors in airway inflammation is poorly understood. TIMs have been implicated in mouse models of asthma (Encinas et al., 2005, Hu et al., 2009, Kearley et al., 2007), Klebsiella pneumoniae infection (Wang et al., 2011a), tuberculosis (Jayaraman et al., 2010) and pulmonary fibrosis (Kim et al., 2010a). TIM receptors expressed on T-cells have also been reported to play a role in human lung disease including asthma (Sonar et al., 2010), sarcoidosis (Idali et al., 2009) and tuberculosis (Wang et al., 2011b) but their role in CF related lung inflammation has not been investigated.

TIM-1 was firstly documented as "kidney injury molecule", KIM-1, due to its expression in injured kidney epithelium (Ichimura et al., 1998) and was later described as a marker of Th-2 cells (Monney et al., 2002). TIM-1 has also been reported to be expressed in mast cells (Nakae et al., 2007), NKT cells (Lee et al., 2010), B cells (Ma et al., 2011) and dendritic cells (Xiao et al., 2011) exhibiting stimulatory properties. More recently, TIM-1 has also been shown to be expressed in mucosal epithelial cells, and in particular in human primary tracheal cells (Kondratowicz et al., 2011). On the other hand, TIM-3 was initially described as a marker of Th-1 cells (Monney et al., 2002) and since then it has been shown to be expressed in a variety of immune cells including Th-17 (Hastings et al., 2009), dendritic cells (Anderson et al., 2007), NK cells (Ju et al., 2010), NKT cells (Khademi et al., 2004), monocytes (Khademi et al., 2004), macrophages (Nakayama et al., 2009, Zhao et al., 2009), and mast cells (Nakae et al., 2007). There is mounting evidence that TIM-3 is a potent regulator of both the adaptive and innate immune response, however, the mechanism involved depends on both cell type and specific disease. Indeed, TIM-3 has exhibited modulatory properties involved in tumour cell proliferation and immune evasion in non-immune cells including endothelial cells (Huang et al., 2010, Wu et al., 2010) and epidermal melanocytes (Wiener et al., 2007). However,
the expression of TIM-3 in bronchial epithelial cells had not been established prior to our study (Vega-Carrascal et al., 2011). Interestingly, TIM-3 function has also been implicated in neutrophil recruitment and tissue injury in several in vivo models (Tzianabos et al., 2008, Uchida et al., 2010, Zhu et al., 2005). As neutrophil-dominated inflammation is one of the main causes of morbidity in CF due to lung tissue destruction by neutrophil derived proteases (Davis, 2006), in the present study we investigated the expression of TIM-3 in bronchial epithelial cells and the deleterious effects of excessive neutrophil derived proteases in TIM-3 mediated mechanisms.

The other member of the TIM family in humans, TIM-4 is mainly expressed in antigen presenting cells but not in lymphocytes. TIM-4 presents important structural differences compared to TIM-1 and TIM-3, notably, the lack of an intracellular signalling motif (Kuchroo et al., 2003). This unique structural feature potentially renders TIM-4 incapable of mediating transmembrane signalling. Indeed, TIM-4 did not transduce downstream signalling in a human kidney cell line despite being directly involved in phagocytosis of apoptotic bodies (Park et al., 2009), and for this reason was not the focus of this study.

3.2 Aims of this chapter

Given the pivotal role of TIM receptors as modulators of the immune response and cellular homeostasis in a varied range of pathological contexts, we hypothesised that TIMs may also play a role in the airway immune response. Furthermore, TIMs function and/or expression may also be altered in CF bronchial epithelial cells contributing to lung disease as it has been shown for other immune receptors such as TLR-4 (Hauber et al., 2005, John et al., 2010). The purpose of this chapter was therefore to characterise TIMs expression in human bronchial epithelium. To this end the following aims were set:

1. To characterize the expression of TIMs in human bronchial epithelial lines and bronchial brushings.
2. To elucidate if CF bronchial epithelial cells exhibit altered TIM expression
3. To determine whether TIM-3 expressed in bronchial epithelial cells is a functional receptor.
3.3 Results

3.3.1 Expression of TIM in airway epithelial cells

In order to determine whether TIMs play a role in airway epithelial cells, we initially set out to characterise the expression of TIM-1 and TIM-3 which are capable of initiating signal transduction due to tyrosine phosphorylation, a feature missing in TIM-4 (Kuchroo et al., 2003). The expression of TIM-1 and TIM-3 was examined in unstimulated HBE and CFBE by RT-PCR (Figure 3.1A) and quantitative RT-PCR (Figure 3.1B). RT-PCR analysis demonstrated the presence of TIM-3 in both cell types. There was a significant (p<0.05) overexpression of TIM-3 in CF cells compared to normal HBE cells (Figure 3.1B). In contrast, TIM-1 expression was detected in CFBE cells (Figure 3.1A) but there was no detectable level of TIM-1 in HBE cells (C>T>35). Next, expression of TIM-1 and TIM-3 was studied in vivo using bronchial brushings obtained from CF patients that were homozygous for the ΔF508 mutation. TIM-3 overexpression was confirmed in vivo by RT-PCR (Figure 3.1C), suggesting a link between CFTR function and TIM-3 upregulation. In contrast, TIM-1 was not detected in either control or CF brushing samples (C>T>35), indicating minimal or negligible expression of TIM-1 in CF bronchial epithelium in vivo.

The expression of TIM-3 protein in HBE and CFBE cells was further confirmed by western blot analysis (Figure 3.2). TIM-3 is a 33 kDa protein but appears as two diffuse bands of 50 and 64 kDa due to glycosylation (Van de Weyer et al., 2006). TIM-3 expression was higher in CFBE compared to HBE cells confirming the upregulation of TIM-3 observed at translational level (Figure 3.1B). TIM-1 presence in HBE or CFBE cells could not be detected by western blot analysis (data not shown) in agreement with the low or negligible levels measured at mRNA level (Figure 3.2A). An immunoblot for actin was used to demonstrate equal protein loading.
Figure 3.1. TIM mRNA expression is upregulated in CF. A) TIM-1 and TIM-3 mRNA expression was determined by RT-PCR. RT-PCR products were resolved in a 1.2% (w/v) agarose gel. Expected size of PCR products: TIM-1, 127 bp; TIM-3, 96 bp; GAPDH, 137 bp. Negative control lane (CTRL), RT-PCR without mRNA. B) Differential TIM-3 mRNA expression was measured by quantitative RT-PCR in HBE and CFBE cells. Results are presented relative to GAPDH and compared to HBE expression. Bars show mean ± SD, n=3. Results are representative of three independent experiments. C) Differential TIM-3 mRNA expression in bronchial brushings from healthy controls (n=7) and CF patients (n=5) analysed by quantitative RT-PCR. Bars show mean ± SD. Statistical significance analysed by t-test. * p<0.05.
Figure 3.2. Analysis of TIM expression by western blotting. Equal amounts (20 µl) of whole cell extracts were analysed by western blotting on 10% (w/v) SDS-PAGE gels. Glycosylated TIM-3 was detected as diffuse bands at 50 and 64 kDa (expected size 33 kDa). An immunoblot for actin served as a protein loading control. Results are representative of three independent experiments.
This overexpression pattern for TIM-3 was also observed at the protein level as revealed by LSC (Figure 3.3A&B). This method was chosen over flow cytometry to quantify the expression of TIM proteins in airway epithelial cells to avoid altered expression caused by epithelial layer disruption. Figure 3.3A illustrates the differences in expression between CFBE and HBE cells with their corresponding controls, further corroborating TIM upregulation in CF cells. TIM-1 was found to be expressed in CFBE but not in HBE cells (Figure 3.3A), supporting the results obtained at transcriptional level (Figure 3.1A). TIM-3 was found to be expressed in both cell types, however, CFBE cells expressed significantly more TIM-3 (50%; p< 0.05) than HBE cells (Figure 3.3B).
Figure 3.3. TIM expression is upregulated in CF bronchial epithelial cells. A) Laser Scanning Cytometry (LSC) of HBE (red) and CFBE (blue) monolayers, labelled with anti-TIM-1 or anti-TIM-3 followed by the corresponding FITC-conjugated secondary antibody. Controls (black) included secondary antibody alone. Histograms are representative of three different measurements. B) Quantification of TIM surface expression in human bronchial epithelial cells by LSC. A minimum of 3000 cells were counted. Bar graph shows mean fluorescence intensity peak values for each point. Data shown as mean ± SEM, n=3. Statistical significance calculated by t-test, * p<0.05. Results are representative of three independent experiments.
3.3.2 Localisation of TIM in bronchial epithelial cell outer surface

In order to establish whether TIM proteins can act as membrane receptors in bronchial epithelial cells, TIM cellular localisation was studied by fluorescence confocal microscopy. This investigation revealed that TIM-1 is present in unstimulated CFBE and HBE cells, although the membrane localisation could not be ascertained (Figure 3.4). In contrast to TIM-1, TIM-3 was clearly localised to the membrane (Figure 3.5, white arrows), further corroborating TIM-3 membrane expression as shown by LSC (Figure 3.3B). In addition, intracellular green fluorescence following a punctuated pattern close to the plasma membrane was also observed in HBE and CFBE cells. This punctuated pattern has previously been noted in HEK cells (Nakayama et al., 2009) and may be indicative of intracellular stores of TIM-3.

As CFBE cells exhibited strong membrane expression, we next further characterised cell surface expression of TIM-3 in CFBE cells. TIM-3 outer surface expression was confirmed by western blot analysis of membrane and cytosolic fractions obtained by subcellular fractionation by sucrose gradient centrifugation (Figure 3.6A) and by biotinylation of the outer membrane cell surface (Figure 3.6B). Of note, the membrane fraction showed a strong band at 64 kDa, the mature glycosylated form reported to be present on the cell surface and the only one capable of acting as a functional receptor (Van de Weyer et al., 2006).
Figure 3.4. Analysis of TIM-1 localisation by immunofluorescence confocal microscopy. HBE or CFBE cells (10^5/well) were grown on cover slips and probed with anti-TIM-1 followed by incubation with corresponding FITC-labelled antibody. Controls included secondary antibody only. Cell nuclei were visualised by DAPI (blue) and expression of TIM was detected by green fluorescence. Images for each antibody treatment were captured using identical image capture parameters at magnification 40 × under oil immersion. Images are representative of three independent experiments.
Figure 3.5. Analysis of TIM-3 localisation by immunofluorescence confocal microscopy. HBE or CFBE cells (10^5/well) were grown on cover slips and probed with anti-TIM-3 followed by incubation with corresponding FITC-labelled antibody. Controls included secondary antibody only. Cell nuclei were visualised by DAPI (blue) and expression of TIM was detected by green fluorescence. White arrows indicate cell surface localisation. Images for each antibody treatment were captured using identical image capture parameters at magnification 40 × under oil immersion. Images are representative of three independent experiments.
Figure 3.6. Immunoblot analysis of TIM-3 cell surface localisation. A) Localisation of TIM-3 in membranes isolated by sucrose gradient subcellular fractionation. CFBE cells (2 confluent 75 cm² culture flasks each) were trypsinised and resuspended in 200 µL of 10% (w/v) sucrose in Lambe's Break Buffer containing protease inhibitors. The membrane-protein rich fraction and the cytosolic fraction were resolved on a 12.5% (w/v) SDS-PAGE gel. Fractions were probed for the presence of TIM-3 by western blotting. B) Localisation of TIM-3 in the outer cell membrane of CFBE cells. Cell surface proteins were isolated using a cell surface biotinylation kit (Pierce Biotechnology) according to the manufacturer's instructions.
3.3.3 Effect of CFTR inhibition on TIM expression

Having observed that TIMs are upregulated in CF, and in particular TIM-3, we further investigated the link between CFTR function and TIM expression in bronchial epithelial cells. HBE cells were serum starved overnight and then treated with 10 μM CFTR inhibitor 172 (CFTRinh 172) in low serum medium for 48 h. The expression of TIM-3 was then measured by qRT-PCR. A significant (p<0.05) upregulation of TIM-3 was observed compared to vehicle controls (0.1% DMSO) (Figure 3.7). These results suggest a link between CFTR chloride channel function and TIM-3 expression. TIM-1 expression did not change with CFTRinh 172 treatment (data not shown).

3.3.4 Modulation of TIM expression by LPS

The results presented so far demonstrate that TIM-3 is overexpressed in unstimulated CFBFBE cells. Additional studies were conducted to determine whether inflammatory conditions present in the CF lung could further influence TIM-3 expression in bronchial epithelial cells. LPS was chosen as a pro-inflammatory stimulus because CF BAL fluid contains considerable levels of LPS of PA origin (Greene et al., 2005). Cells were treated with LPS (10 μg/ml) and TIM expression was measured by LSC. These conditions have previously been shown to induce a strong inflammatory response in HBE and CFBFBE cells (Greene et al., 2005). After 24 h treatment, LPS (10 μg/ml) induced a 2-fold increase in TIM-3 expression in HBE cells and a 3-fold increase in CFBFBE cells (Figure 3.8A).
Figure 3.7. Effect of CFTR inhibition on TIM-3 expression. HBE cells ($10^5$) were serum starved overnight and then treated with 10 μM CFTR inhibitor 172 (CFTRinh 172) in low serum medium for 48 h. DMSO 0.1% (v/v) was used as a vehicle control. The expression of TIM-3 was then measured by qRT-PCR. Results are presented relative to GAPDH and compared to the expression in controls. Bars show mean ± SD, n=3, statistical significance analysed by t-test. * p<0.05. Results are representative of two independent experiments.
Figure 3.8. Effect of LPS on TIMs expression. HBE cells (white bars) and CFBF (black bars) (1 × 10^5) were treated with LPS (10 μg/ml) for 24 h or low serum media as control (ctrl). TIM-3 (A) or TIM-1 (B) expression was analysed by laser scanning cytometry. A minimum of 3000 cells were counted. Bar graph shows mean fluorescence intensity peak values for each point. Data shown as mean ± SEM, n=3. Statistical significance calculated by t-test, * p<0.05. Results are representative of three independent experiments.
These data reveal that inflammatory stimuli can modulate TIM-3 overexpression in bronchial epithelial cells. Furthermore, TIM-3 expression is more susceptible to LPS induced upregulation in CF than in normal bronchial epithelial cells. Interestingly, the same treatment in CFBF cells showed that TIM-1 expression is downregulated by LPS. After 24 h LPS exposure, TIM-1 expression on CFBF cell outer surface was almost completely abrogated (Figure 3.8B). HBE cells were not included in the study as unstimulated cells do not express TIM-1. The effect of LPS on TIMs expression in CFBF is consistent with the results obtained in patient samples (Figure 3.1C). All CF patients recruited in this study were PA colonised which could result in high levels of LPS in the lung. In these patients, TIM-3 was overexpressed compared to non CF (p>0.05) (Figure 3.1C). Conversely, TIM-1 expression was not detected in controls or CF brushings, in agreement with the inhibitory effect of LPS in CFBF cells in vitro (Figure 3.8B).

3.3.5 Effect of galectin-9 on bronchial epithelial cell viability

In order to explore the TIM-3/galectin-9 signalling mechanisms in bronchial epithelium we first: optimised the experimental conditions for galectin-9 in vitro treatments. For this purpose we used a recombinant galectin-9 that has been modified to be structurally proteolytically stable (Nishi et al., 2005). This form of galectin-9 has been found to bind to TIM-3 and modulate immune responses (Anderson et al., 2007). CFBF cells were incubated with galectin-9 (0, 1, 5, 50 and 500 nM) in low serum and complete media for 24 h. Cell viability was assessed by MTT assay. Of the tested galectin-9 concentrations, only 500 nM in low serum media was cytotoxic after 24 h as indicated by reduced cell viability compared to untreated cells by MTT assay (Figure 3.9). Interestingly, the same concentration was nontoxic in media containing 10% (v/v) FCS. Furthermore, treatments with galectin-9 concentrations below 500 nM did not negatively affect cell proliferation as indicated by percentages of cell viability above 100% (compared to untreated cells).

Subsequent experiments were carried out with 50 nM galectin-9 in low serum media for cytokine production or 500 nM in complete media for the TIM-3 activation study, as these concentrations were proved nontoxic to CFBF cells.
Figure 3.9. Effect of galectin-9 on bronchial epithelial cell viability. CFBE (1 × 10⁴) cells were plated in complete media in a 96-well plate. Cells were treated with galectin-9 at the indicated concentrations in media containing 1% (v/v) FCS (white bars) or 10% (v/v) FCS (black bars). Cell viability was measured using an MTT assay after 24 h incubation. Data are mean ± SD n=3. * statistically significant versus 1% (v/v) FCS control; statistically significant versus 10% FCS control. p< 0.05 by t-test.
3.3.6 Activation of TIM-3 by galectin-9 in bronchial epithelial cells

We next investigated if TIM-3 expressed on bronchial epithelial cells was a functional receptor for galectin-9. For this purpose, CFBE or HBE cells were treated with galectin-9, a TIM-3 ligand known to activate TIM-3 by phosphorylation of the tyrosine motif Y265 in the cytosolic domain (Van de Weyer et al., 2006). Since galectin-9 induced signalling has never been documented in CFBE or other epithelial cells, we employed 500 nM galectin-9. This dose has been reported to induce TIM-3-mediated responses in Th cells (Zhu et al., 2005). This experiment required optimisation in terms of galectin-9 dose and length of exposure time required to induce phosphorylation. The protocol outlined in the materials and methods section was fully optimised in this study and the data obtained following the described methodology are reproducible. In brief, a standard immunoprecipitation protocol with whole cell lysates was adopted initially but the levels of immunoprecipitated TIM-3 were insufficient for phosphorylation studies. Subsequently, several steps were taken to achieve the optimal immunoprecipitation, including testing different bead types (acrylic and magnetic coated with protein A or protein G). Non specific binding was reduced by pre-clearing with normal IgG preadsorbed with human and mouse serum and employing crude membrane extracts, rather than whole cell lysates. The TIM-3 protein recovery was improved by increasing starting cell numbers and concentrating membrane-bound TIM-3 by isolating membrane extracts by ultracentrifugation. All steps were carried out at 4°C and incubation times to precipitate the immunocomplex were reduced from 24 h to 2 h to minimise TIM-3 degradation. The immunoprecipitation was carried out with two different anti-TIM-3 antibodies raised against different epitopes with similar results.

After 15 min treatment with galectin-9 (500 nM in complete media), TIM-3 was immunoprecipitated from CFBE or HBE cell membrane extracts. Western blot analysis of immunoprecipitates revealed that TIM-3 exhibited a substantial increase in the level of tyrosine phosphorylation compared to untreated controls (Figure 3.10, upper panel) in response to galectin-9 treatment. Background levels of phosphorylation were detected suggesting constitutive activation of TIM-3, possibly due to constitutively produced galectin-9. A blot probed with rabbit anti-TIM-3 served as a control for equal amounts of immunoprecipitated TIM-3 (Figure 3.10, lower panel). This result shows that TIM-3 in
bronchial epithelial cells can become activated by galectin-9 challenge and subsequently engage in intracellular signal transduction pathways by tyrosine phosphorylation of the receptor.
Figure 3.10. TIM-3 in CFBE cells is a functional receptor. CFBE or HBE cells were either left untreated or treated with 500 nM galectin-9 (Gal-9) for 15 min. TIM-3 was immunoprecipitated (IP) from cell membranes with goat anti-TIM-3 antibody. Normal goat-IgG was used as a control of non-specific binding. Immunoprecipitates were resolved in a 12.5% (w/v) SDS-PAGE gel. Activation of TIM-3 was analysed by immunoblotting using an anti-phosphotyrosine (p-Tyrosine) antibody (upper panel). Blots were stripped and reprobed with rabbit anti-TIM-3 antibody to confirm equal levels of immunoprecipitated TIM-3 (lower panel). The image is representative of two independent experiments.
3.4 Discussion

Within Chapter 3, experiments focused on the characterisation of TIM expression in bronchial epithelial cells. The data presented demonstrate that TIMs are expressed and upregulated in CF both in bronchial epithelial cell lines and in bronchial brushings obtained from CF patients. HBE and CFBE cell lines have long been established as in vitro models of normal and CF epithelium respectively. CFBE cells were immortalised from a CF patient homozygous for the most common CF mutation, ΔF508 (Bruscia et al., 2002), representing the model of choice for this study. Although the use of primary cultures instead of immortalised cell lines in CF research is sometimes preferred, we confirmed TIM expression in bronchial brushings from CF and control patients by RT-PCR. Furthermore, all the selected patients were PA positive, a clinically relevant feature difficult to accurately replicate ex-vivo.

TIM-3 expression has been previously reported in lung tissue (van de Weyer et al., 2006) and in murine bronchial epithelial cells (DeKruyff et al., 2010), data supporting our results in human bronchial epithelial cells. In contrast, TIM-1 is believed to be only expressed in damaged epithelium, as shown in a model of kidney injury (Ichimura et al., 1998). Our work demonstrates that TIM-1 is expressed in unstimulated CF bronchial epithelial cells. In addition, we have made the novel observation that both TIM-1 and TIM-3 are constitutively up-regulated in CFBE cells. This finding provides a new insight into the mechanisms involved in CF pathology given the role of TIM as modulators of the immune response (Kane, 2010).

Of interest, this also constitutes the first report of TIM overexpression linked to a specific genetic mutation. The data from CFTR inhibition experiments suggest a link between CFTR function and TIM-3. Pharmacological inhibition of CFTR with CFTRinh172 resulted in an upregulation of TIM-3 expression. It has been proposed that loss of wild type CFTR cell membrane expression suppresses NFkappaB (NFκB) mediated inflammation (Hunter et al., 2010), and that functional CFTR is required for such an inhibitory effect (Hunter et al., 2010, Vij et al., 2009). Inhibition of CFTR activity after CFTRinh172 treatment caused
an increase in NFκB activity in HBE cells (Hunter et al., 2010). Conversely, activation of CFTR activity following forskolin treatment reversed this effect. Given that NFκB has been predicted to have several binding sites in the TIM-3 promoter region (Anderson et al., 2010), the mechanism behind the upregulation of TIM-3 after CFTR pharmacological inhibition may involve modulation of NFκB activity.

Pharmacological inhibition with CFTRinh 172 is an accepted model of CF (Perez et al., 2007). Other CFTR blockers such as the glycine hydrazide GlyH-101 (Muanprasat et al., 2004) could be employed to ascertain the effect of CFTR function in TIM-3 expression. An alternative approach could also involve the use of isogenic cell lines where the defective CFTR channel expression has been corrected. Nevertheless, our findings in CFTR inhibition studies are in agreement with the data obtained from CF patient bronchial brushings where TIM-3 was clearly upregulated compared to matching controls. However, the mechanism by which the CFTR mutation results in up-regulation of TIM remains unknown and has yet to be explored.

The observed constitutive overexpression of TIM-3 in bronchial epithelial cells prompted us to explore the role of TIM-3 in the CF lung. TIM function in epithelial cells has not been studied to date therefore we sought to characterise in detail TIM expression in bronchial epithelial cells prior to investigating possible TIM-3/galectin-9 signalling mechanisms. Firstly, we investigated whether TIMs were present on the surface of epithelial cells to facilitate the binding of the TIM-3 ligand, galectin-9 (Zhu et al., 2005) or indeed any other putative ligand present within the CF airways. The presence of TIM-3 in CFBE and HBE cell membranes was confirmed by fluorescence confocal microscopy and western blot analysis of isolated membranes. Confocal imaging revealed a clear membrane localisation of TIM-3 in both CFBE and HBE cells, and also a cytosolic presence following a punctuated pattern. This cytosolic distribution was also observed in HEK293T cells transiently transfected with an expression vector for mouse TIM-3 fused to green fluorescent protein (Nakayama et al., 2009). In this model, TIM-3 was recruited to the phagocytic cup in the membrane after stimulation. Thus, this could indicate that TIM-3 is stored in the cytosol of bronchial epithelial cells and, upon cell stimulation, can be
recruited to the membrane from intracellular vesicle stores. Expression of TIM-3 on the outer cell membrane surface was also quantified by LSC corroborating the overexpression of TIM-3 on the surface of unstimulated CFBE compared to HBE cells. Of note, western blot analysis of TIM-3 in whole cell lysates and isolated membranes demonstrated that the fully glycosylated form of TIM-3, represented by a diffuse band of approximately 64 kDa, was present in CFBE cells. Only fully glycosylated TIM-3 can be inserted into the plasma membrane of cells (Van de Weyer et al., 2006). Although a recent publication reports the expression of TIM-1 in primary human tracheal epithelium (Kondratowicz et al., 2011), in our hands TIM-1 was found to be expressed only in CFBE cells by LSC, in agreement with the RT-PCR results, but not in non-CF control cells. Confirmation of TIM-1 localization on CFBE cell membranes was also analysed by western blotting, but results were negative, possibly due to low protein abundance.

The focus of the second part of this chapter was to determine if TIM-3 expressed in bronchial epithelial cells is a functional receptor. Given that TIM-3 signalling has never been reported in epithelial cells, no downstream targets could be monitored to assess TIM-3 function. However, galectin-9 has been shown to induce tyrosine phosphorylation in several cell types (Van de Weyer et al., 2006, Anderson et al., 2007, Cho et al., 2011). Therefore, we examined the effect of galectin-9 on TIM-3 phosphorylation by immunoprecipitation. Following the optimised protocol, TIM-3 phosphorylation upon galectin-9 stimulation was demonstrated in CFBE cells, indicating the presence of a functional TIM-3 receptor. This observation is of extreme importance as it suggests that epithelial TIM-3 plays an active role and it is not a mere scavenger receptor as Ichimura and colleagues suggested for TIM-1 in kidney cells (Ichimura et al., 2008).

We also evaluated TIM expression under pro-inflammatory conditions. Cells were treated with LPS (10 µg/ml) from PA, a well characterised component of CF BAL fluid capable of inducing an inflammatory response in HBE and CFBE cells (Greene et al., 2005). This elevated concentration of LPS has physiological relevance as it has been estimated that CF lung surface can contain LPS levels as high as 40-70 µg/ml (Greene et al., 2005). LPS treatment stimulated TIM-3 expression in HBE cells and particularly increased TIM-3
levels in CFBE cells. In contrast, LPS treatment resulted in almost a complete abrogation of TIM-1 expression on CFBE cells suggesting an antagonistic role for TIM-1 and TIM-3 under inflammatory conditions. Interestingly, LPS treatment downregulated TIM-3 expression in dendritic cells (Sui et al., 2006) and monocytes (Zhang et al., 2011), whereas LPS exposure upregulated TIM-3 expression in endothelial cells (Wu et al., 2010) indicating a cell-type specific response. Of note, another TLR-4 agonist, the alarmin high mobility group box 1 (HMGB1) also upregulated TIM-3 expression in endothelial cells in an NFκB and AP-1 dependent manner (Wu et al., 2010). Other TIM receptors modulated by bacterial toxins include TIM-4 which has been shown to be upregulated by Staphylococcal enterotoxin B (Liu et al., 2007) and cholera toxin (Feng et al., 2008). In addition, the effect of other inflammatory agents on TIM expression would be of benefit and serve to increase our knowledge of TIM function under physiological conditions that better resemble the CF lung milieu.

The observation that TIM-1 was downregulated by LPS while TIM-3 was upregulated was confirmed in patient samples. All the patient samples were colonised with PA which is an important source of LPS in the CF lung. In these bronchial brushings TIM-3 was overexpressed in CF patients whereas TIM-1 expression was not detected in bronchial brushings.

To summarize, our data report for the first time that TIMs are present and upregulated in CF bronchial epithelial cells, even under unstimulated conditions. Furthermore, we have provided evidence for a role in CFTR function in the expression of TIM since pharmacological inhibition of CFTR induced an upregulation of TIM-3. Additionally, the membrane localisation of a functional TIM-3, responsive to galectin-9 stimulation, further substantiates the role of this protein as a receptor in the human bronchial epithelium. We also initiated studies of TIM expression in the CF lung context, demonstrating that proinflammatory mediators highly present in CF BAL, such as LPS, can modulate TIM expression. Furthermore, we have established that TIM-3 may play a potential role in the inflammatory response within the CF lung as it is constitutively upregulated in the CF bronchial epithelium and LPS can further increase its expression. The physiological
relevance of TIM-3 overexpression in CF bronchial epithelium is studied in detail in Chapter 4.
CHAPTER 4: DYSREGULATION OF TIM-3/GALECTIN-9 SIGNALLING IN CF AIRWAY EPITHELIUM

4.1 Introduction

Experiments described in Chapter 3 have revealed the presence of TIMs in the lung. Our results particularly highlight TIM-3 as a potentially novel mediator in the CF chronic inflammatory context since TIM-3 high basal expression was further enhanced by LPS, in contrast, TIM-1 expression was downregulated. Moreover, TIM-3 may play a predominant role over TIM-1 in the CF lung once chronic bacterial colonisation of gram-negative bacteria (e.g. PA) takes place and LPS levels in the airways rise.

The initial function attributed to TIM-3 was downregulation of Th-1 mediated responses in a mouse model of experimental autoimmune encephalomyelitis (Monney et al., 2002). This downregulation was achieved due to induction of Th-1 cell apoptosis via galectin-9 interaction (Zhu et al., 2005) in mice, and via reduction of cytokine production from Th1 and Th17 cells in humans rather than direct apoptosis (Hastings et al., 2009). TIM-3 has also been shown to be crucial in T-cell exhaustion (reviewed in (Zhu et al., 2011)). More recently, a contrasting effect for TIM-3 function has been described in innate immune cells. Rather than inhibiting the production of inflammatory factors, TIM-3 engagement resulted in enhanced inflammatory responses in dendritic cells and macrophages (Anderson et al., 2007). In particular, galectin-9 treatment in the presence of LPS triggered a potent TNF-α response. Activation of murine mast cells with an agonist TIM-3 antibody also enhanced cytokine production (Nakae et al., 2007). More surprisingly, the immunomodulatory properties of TIM-3 have been extended to non immune cells. For instance TIM-3 expressed in melanoma cells (Wiener et al., 2007) and endothelial cells (Huang et al., 2010, Wu et al., 2010) have recently been shown to be capable of inhibiting the local tumour immune response facilitating tumour progression. TIM-3 function is not limited to the modulation of the immune response. TIM-3 can also recognise phosphatidylserine receptors on the surface of apoptotic cells and mediate efferocytosis (Nakayama et al., 2009). Furthermore, TIM-3 has been postulated as a Hepatitis A virus cellular receptor (Sui et al., 2006).
Given the broad range of putative TIM-3 functions in non-epithelial cells, we decided to explore the immunomodulatory properties of TIM-3 in human bronchial epithelium. Airway epithelial cells constitute the first line of the host immune defence in the lung. Not only do these cells form a physical barrier against pathogen invasion, they also provide an active role in preventing infection by secreting a wide variety of antimicrobial agents such as mucins (reviewed in (Voynow and Rubin, 2009)), ROS, antimicrobial peptides (reviewed in (Rogan et al., 2006) and larger antimicrobial proteins including lactoferrin, SLPI and elafin (Sallenave et al., 1994, van Wetering et al., 2000). Furthermore, epithelial cells also regulate the inflammatory response in the airways via generation of pro and anti-inflammatory mediators (Bedard et al., 1993), namely cytokines such as IL-6, GM-CSF, TGFβ and most notably, the chemokine IL-8 (Greene et al., 2005), a potent neutrophil chemotactic agent highly abundant in the CF lung (McElvaney et al., 1992). Therefore, the question that remained unanswered was whether the TIM-3/galectin-9 signalling axis in bronchial epithelium plays a role in CF airway inflammation.

4.2 Aims of this chapter

The expression of functional TIM-3 on the surface of CF bronchial epithelial cells prompted us to explore the role of TIM-3 signalling in CF bronchial epithelium. Specifically, TIM-3 phosphorylation post galectin-9 challenge in bronchial epithelial cells suggested a potential role for TIM-3 in triggering an inflammatory response. We hypothesised that TIM-3/galectin-9 played a role in modulating inflammatory responses in the airways but that this function may be blunted by proteolytic action in the CF lung. The aims of this chapter were threefold:

1. To determine if TIM-3 ligand, galectin-9 is expressed in the airways
2. To examine the role of TIM-3/galectin-9 interactions in the bronchial epithelial cell inflammatory response and in particular, the production of pro-inflammatory chemokines and cytokines
3. To evaluate the role of proteases in TIM-3/galectin-9 function, most notably neutrophil derived proteases
4.3 Results

4.3.1 Galectin-9 is upregulated in CF

Galectin-9 is widely expressed in mast cells, T cells, B cells, macrophages, endothelial cells and fibroblasts (Liu et al., 2010). However, whether CF affects galectin-9 expression in bronchial epithelial cells was not known. The constitutive phosphorylation of TIM-3 in CFBE cells (Figure 3.10) suggested that galectin-9 was also expressed in this cell type. We confirmed the expression of galectin-9 in HBE and CFBE cells by qRT-PCR (Figure 4.1A). Of note, galectin-9 was significantly upregulated in CF cells compared to controls (p<0.05). We next tested if the inhibition of CFTR function using the pharmacological inhibitor CFTRinh 172 had an effect on galectin-9 expression in normal bronchial epithelial cells. Figure 4.1B shows that CFTRinh 172 (10 μM) upregulates galectin-9 expression after 48 h. This suggested that the TIM-3/galectin-9 axis is upregulated in CF bronchial epithelium due to lack of CFTR function.
Figure 4.1 Galectin-9 expression in CF bronchial epithelial cells. A) Galectin-9 expression in unstimulated HBE and CFBE was measured by real-time RT-PCR. Results are presented relative to GAPDH and compared to HBE expression. B) Effect of CFTR inhibition on galectin-9 expression. HBE cells (1 x 10^5) were treated with 10 µM CFTR inhibitor 172 (CFTRinh 172) in low serum medium for 48 h. DMSO (0.1%, w/v) was used as a vehicle control. Bars show mean ± SD, n=3, statistical significance analysed by t-test. * p<0.05. Results are representative of two independent experiments.
4.3.2 Effect of galectin-9 on cytokine production

TIM-3 has been involved in regulation of neutrophil recruitment via modulation of neutrophil chemokine expression (Tzianabos et al., 2008). Thus, we investigated whether TIM-3 could modulate the immune response in the CF lung and in particular, the production of chemokines and cytokines by bronchial epithelial cells in response to galectin-9. To determine if TIM-3 could modulate the immune response in the CF lung, IL-8 and IL-17A production upon galectin-9 stimulation was tested. In CF, bronchial epithelium secretes IL-8, a potent neutrophil chemoattractant, which results in neutrophils being recruited into the lung. These neutrophils in turn release NE that further induces IL-8 generation with a subsequent massive neutrophil infiltration that perpetuates the inflammatory response in the CF lung (Nakamura et al., 1992, McElvaney et al., 1992). IL-17A has also been implicated in neutrophil recruitment to the airways by inducing IL-8 production in bronchial epithelial cells (Laan et al., 1999).

4.3.2.1 Effect of galectin-9 on IL-8 production

We examined IL-8 production in CFB E cells upon galectin-9 stimulation. Galectin-9 (50 nM) promoted IL-8 production in CFB E cells as measured in cell culture supernatants after 24 h (Fig 4.2A). To determine whether IL-8 production was due to TIM-3 signalling upon galectin-9 ligation, cells were exposed to galectin-9 pretreated with lactose (30 mM) or rhTIM-3-Fc (100 ng/ml) which have been shown to prevent galectin-9 binding to TIM-3 (Negehera et al., 2008). Blockade of the TIM-3/galectin-9 interaction by lactose or rhTIM-3-Fc suppressed IL-8 production in CFB E cells (Fig. 4.2B). These results suggest that galectin-9 is a natural agonist for TIM-3 in bronchial epithelial cells, inducing production of IL-8. Subsequently, we set out to establish the localization of TIM-3 in polarized cells. Apical treatment of CFB E cells with galectin-9 resulted in a 2-fold increase in IL-8 release demonstrating that TIM-3 is localized apically in CFB E cells (Fig 4.2C). Basolateral treatment also induced IL-8 release but to a lesser extent (1.5-fold increase), suggesting TIM-3 expression is minimally localized to the basolateral compartment. The results of
these experiments were two-fold: firstly galectin-9 induced production of IL-8 and secondly, expression of TIM-3 is predominantly apical.

Figure 4.2 Effect of galectin-9 on IL-8 production. Galectin-9 induces IL-8 production via TIM-3 in CFBE cells. A) CFBE cells ($1 \times 10^5$) were treated with 50 nM galectin-9 (gal-9) or left untreated (ctrl). After 24 h IL-8 levels were determined in cell culture supernatants by ELISA. Results are expressed as mean ± SD, n=3. Statistical significance analyzed by Mann-Whitney test. * p<0.05. B) Blockade of TIM-3/galectin-9 interaction abrogates IL-8 production. CFBE cells ($10^5$) were treated with 50 nM galectin-9 (gal-9) alone or in combination with 30 mM lactose (gal-9 + lact), or 100 ng/ml recombinant humanTIM-3 fusion protein (gal-9 + rhTIM-3). IL-8 levels were determined in cell culture supernatants by ELISA and expressed as percentage increase compared to control. Results are expressed as mean ± SD, n=3. Statistical significance analyzed by Kruskal-Wallis test. * p<0.05. C) CFBE cells were grown at a liquid/liquid interface as polarized monolayers. Cells were treated with 50 nM galectin-9 (black bars) or left untreated (white bars). After 24 h IL-8 levels were determined in the apical and basolateral compartment by ELISA. Statistical significance analyzed by Mann-Whitney test. * p<0.05. Results are representative of at least three independent experiments for monolayers (A and B) or two experiments for polarized cells (C).
4.3.2.2 Effect of galectin-9 on IL-17A production

In addition to IL-8, IL-17A has been shown to be involved in neutrophil recruitment to the lung (Laan et al., 1999). Furthermore, elevated IL-17 levels have been documented in stable CF patients (Decraene et al., 2010) and during exacerbations (Dubin et al., 2007). Thus, we investigated the effect of galectin-9 on IL-17A generation. Galectin-9 treatment induced a significant increase in IL-17A mRNA (Figure 4.3A). IL-17A was not detected in cell culture supernatants although a statistically significant increase was detected in cell lysates ($p<0.05$), indicating elevated intracellular production (Figure 4.3B).
Figure 4.3 Effect of galectin-9 on IL-17A production. A) Effect of galectin-9 treatment on IL-17A mRNA expression. CFBE cells (1 x 10^6) were treated with galectin-9 (gal-9; 50 nM) or left untreated (control) for 24 h. IL-17A expression was measured by qRT-PCR. Results expressed as mean ± SD. Results are representative of three independent experiments each obtained from triplicate wells. B) Effect of galectin-9 treatment on IL-17A protein expression. CFBE cells (1 x 10^6) were treated with galectin-9 (gal-9; 50 nM) or left untreated (control). After 24 h, cells were washed with 1 ml ice-cold PBS and lysed in 250 µl cell lysis supplemented with protease inhibitors. IL-17A content in cell lysates was quantified by ELISA and protein content determined by BCA assay. Results are normalised to protein content. Results expressed as mean ± SD. Results are representative of two independent experiments each obtained from triplicate wells. Statistical significance analysed by t-test. * p<0.05.
4.3.3 Determination of galectin-9 levels in BAL

Prompted by the results of in vitro experiments suggesting an upregulation of galectin-9 in CF (Figure 4.1A), we next measured the levels of galectin-9 in CF BAL by ELISA to determine whether galectin-9 function has relevance in vivo. Galectin-9 was not detected in any of the CF BAL samples tested, whereas different levels of galectin-9 were found in healthy controls. Non-CF bronchiectasis and COPD were also included in the study as controls of inflammatory lung disease with a strong neutrophilic component. COPD and non-CF Bronchiectasis BAL samples showed varied levels of galectin-9 ranging from 0-1000 pg/ml (Figure 4.4A). As CF BAL fluid is considered to be 25 to 100-fold diluted compared to epithelial lining fluid (Greene et al., 2005, Rennard et al., 1986), these values indicate that galectin-9 can be present in the nanomolar range on the lung surface in vivo.

Galectin-9 has been shown to be highly susceptible to rapid proteolytic degradation by NE (Nishi et al., 2005). Because this protease is abundantly present in CF BAL (Reeves et al., 2010b), we measured galectin-9 levels in CF infants and children’s BAL where the levels of neutrophil infiltration are lower than in adult CF patients, and therefore, the levels of neutrophil-derived proteases are low (Table 2.3). Galectin-9 was detected in BAL from infant (0-2 year old) CF patients (Fig. 4.4B), however, galectin-9 levels were markedly decreased in young CF children (3-8 year old). Of major importance, galectin-9 levels correlated inversely with NE (Fig. 4.4C). Indeed, no galectin-9 was detected in any adult CF sample indicating that galectin-9 is abundantly produced in the CF lung from an early age, but the levels dramatically drop as the disease progresses and do not recover later on in life. Collectively, these data suggest that galectin-9 in the lung undergoes degradation by proteases, possibly neutrophil-derived elastase.
Figure 4.4 Determination of galectin-9 levels in BAL. A) Galectin-9 levels were measured by ELISA in BAL from non-CF Bronchiectasis (Bron) (n=10), adult cystic fibrosis (CF) (n=14), Chronic Obstructive Pulmonary Disease (COPD) (n=5) and healthy controls (HC) (n=5). B) galectin-9 levels were measured in CF patients aged 0-2 years old (n=4), 3-8 years old (n=5) and >18 years old (n=14). Statistical significance calculated by t-test. * p<0.05. C) Correlation of galectin-9 levels and NE activity in BAL by one-tailed Spearman correlation (r=-0.9384, p=0.0002).
4.3.4 Degradation of TIM-3 in CF lung

As we had confirmed the lack of galectin-9 in the adult CF lung due to proteolytic degradation, we next investigated the effect of the proteolytic burden of the CF lung on the integrity of galectin-9 receptor, TIM-3.

4.3.4.1 Proteolytic degradation of TIM-3 in BAL

Recombinant human TIM-3 (rhTIM-3-Fc) was used to investigate the stability of TIM-3 in the CF lung. rhTIM-3-Fc was completely degraded after 24 h incubation at 37°C in CF BAL compared to the PBS control, as evidenced by the disappearance of the 64 kDa band on western blot analysis (Figure 4.5A). In contrast, TIM-3 was only partially degraded in non-CF bronchiectasis BAL, possibly due to lower abundance of proteases compared to CF BAL. A time course of TIM-3 proteolytic degradation showed that after only 10 min incubation, proteolytic degradation of the 64 kDa band commenced and TIM-3 was rapidly cleaved into smaller fragments of approximately 60 and 36 kDa and (Figure 4.5B).

Longer incubation times revealed a further fragmentation of TIM-3, suggesting the involvement of various proteases at different stages of degradation. The 64 kDa band corresponding to the fully mature form of TIM-3 expressed on the membrane was almost completely degraded after 4 h. After 24 h virtually no TIM-3 fragments were detected by western blot. In contrast rhTIM-3-Fc was stable for 24 h in non-CF BAL (Figure 4.5C) or PBS control (Figure 4.5D) experiments. Degradation of cell surface receptors by proteases has been proven to be detrimental in the CF lung as the proteolytic fragments were shown to increase inflammation by induction of IL-8 production by bronchial epithelial cells (Hartl et al., 2007). However, the presence of TIM-3 fragments in pooled CF BAL was not detected by ELISA or by western blot analysis (data not shown).
Figure 4.5 Proteolytic degradation of TIM-3 in CF BAL. (A) TIM-3 degradation was analysed by western blotting. 300 ng of human recombinant TIM-3 fusion protein (rhTIM-3) was incubated for 24h at 37°C with 10 μl of CF or non CF bronchiectasis (Bron) BAL. PBS was used as a negative control. B) 100 ng of rhTIM-3-Fc was incubated for the indicated times at 37°C with 10 μl of CF BAL, (C) non-CF BAL or (D) PBS as a controls. Results are representative of two independent experiments.
4.3.4.2 Identification of proteases responsible for degrading TIM-3 in CF BAL

Since proteolytic degradation of TIM-3 has never been reported, a systematic approach was adopted to identify the proteases involved in TIM-3 fragmentation. CF pooled BAL aliquots were preincubated with specific protease inhibitors for each class of enzyme for 1 h at 4°C before adding rhTIM-3-Fc. After 2 h incubation at 37°C, samples were analysed by western blot to identify which protease inhibitor could prevent degradation of TIM-3.

Firstly, non specific protease inhibitors targeting the main protease families were employed: E64 is an inhibitor of cysteiny1 proteases, Pepstatin A (Pep A) is an aspartic protease inhibitor, GM6001 is a general metalloprotease inhibitor and Pefabloc (PEFA) is a potent serine protease inhibitor. Only Pefabloc, and to a lesser extent Pepstatin A, prevented the degradation of full length TIM-3 implicating a serine and an aspartic protease in TIM-3 cleavage (Figure 4.6A). Aprotinin (Apro) and soya bean trypsin inhibitor (SBTI) are inhibitors of the serine protease chymotrypsin and trypsin families respectively, but only SBTI appeared to have an inhibitory effect on TIM-3 degradation. These data implicated a serine protease or an aspartic protease in TIM-3 degradation.

As metalloproteases have been implicated in TIM-1 shedding (Bailly et al., 2002) a wider range of metalloprotease inhibitors were tested in comparison with GM6001. The metalloprotease inhibitors used included o-phenanthroline (OP), TAPI-1, EDTA and phosphoramidon (Phos). Results in Figure 4.6B revealed that none of the metalloprotease inhibitors employed were capable of inhibiting the degradation of the 64 kDa band corresponding to full size TIM-3 (Figure 4.6B). This experiment excludes metalloproteases from the list of candidate proteases involved in TIM-3 degradation in CF BAL.
In addition to Pefabloc, several serine protease inhibitors were used to classify more precisely the serine protease involved in TIM-3 cleavage (Figure 4.6C). MeOSuc-AAPV-CMK (CMK), a potent NE inhibitor completely prevented TIM-3 cleavage. In contrast, the trypsin-like serine protease inhibitor (TPCK) or (A1CT) had little or no effect in TIM-3 degradation respectively. Similarly, the chymotrypsin-like serine protease inhibitor benzamidine (BENZ) had no effect and TLCK had only a moderate effect in preventing TIM-3 degradation. This set of experiments revealed neutrophil serine proteases as the most likely group of enzymes involved in TIM-3 degradation in CF BAL.

Neutrophil serine proteases comprise NE, PR3 and Cath G. The natural serine protease inhibitors alpha-1-anti-trypsin (A1AT), elafin (ELA) and serine leukoprotease inhibitor (SLPI) were used to further identify the neutrophil derived serine proteases responsible for the rapid cleavage of TIM-3 (Figure 4.6D). A1AT blocked TIM-3 degradation completely. A1AT is a potent inhibitor of NE, therefore these results suggest that NE is implicated in TIM-3 degradation. Elafin also blocked TIM-3 degradation completely. The fact that elafin prevented TIM-3 degradation indicates that Cath G plays a minor role in the initial cleavage of TIM-3 as elafin inhibits NE and PR3 but not Cath G (Wiedow et al., 1990). This result is further confirmed by the lack of effect of A1CT which also inhibits Cath G. In contrast, SLPI only partially inhibited TIM-3 degradation, suggesting that PR3 can also cleave TIM-3 as SLPI inhibits NE and Cath G, but not PR3.

Collectively, these results suggest that NE and PR3 are the main serine proteases involved in TIM-3 rapid cleavage in the CF lung. However, this finding does not exclude the involvement of other proteases in further degradation of TIM-3 at later stages, alone or in combination with serine proteases.
Figure 4.6 Identification of proteases degrading TIM-3 in CF BAL. CF BAL (20 μL) were pre-incubated with 1 μl protease inhibitors at 4°C for 1 h. Subsequently, samples were incubated with 100 ng of rhTIM-3-Fc at 37°C for 2 h. A) non specific inhibitors, aprotinin – Apro (1.1mg/ml), soya bean trypsin inhibitor – SBTI (5mg/ml), pefabloc – PEFA (0.2 M), pepstatin A-Pep A (10 mg/ml), GM6001 (2.5 mM), E64 (5 mg/ml). B) Metalloprotease inhibitors, GM6001 (2.5 mM), o-phenantroline-OP (20 mg/ml), TAPI-1 (10 mg/ml), E61A (0.5 M), Phosphoramidon-Phos (2mg/ml). C) serine protease inhibitors, CMK (3 mM), A1CT (1mg/ml), TPCK (2mg/ml), BENZ (10 mg/ml), TLCK (10 mg/ml). D) natural neutrophil serine protease inhibitors, alpha1-antitrypsin- A1AT (1mg/ml), elafin – ELA (1mg/ml), serine leukoprotease inhibitor– SLPI (5mg/ml). Control shows rhTIM-3-Fc incubated in PBS. Results are representative of three independent experiments.
4.3.4.3 Cleavage of TIM-3 by neutrophil serine proteases *in vitro*

The data obtained by use of serine protease inhibitors provided evidence for a role for neutrophil derived serine proteases in TIM-3 degradation in the CF lung. In order to further confirm this role, the ability of purified human neutrophil serine proteases to cleave TIM-3 was examined *in vitro*. A dose-response experiment was carried out with rhTIM-3-Fc incubated for 2 h at 37°C with physiologically relevant concentrations of each protease (10⁻⁷ – 10⁻⁸ M). Western blot analysis of the samples revealed that all serine proteases, but particularly NE and PR3 cleaved TIM-3 in a dose-dependent manner (Figure 4.7). Interestingly, each protease produced fragments of varied sizes suggesting different TIM-3 cleavage sites for each protease. NE produced a single cleavage product corresponding to a fragment of between 36 and 50 kDa, Cath G produced a clear fragment of approximately 36 kDa and a weak band corresponding to a fragment of approximately 40 kDa. PR3 generated a band of approximately 50 kDa and a weaker band of 36 kDa. In order to further identify the cleavage sites, the proteolytic products were analysed by N-terminal sequencing Edman degradation. NE was found to cleave TIM-3 at Ser-186 — Arg-187 whereas PR3 cleaved TIM-3 at Ile-199 — Arg-200 (Figure 4.8). Both NE and PR3 were capable of cleaving TIM-3 in the extracellular region of TIM-3 which would result in the receptor inactivation *in vivo*. This TIM-3 fragments would then undergo further degradation by proteases present in BAL. Indeed no TIM-3 fragments were detected in CF BAL by western blot analysis (data not shown). These results prove that neutrophil proteases initiate the inactivation of TIM-3 receptor in bronchial epithelial cells by proteolytic cleavage, although this soluble fragment would undergo further degradation.
Figure 4.7 Effect of purified neutrophil serine proteases on TIM-3 cleavage. A) rhTIM-3 (100 ng) was either left untreated (ctrl) or exposed to purified neutrophil elastase (NE), cathepsin G (Cath G) or proteinase-3 (PR3) at 37°C for 4 h. TIM-3 degradation was analysed by western blotting. Image is representative of two independent experiments.
Figure 4.8 Schematic representation of NE and PR-3 cleavage sites in the amino-acid sequence of TIM-3. Recombinant human TIM-3 proteolytic fragments were analysed by N-terminal sequencing Edman degradation to identify the cleavage site for each protease. Arrows represent cleavage sites by NE and PR-3. NE cleaves TIM-3 at Ser-186 — Arg-187 and PR3 at Ile-199 — Arg-200.
4.3.4.4 Cleavage of TIM-3 by neutrophil serine proteases on CFBE cell outer surface

As the experiments described so far were conducted *in vitro* using recombinant TIM-3, we next evaluated the ability of NE and PR3 to cleave native TIM-3 expressed on the cell surface of CF bronchial epithelial cells. CFBE cells were treated with $10^{-7}$ M of NE, PR3 or a combination of both, for 2 h at 37°C in low serum media. The remaining levels of TIM-3 on the cell outer surface were measured by LSC. Both NE and PR3 decreased the levels of TIM-3 cell outer surface expression. Furthermore, simultaneous addition of both proteases resulted in an almost complete reduction of TIM-3 expression on CFBE cells (Figure 4.9). These results confirm that neutrophil serine proteases can degrade native TIM-3 and therefore have the potential to degrade TIM-3 in bronchial epithelial cells in the CF lung *in vivo*. 
Figure 4.9 TIM-3 is cleaved on the cell surface of CF bronchial epithelial cells by NE and PR-3. CFBSE cells were treated with 10^{-7} M of NE, PR3 or a combination of both, for 2 h at 37°C in MEM containing 1% (v/v) FCS. Levels of TIM-3 on the cell surface were measured by laser scanning cytometry. Data shown as mean ± SEM. Statistical significance calculated by t-test. * p<0.05. Results are representative of two independent experiments each obtained from quadruplicate wells.
4.4. Discussion

Having clearly established the expression of TIM-3 in CF bronchial epithelial cells in Chapter 3, we proceeded to examine the functional significance of TIM-3 signalling in the CF lung in Chapter 4. Although TIM-3 has been shown to exhibit a wide variety of functions ranging from clearance of apoptotic bodies (DeKruyff et al., 2010, Nakayama et al., 2009) to promotion of Hepatitis A cellular infection (Sui et al., 2006), we focused on the immunomodulatory properties of TIM-3. Our aim was to determine whether epithelial TIM-3 played a role in modulating the immune response in the CF lung. To study the functional effect of TIM-3 we first confirmed the expression of galectin-9 in bronchial epithelial cells. RT-PCR analysis confirmed that this cell type can produce galectin-9. Of note, the constitutive overproduction of galectin-9 puts forward an explanation for the basal levels of TIM-3 phosphorylation observed in immunoprecipitation studies. Interestingly, similar to TIM-3, galectin-9 was also upregulated in CFBE cells compared to HBE cells, suggesting a link between CFTR chloride channel function and galectin-9 expression. Pharmacological inhibition of CFTR induced a significant upregulation of galectin-9 production in HBE cells, substantiating the idea of constitutive upregulation of the TIM-3/galectin-9 axis in CF.

Since galectin-9 is upregulated under inflammatory conditions (Kasarratsu et al., 2005, Ishikawa et al., 2004, Imaizumi et al., 2002) and we have demonstrated that TIM-3 becomes upregulated by LPS, we hypothesised that TIM-3/galectin-9 plays a role in CF inflammation. Furthermore, constitutive upregulation of this receptor and its ligand may reflect the previously reported constitutive proinflammatory state of CF bronchial epithelial cells (Vij et al., 2009, Hunter et al., 2010). To this end, we investigated whether galectin-9 had an effect on the production of the potent neutrophil chemoattractant IL-8 in CFBE cells. The galectin-9 used in this study is a modified recombinant protein (Nishi et al., 2005). Although lacking the peptide linker, this recombinant galectin-9 maintains the carbohydrate recognition domains of the native form and its use has been reported to induce TIM-3 activation both in vitro and in vivo (Seki et al., 2008, Arikawa et al., 2009, Nagahara et al., 2008, Dardalhon et al., 2010). This form of galectin-9 was selected
because it is resistant to proteolytic cleavage. Galectin-9 treatment at physiological concentrations stimulated IL-8 secretion by CFBE cells, an effect reversed by pretreatment of galectin-9 with lactose (30 mM) suggesting that galectin-9 induction of IL-8 production depends on its β-galactosidase-binding properties. Additionally, pretreatment with recombinant human TIM-3 (100 ng/ml) also returned IL-8 production to control levels, indicating engagement of TIM-3 by galectin-9. These results demonstrate that galectin-9 induces IL-8 secretion via TIM-3 interactions in CFBE cells. Similarly, galectin-9 treatment also upregulated expression of IL-17A, a cytokine with potent effects on neutrophil recruitment in the lung (Laan et al., 1999) that has recently been implicated in CF (Dubin et al., 2007). Collectively, these results indicate that galectin-9 signalling via TIM-3 may play a role in the pathogenesis of CF by promoting neutrophil recruitment. Furthermore, in fully polarised cells, IL-8 was mainly secreted apically, in agreement with the reported apical localisation of TIM-3 in human and porcine tracheal epithelium (Kondratowicz et al., 2011). The luminal airway localisation of TIM-3 may be of high clinical relevance since chronic infection and aberrant immune response is predominantly confined to the lung in CF patients.

The observation that galectin-9 was upregulated in CF prompted us to examine the levels of galectin-9 in CF BAL. Galectin-9 was not detected in any adult CF BAL sample tested. However, galectin-9 was detected in other BAL samples from patients with respiratory diseases such as COPD and non-CF bronchiectasis, which display neutrophilic infiltration but to a lesser extent. Examination of the NE levels revealed that galectin-9 expression correlated inversely with NE activity. Furthermore, galectin-9 is present in CF infants with low neutrophil infiltration and therefore very low or non-detectable levels of NE activity. Subsequently, galectin-9 levels gradually decrease as patient age increases. Since this protease is abundantly present in CF BAL (Reeves et al., 2010b, Greene et al., 2005), the data indicate that in older patients with chronic neutrophil infiltration, galectin-9 is degraded, at least partly, by neutrophil derived proteases. This finding is in agreement with previous reports of the ability of proteases, including elastase, to rapidly cleave galectin-9 (Nishi et al., 2005). Examination of the stability of the galectin-9 receptor in the CF lung demonstrated that TIM-3 is cleaved in CF BAL within 10 min and the fully
glycosylated receptor is totally degraded in less than 2 h. A systematic study of the proteases involved in TIM-3 degradation revealed that serine proteases, specifically NE and PR3, are involved in the initial cleavage of TIM-3 in the extracellular domain, an effect that would lead to inactivation of the receptor. Furthermore, the complex fragmentation patterns suggest that various proteases are involved in this degradation at different stages. Noteworthy, physiologically relevant concentrations of NE and PR3 completely abolished TIM-3 presence on CFBE cell outer surface. Neutrophil derived proteases, in particular NE and Cathepsin G, have been reported to deactivate the proteinase-activated receptor-2 on the surface of bronchial epithelial cells due to proteolytic action (Dulon et al., 2003). In addition, the unopposed action of serine proteases has been shown to cleave a variety of cell surface receptors in the CF lung, including the phosphatidylserine receptor in macrophages (Vandivier et al., 2002), causing defective removal of apoptotic neutrophils and exacerbating inflammation. The CXCR1 chemokine receptor in neutrophils has also been shown to be cleaved by serine proteases, reducing bacterial killing and inducing IL-8 production which led to further inflammation (Hartl et al., 2007). Of major importance, the extraordinarily high protease burden in the CF lung also causes the degradation of the natural protease inhibitors A1AT, elafin and SLPI (reviewed in (Greene and McElvaney, 2009)) exacerbating the deleterious effect of neutrophil derived proteases, including NE.

In this chapter we have examined the role of TIM-3 signalling in the CF lung and demonstrated that CFBE cells release IL-8 and IL-17A upon galectin-9 stimulation at physiological concentrations. However, both TIM-3 and its ligand galectin-9 get rapidly degraded in the lung by neutrophil derived proteases. We have shown that, like TIM-3, galectin-9 is constitutively upregulated in CF implying a role for CFTR function since pharmacological inhibition of normal cells resulted in upregulation of galectin-9. Galectin-9 signalling via TIM-3 receptor on CFBE cells induced IL-8 and IL-17A production in vitro which may contribute to the early influx of neutrophils in vivo. However, this mechanism of constitutive recruitment of neutrophils to the airways would be predominantly active in young children and become redundant in adult CF patients where IL-8 production is kept high due to the presence of many other proinflammatory agents including activation of
bronchial epithelial cells by NE (Walsh et al., 2001) or other proteases (Cosgrove et al., 2011). Indeed, galectin-9 levels were completely depleted in BAL from adult CF patients, most likely due to neutrophil derived proteases. This finding was supported by the fact that galectin-9 was detected in infant CF BAL but decreased with age in a NE-dependent manner. Neutrophil derived proteases, in particular NE and PR3 were also found to cleave TIM-3 at physiological concentrations which would cause inactivation of TIM-3 signalling pathways.
CHAPTER 5: ROLE OF TIM-3 IN THE NEUTROPHIL

5.1 Introduction

Within Chapter 3 and 4 of this study a previously unidentified role for TIM-3 and galectin-9 in airway epithelial cells was described. The data presented implicated TIM-3 in the initiation of the airway inflammatory response via production of neutrophil chemoattractants, including IL-8. Given the paramount importance of neutrophils in CF lung disease, we next considered whether TIM-3 and its ligand galectin-9 played a role in neutrophil function. Any potential TIM-3 mediated neutrophil activity would inevitably be abrogated by proteolytic degradation in the CF lung. Surprisingly, neither TIM-3 expression nor function in neutrophils has previously been reported in the literature. Furthermore, only a small number of studies on the role of galectin-9 in neutrophil function exist. Galectin-9 was found not to be chemotactic for human neutrophils in vitro (Matsumoto et al., 1998), although in vivo galectin-9 treatment in a mouse model of sepsis induced neutrophil recruitment (Tsuboi et al., 2007). Additionally, neutrophil apoptosis has not been directly investigated although galectin-9 has been found to induce apoptosis of HL-60 cells (Kuroda et al., 2010). Conversely, the role of TIM-3 in macrophage function has attracted more attention. For instance, TIM-3/galectin-9 interactions have been implicated in macrophage activation (Monney et al., 2002), regulation of cell surface expression of co-stimulatory proteins CD80 and CD86 (Frisancho-Kiss et al., 2007), phagocytosis and antigen presentation (Nakayama et al., 2009), bacterial killing (Jayaraman et al., 2010, Zhao et al., 2009) and modulation of production of inflammatory mediators such as IL-1β (Jayaraman et al., 2010), NO (Zhao et al., 2009) and TNFα (Anderson et al., 2007).

Identification of TIM-3 expression in the neutrophil would widen the scope of TIM-3 putative functions in innate immune cells. The main neutrophil physiological functions comprise neutrophil chemotaxis and migration, NADPH oxidase activity, phagocytosis, degranulation of proteases and antimicrobial agents and inflammatory mediators and apoptosis (reviewed in (Witko-Sarsat et al., 2000)). Several of these functions have been found to be intrinsically abnormal in CF neutrophils, or altered due to chronic
inflammation (see (Hayes et al., 2011) for a comprehensive review). For instance, CF neutrophils exhibited reduced chemotaxis due to reduced responsiveness to several chemoattractants (Hayes et al., 2011), increased O$_2^-$ production (Brockbank et al., 2005) and reduced PA killing due to defective intravacuolar chlorination (Painter et al., 2006). Degranulation defects have also been reported in CF neutrophils, for instance TNFα and IL-8 stimulated a higher release of primary granules by CF peripheral blood neutrophils (Taggart et al., 2000). In addition, CF lung neutrophils showed reduced phagocytic capacity in the lung, likely due to the cleavage of receptors by proteases, resulting in reduced bacterial killing (Hartl et al., 2007). Most of these neutrophil functions start with activation of cell surface receptors which triggers transmembrane signal transduction leading to mobilisation of intracellular calcium stores and tyrosine phosphorylation signalling cascades. In particular the involvement of calcium (Kim-Park et al., 1997) and tyrosine phosphorylation (Rollet et al., 1994) have been shown to be crucial for priming and activation of the NADPH oxidase system in neutrophils.

Given the potentially deleterious effects of fully active neutrophils to the surrounding tissue, neutrophil activation is tightly controlled. Indeed, neutrophils can be found in different states of activation. Resting neutrophils do not show production of reactive oxygen species unless challenged with bacterial agents such as fMLP. Certain agents, including LPS (Guthrie et al., 1984), IL-8 (Daniels et al., 1992) and TNFα (Berkow et al., 1987), can trigger a series of mechanisms that lead to enhanced NADPH oxidase activation following exposure to stimulants, also known as "neutrophil priming". A key feature of priming agents is they on their own they can not elicit O$_2^-$ production. Interestingly, each priming agent can exert these effects through different signal transduction mechanisms leading to the assembly of the different components required for the activation of the NADPH oxidase system. Additionally, priming usually involves other complementary processes that amplify the role of neutrophil stimulating agents. Such processes include, but are not limited to, translocation to the membrane of agonist receptors such as fMLP (O’Flaherty et al., 1991, Roberts et al., 1993), adhesion receptors such as CD11b+ (Roberts et al., 1993) and degranulation. All these processes collectively would enhance the antimicrobial response.
Although reports on galectin-9 function in neutrophils are limited, galectin-3 has been shown to interact with neutrophils leading to modulation of neutrophil activity. For instance, galectin-3 treatment induced $O_2^{•−}$ production within the micromolar range (Yamaoka et al., 1995) but nanomolar concentrations of galectin-3 only induced $O_2^{•−}$ production in exuded neutrophils (Karlsson et al., 1998) since mobilisation of galectin-3 receptors CD66a and CD66b from intracellular granules was a requirement (Feuk-Lagerstedt et al., 1999). Galectin-1 also was also shown to activate NADPH in extravasated neutrophils but not in circulating neutrophils (Almkvist et al., 2002). LPS (Almkvist et al., 2001) and other bacterial wall components (Almkvist et al., 2004, Faldt et al., 2001) have been shown to mobilise galectin-3 receptors from secretory vesicles and gelatinase granules. Additionally, galectin-3 treatment was found to be sufficient to increase neutrophil degranulation, phagocytic activity and apoptosis (Fernandez et al., 2005). Galectin-3 was also shown to induce L-selectin shedding and IL-8 production in human neutrophils (Nieminen et al., 2005). Given the broad accumulating evidence revealing galectin-3 as a modulator of neutrophil function, it is tempting to speculate that galectin-9 may also be capable of modulating neutrophil activity and that some of those functions may be TIM-3 mediated.

5.2 Aims of this chapter

There is mounting evidence suggesting that TIM-3 can modulate the function of several immune cell types. However, neither TIM-3 neutrophil expression nor function has been reported to date. Furthermore, there are virtually no studies on the role of galectin-9 in neutrophils. Therefore within this chapter we investigated whether TIM-3 was expressed in neutrophils and what function it may have in the normal neutrophil, which would be necessarily defective in the adult CF lung due to inactivation by neutrophil derived proteases. To this end, the following aims were set for this chapter:

1. To characterise TIM-3 expression in circulating neutrophils
2. To determine whether TIM-3 expression is intrinsically altered in the CF neutrophil
3. To examine whether TIM-3 plays a role in neutrophil function, specifically in response to galectin-9 treatment
5.3 Results

5.3.1 Characterisation of TIM-3 expression in circulating neutrophils

5.3.1.1 TIM-3 is expressed in the neutrophil

In order to determine whether TIM-3 plays a role in neutrophil function, we firstly characterised the expression of TIM-3 in resting neutrophils obtained from blood from healthy individuals. Because we have found that TIM-3 is rapidly degraded in the presence of neutrophil derived proteases, namely NE and PR3 (Vega-Carrascal et al., 2011) the expression of TIM-3 was examined in whole cell lysates obtained by precipitation with TCA. This method has previously been reported to successfully preserve neutrophil proteins such as CFTR that otherwise are readily cleaved by neutrophil proteases (Painter et al., 2006). Western blot analysis of whole neutrophil lysates identified three bands indicating TIM-3 expression in human neutrophils (Figure 5.1A). In contrast with the bands obtained in human bronchial epithelial cells at 64 and 50 kDa (Vega-Carrascal et al., 2011), neutrophil samples revealed bands at 64, 36 and 16 kDa. Fully glycosylated TIM-3 was found to be present in neutrophils (64-kDa band), and also the non glycosylated form at approximately 36 kDa. Interestingly a weak band at approx. 16 kDa was also observed in neutrophils. It is likely that this band corresponds to a proteolytic fragment.

Western blot analysis of outer cell membrane proteins obtained by cell surface biotinylation of neutrophils, revealed only two size bands, a weak band corresponding to mature TIM-3 at 64 kDa and a stronger band at 16 kDa (Figure 5.1B). The presence of this 16 kDa band suggested that TIM-3 may be cleaved on the surface, possibly by neutrophil derived proteases, leaving behind a residual 16 kDa fragment in the neutrophil plasma membrane. Interestingly, an estimation of the size of the proteolytic fragment using the previously identified TIM-3 cleavage sites for NE and PR3 yields a theoretical size of approximately 16 kDa for the fragment containing the cytosolic domain. This observation has important implications as it suggests that TIM-3 may be cleaved off the
surface of neutrophils by neutrophil derived proteases leaving the cytosolic domain embedded in the plasma membrane and releasing a fragment containing both the IgV and the mucin domain, raising questions about the potential role of soluble TIM-3 produced by neutrophils.
Figure 5.1 TIM-3 is expressed in circulating neutrophils. (A) Neutrophil protein was obtained by TCA precipitation. Protein samples equivalent to $4 \times 10^6$ neutrophils were resolved on a 12.5% (w/v) SDS-PAGE gel, transferred to a nitrocellulose membrane and TIM-3 expression analysed by western blotting. TIM-3 expression was detected as bands at 64, 36 and 16 kDa. Image shown is representative of results obtained from five different neutrophil donors. (B) Cell surface proteins were isolated using a cell surface biotinylation kit (Pierce Biotechnology) according to the manufacturer's instructions. Membrane protein samples equivalent to $1.5 \times 10^6$ neutrophils were resolved in a 12.5% (w/v) SDS-PAGE gel and probed for the presence of TIM-3 by western blotting. TIM-3 expression was detected indicated by arrows as bands at 64 and 16 kDa (expected size 33 kDa). Image shown is representative of results obtained from three different neutrophil donors.
Because we had established the expression of TIM-3 in circulating neutrophils, we next considered whether TIM-3 would be expressed on airway infiltrating neutrophils. TIM-3 expression has previously been reported in elicited macrophages (Jayaraman et al., 2010), although TIM-3 expression was not detected in resident peritoneal macrophages (Nakayama et al., 2009). Therefore, expression of TIM-3 in resting neutrophils does not automatically grant expression in extravasated neutrophils. In order to confirm that TIM-3 could still be present on the neutrophil membrane once the neutrophil has been recruited to the site of infection, TIM-3 expression was examined in thioglycollate elicited neutrophils using a mouse model. Female BALB/c mice were injected intraperitoneally with sterile thioglycollate broth. After 6 h, elicited neutrophils were recovered from the peritoneal cavity by lavage. Neutrophils were washed and fixed with paraformaldehyde before staining with PE-labelled anti-mouse TIM-3 antibody. As illustrated in Figure 5.2, elicited neutrophils retained TIM-3 surface expression. This observation suggested that TIM-3 would still be expressed on neutrophils at the site of infection and be capable of exerting a putative function once migration into the airways from the systemic circulation has occurred.
Figure 5.2 TIM-3 expression is preserved after neutrophil migration. Neutrophils were elicited in BALB/c mice by intraperitoneal injection of 3% (w/v) thioglycolate. After 6 h neutrophils were recovered from the peritoneal cavity by lavage, fixed in 2% (w/v) paraformaldehyde, blocked and stained with 1 μg/10⁶ cells with PE-anti-TIM-3 antibody (green) or isotype control (blue). TIM-3 expression was analysed by flow cytometry. Image shown illustrates histogram representative of three independent experiments.
5.3.1.2 TIM-3 expression is not altered in CF

Having established that TIM-3 is expressed in circulating neutrophils, we next asked if the expression of this receptor was abnormal in CF patients. Altered TIM-3 expression in peripheral monocytic cells has been reported in a number of diseases, including sarcoidosis where a decrease in TIM-3 expression in CD4+ cells both in blood and in BAL correlated with a prototypical elevated Th-1 response (Idali et al., 2009). Furthermore, we have previously reported that TIM-3 expression is constitutively upregulated in human bronchial epithelial cells and that this upregulation was CFTR-dependant (Vega-Carrascal et al., 2011). Using qRT-PCR, we detected a slight decrease in TIM-3 mRNA expression in neutrophils from CF patients homozygous for the ΔF508 mutation that did not reach statistical significance (Figure 5.3).

In order to further evaluate differential expression of TIM-3 on circulating neutrophils from healthy and CF subjects at a protein level, a second approach was adopted. Whole cell lysates obtained by TCA precipitation were obtained from healthy and ΔF508 homozygous CF patients. TIM-3 expression of mature TIM-3 (64kDa band) was analysed by densitometric analysis of immunoblots. As illustrated in Figure 5.4, no significant difference in TIM-3 expression was detected by this method.
Figure 5.3 TIM-3 gene expression is not altered in CF circulating neutrophils. Differential TIM-3 mRNA expression in peripheral blood neutrophils from healthy controls (ctrl; n=20), stable ΔF508 homozygous CF patients (F508; n=13) or stable CF patients carrying at least one ΔF508 allele (CF; n=26) were analysed by quantitative RT-PCR. TIM-3 mRNA levels were not significantly different among groups. Horizontal bars indicate median values. Data analysed by non-parametric one-way ANOVA. ns, non significant p>0.05.
Figure 5.4 TIM-3 protein expression is not altered in CF neutrophil whole cell lysates. Neutrophil protein obtained by TCA precipitation from healthy individuals (HC; n=4) or stable ΔF508 homozygous CF patients (CF; n=4). Protein samples equivalent to 4 x 10^5 neutrophils obtained from each individual were resolved on a single 12.5 % (w/v) SDS-PAGE gel, transferred to nitrocellulose membrane and TIM-3 expression analyzed by western blotting using polyclonal goat TIM-3 antibody. A representative immunoblot from one healthy individual and one CF patient is shown. Bar graph shows densitometric analysis of TIM-3 expression in healthy individuals (n=4) and CF patients (n=4). Bar show mean ± SD. Data analysed by t-test, ns, non significant p>0.05.
Next, TIM-3 expression on the neutrophil surface was studied by flow cytometric analysis. Since standard staining methods of live cells may exceed the half life of TIM-3 in the presence of proteases (<1 h), all neutrophils were fixed in paraformaldehyde prior to incubation with TIM-3 antibody as detailed in the Materials and Methods section in Chapter 2. TIM-3 expression was detected on the membranes of peripheral blood neutrophils from CF patients and healthy controls (Figure 5.5). There was no significant difference in the number of TIM-3 positive cells. This result is in clear contrast with the results obtained in bronchial epithelial cells and highlights the cell-specific dependence of TIM-3 expression.

Collectively, our novel results demonstrated that TIM-3 is expressed in resting circulating and extravasated neutrophils. In addition, we also showed that, although the surface expression varies from individual to individual, TIM-3 expression is not altered in CF neutrophils obtained from peripheral blood. This finding may have important clinical consequences as all TIM-3 mediated functions would be present in CF neutrophils.
Figure 5.5 TIM-3 cell surface expression is not altered in CF circulating neutrophils. Neutrophils from healthy controls (HC; n=16) or stable CF patients (CF; n=11) were isolated from blood, fixed with 2% (w/v) paraformaldehyde, blocked and incubated for 30 min with PE-labelled anti-TIM-3 antibody or PE isotype control. TIM-3 expression was analysed by flow cytometry. Graph shows the percentage of TIM-3 positive neutrophils. Each dot represents one subject. Horizontal bars indicate the median. Data analysed by t-test, ns, non significant.
5.3.2 Effect of galectin-9 on neutrophil function

5.3.2.1 Galectin-9 induces intracellular calcium mobilisation

Because TIM-3 was found to be present in resting neutrophils, and still present on the surface of transmigrated cells, we set out to investigate whether TIM-3 plays a role in neutrophil function. Within this chapter we focused on mechanisms leading to neutrophil activation. Specifically, we examined whether TIM-3 is involved in the initiation or mediation of the mechanisms underlying neutrophil priming.

An increase in intracellular calcium stores is a regular feature in neutrophil priming and activation (Kelher et al., 2003). Moreover, galectin-9, the TIM-3 ligand, has been shown to promote intracellular calcium mobilisation via TIM-3 in mouse Th-1 cells (Zhu et al., 2005) and a number of human cell lines including HL-60 (Kashio et al., 2003), although the involvement of TIM-3 was not confirmed in human cells. To determine whether galectin-9 can trigger a calcium flux in neutrophils, intracellular calcium mobilisation was monitored following stimulation with 500 nM galectin-9 (Figure 5.6). A rapid increase in cytoplasmatic calcium was observed immediately after addition of galectin-9. This augmentation reached a maximum after 50 sec and was then sustained for over 7 min without returning to basal levels (data not shown). To confirm that this effect was caused by galectin-9 binding to β-galactoside in the TIM-3 receptor, neutrophils were preincubated with lactose (30 mM) or sucrose (30 mM) as a negative control. Co-treatment with lactase significantly decreased galectin-9-evoked intracellular calcium flux, whereas control sucrose treatment did not reverse galectin-9 effects (Figure 5.7).
Figure 5.6 Galectin-9 induces intracellular calcium mobilisation. Neutrophils (1.25 × 10⁶/100 μl) were incubated with Fluo-4 (5 mM) according to the manufacturer's instructions for 30 min at 37°C. Intracellular calcium mobilisation was monitored after addition of 500 nM galectin-9 (gal-9). Data shown are representative of three independent experiments.
Figure 5.7 Galectin-9 induced intracellular calcium mobilisation is inhibited by lactose. Neutrophils (1.25 × 10^6/100 μl) were incubated with Fluo-4 (5 mM) according to the manufacturer's instructions for 30 min at 37°C. Neutrophils were then preincubated in the presence or absence of lactose (30 mM) or sucrose (30 mM) before addition of galectin-9 (500 nM). Intracellular calcium mobilisation was monitored after addition galectin-9 (gal-9). Blockade of galectin-9 with lactose abrogated the galectin-9 evoked intracellular calcium response in neutrophils. Data shown are representative of three independent experiments.
Although the dose employed to stimulate neutrophils (500 nM) is half the previously reported one for other human immune cells (Kashio et al., 2003), it is still much higher than the values documented in the lung (Katoh et al., 2010, Vega-Carrascal et al., 2011). Therefore we asked if the calcium mobilisation was a dose-dependent effect in neutrophils. To this end, neutrophils were treated with increasing levels of galectin-9 (50, 100 or 500 nM). All galectin-9 treatments triggered immediate intracellular calcium mobilisation (Figure 5.8). Interestingly, the higher doses of galectin-9 resulted in a sustained elevation of cytosolic calcium that lasted over 7 min and never returned to basal levels. On the other hand, a lower, more physiological dose of galectin-9 (50 nM), induced a rapid calcium spike that returned to near basal intracellular calcium levels after 20-30 sec (Figure 5.8). These results not only indicated that galectin-9 may activate neutrophils at physiological concentrations, but they also suggested potentially different intracellular signalling mechanisms initiated by different levels of galectin-9 as evidenced by differences in cytosolic calcium fluxes. Collectively our data indicate that galectin-9 can stimulate neutrophils, putatively via TIM-3 interactions.
Figure 5.8 Galectin-9-evoked intracellular calcium mobilisation is dose dependent. Neutrophils (1.25 × 10^6/100 µl) were incubated with Fluo-4 (5 mM) according to the manufacturer's instructions for 30 min at 37°C. Calcium mobilisation was monitored after addition of galectin-9 (0, 50, 100 or 500 nM). Data shown are representative of three independent experiments.
5.3.2.2 Galectin-9 induces intracellular signalling events in neutrophils

Release of intracellular calcium stores into the neutrophil cytosol has been shown to be involved in a series of signalling events leading to actin polymerisation, translocation of fMLP receptors to the plasma membrane, NADPH oxidase assembly, degranulation, etc. Therefore, we asked if galectin-9-evoked calcium flux could trigger known signalling cascades and in particular tyrosine phosphorylation of intracellular and membrane proteins, an early event in neutrophil signal transduction. To test this point, whole cell aliquots were taken from unstimulated neutrophil samples or following neutrophil stimulation with galectin-9 (50 nM) at 0, 1, 2, 5 and 10 min to study the role of galectin-9 in early signalling events in the neutrophil. Samples were immediately dissolved and boiled in sample loading buffer and the level of tyrosine phosphorylation was evaluated by western blot analysis using the anti-phosphotyrosine antibody 4G10. The results from this experiment illustrated in Figure 5.9 revealed that galectin-9 treatment elicited a potent increase in the levels of tyrosine phosphorylation evident after 1 min. The maximum level of phosphorylation was achieved at 2 min post-treatment and phosphorylation then decreased slowly without reaching control levels by 10 min. Prominent tyrosine phosphorylation bands were detected at approx. 20, 38, 50-60 kDa in galectin-9 treated neutrophils. As expected, constitutive levels of phosphorylation were observed in resting neutrophils which increased slowly with time in the absence of treatment (left panel) although not to the levels seen in galectin-9 stimulated neutrophils (right panel). The galectin-9-evoked phosphorylation pattern differs from tyrosine phosphorylation via TIM-3 receptor reported in other cell types (Anderson et al., 2007, Cho et al., 2011) suggesting that signal transduction mechanisms initiated by galectin-9 are cell-specific.
Figure 5.9 Galectin-9 induces tyrosine phosphorylation. Neutrophils were incubated at 37 °C in the presence of galectin-9 (0 and 50 nM). Aliquots were taken at 0, 1, 2, 5 and 10 min, centrifuged and cell pellets were lysed in 2 × sample loading buffer containing protease and phosphatase inhibitors. Whole cell lysates (corresponding to 3 × 10^6 cells) were loaded onto 4-12% (w/v) SDS-PAGE gradient gels and induction of tyrosine phosphorylation was analysed by immunoblotting with anti-phosphotyrosine antibody 4G10. Arrows indicate bands corresponding to differential tyrosine phosphorylation. Blots shown are representative of results obtained from four different neutrophil donors.
Having established that galectin-9 was capable of inducing tyrosine phosphorylation, we next investigated if the MAPKs p38 and ERK 1/2 were also activated by tyrosine phosphorylation following galectin-9 treatment. MAPK are widely known to be involved in multiple neutrophil functions. Of interest, they have been identified as mediators of neutrophil degranulation, NADPH activation and phagocytosis (Simard et al., 2010, Simard et al., 2011), key features of neutrophil antimicrobial activity. The time course of the experiment for p38 is shown in Figure 5.10 and it is similar to that illustrated in Figure 5.9 for total tyrosine phosphorylation. Galectin-9 treatment (50 or 500 nM) induced a rapid phosphorylation of p-38. Increased p-38 phosphorylation was detected with all treatments at 1 min and peaked at 2 min after neutrophil stimulation with galectin-9, subsequently, p-38 phosphorylation decreased to levels below basal phosphorylation. An immunoblot for total p-38 was included to prove equal loading. As expected, untreated cells also exhibited basal p-38 phosphorylation that decreased at 10 min, but no sharp peak in p38 phosphorylation at 2 min in the absence of galectin-9. Interestingly, another member of the galectin family, galectin-3 (Abedin et al., 2003) has been found to induce neutrophil phagocytosis and degranulation in a p-38 dependent manner (Fernández et al., 2005). Thus these results suggest that galectin-9 may stimulate neutrophil function via p-38 phosphorylation.

The role of ERK 1/2 in neutrophil priming is not that clear, p-38 and JNK phosphorylation was shown to be involved in TNFα and LPS priming. However, TNFα and LPS priming was found to be ERK 1/2 independent (Ward et al., 2000). Furthermore, inhibition of p-38 and JNK but not ERK 1/2 has recently been demonstrated to block neutrophil degranulation (Simard et al., 2010). Thus, ERK 1/2 appeared to play a prominent role in microbial killing due to phagocytosis rather than degranulation or neutrophil oxidative burst (Simard et al., 2011). Because each branch of the MAPKs is implicated in different neutrophil functions depending on stimulus, we examined the role of ERK 1/2 in neutrophil activity. To this end, ERK 1/2 phosphorylation was monitored by western blot analysis following galectin-9 treatment. An immunoblot for total ERK2 was used as a loading control. The same samples employed in total tyrosine and p-38 phosphorylation were used for this purpose. The levels of ERK 1/2 phosphorylation followed a rolling
pattern that peaked at 1 min following galectin-9 treatment (Figure 5.11) although the extent of the phosphorylation was more modest than the one observed for p-38 (Figure 5.10). Collectively, the data presented from the phosphorylation studies constitute interesting preliminary data of galectin-9 as a stimulant of neutrophil signal transduction. In particular these data reveal MAPKs as likely mediators of galectin-9 induced intracellular signalling in the neutrophil and anticipate a role for galectin-9 in multiple neutrophil functions.
Figure 5.10 Galectin-9 induces p38 phosphorylation. Neutrophils were incubated at 37 °C in the presence of galectin-9 (gal-9; 0, 50 or 500 nM). Aliquots were taken at 0, 1, 2, 5 and 10 min, centrifuged and cell pellets were lysed in 2 x sample loading buffer containing protease and phosphatase inhibitors. Whole cell lysates (corresponding to 3 x 10^6 cells) were loaded onto 10% (w/v) SDS-PAGE gradient gels and induction of p38 phosphorylation was analysed by immunoblotting with the anti-phospho-p38 (p-p38). Blots were stripped and reprobed with an antibody against total p38 (p38) as a loading control. Blots shown are representative of results obtained from four different neutrophil donors.
Figure 5.11 Galectin-9 induces ERK1/ERK 2 phosphorylation. Neutrophils were incubated at 37 °C in the presence of galectin-9 (gal-9; 0, 50 or 500 nM). Aliquots were taken at 0, 1, 2, 5 and 10 min, centrifuged and cell pellets were lysed in 2 × sample loading buffer containing protease and phosphatase inhibitors. Whole cell lysates (corresponding to 3 x 10⁶ cells) were loaded onto 10% (w/v) SDS-PAGE gradient gels and induction of ERK1/ERK2 phosphorylation was analysed by immunoblotting with the anti-phospho-ERK1/ERK2 (p-ERK1/2). Blots were stripped and reprobed with an antibody against ERK2 (ERK2) as a loading control. Blots shown are representative of results obtained from four different neutrophil donors.
5.3.2.3 Galectin-9 is a novel neutrophil priming agent

Although neutrophil extracellular microbial killing has been described in the form of "neutrophil extracellular traps" known as NETS, upon phagocytosis, killing occurs mainly within the phagocytic vacuole. Intravacuolar killing in neutrophils occurs as a combination of oxidants produced by NADPH oxidase and the action of proteases and antimicrobial proteins released from intracellular granules. Both mechanisms are regulated by complex signalling cascades that involve MAPK among other mediators. Prompted by the data suggesting a role for galectin-9 as a novel neutrophil stimulus of MAPK signalling cascade, we next focused on the role of galectin-9 in NADPH activation and neutrophil respiratory burst.

NADPH oxidase constitutes an enzymatic complex with multiple cytosolic (p40phox, p47phox and p67phox) and membrane components (p22phox and gp91phox, a heterodimer also known as cytochrome b558). Neutrophil activation leads to phosphorylation of p47phox and translocation of the cytosolic components to the membrane. Following association with cytochrome b558, the NADPH oxidase system is fully assembled and activated by several stimuli that trigger production of $O_2^-$ (Babior, 1984). Since this process requires a substantial amount of oxygen consumption, it has been termed "respiratory burst". $O_2^-$ rapidly dismutates into $H_2O_2$ and becomes chlorinated by MPO to produce hypochlorous acid, a potent oxidant. This system can be studied in vitro by stimulation of NADPH oxidase activation on the plasma membrane using different agents and measuring extracellular $O_2^-$ release. Among the different agents, phorbol 12-myristate 13-acetate (PMA) has been shown to be a potent inducer of the respiratory burst. PMA structure mimics diacylglycerol, a natural activator of PKC (protein kinase C) and can directly activate PKC to phosphorylate p47phox and consequently induce NADPH oxidase activation. PMA (500 ng) was chosen as a positive control in which to examine the role of galectin-9 as a direct activator of NADPH oxidase in the neutrophil. Neutrophils were stimulated with increasing concentrations of galectin-9 (50, 100, 250 and 500 nM) and $O_2^-$ generation was monitored over 30 min by the cytochrome C assay (Figure 5.12).
Figure 5.12 Effect of galectin-9 on $O_2^-$ production. $O_2^-$ production was analysed in neutrophils stimulated in the presence or absence of galectin-9 (gal-9; 0, 50, 100, 250 or 500 nM). PMA (500 ng) was used as a positive control. Reduction of cytochrome C was measured at 550 nm every min. Results shown are average ± SD of n=3 independent experiments. $O_2^-$ production following gal-9 treatment (50 nM) was not significantly different from control. All other treatments were significantly different. Differences among treatments were analysed by 2-way ANOVA compared to unstimulated control, * p<0.05.
As expected, PMA induced a robust NADPH oxidase activation with a maximal production of $O_2^-\}$ at 10 min that remained elevated for another 20 min. This positive control was necessary to confirm adequate functioning of the NADPH oxidase complex in our system. In contrast, $O_2^-\}$ generation was not detected in unstimulated neutrophils. Low galectin-9 treatment (50 nM) was not sufficient to induce $O_2^-\}$ production, indicating that physiologically relevant levels of extracellular galectin-9 do not induce oxidative burst in human neutrophils. Interestingly, higher doses of galectin-9 (100-500 nM) induced a significant ($p<0.05$) extracellular production of $O_2^-\}$ in a dose-dependent manner compared to unstimulated neutrophils. These results are in line with the observed intracellular calcium sustained elevation induced by galectin-9.

Because galectin-3 has been shown to prime neutrophils for $O_2^-\}$ production (Yamaoka et al., 1995), we next tested if galectin-9 could also act as a priming agent. Neutrophils were treated for 10 min with increasing levels of galectin-9 (0, 50, 100, 250 and 500 nM), then fMLP (1µM final concentration) was added and cytochrome C reduction was monitored for 25 min to measure $O_2^-\}$ generation (Figure 5.13). In line with previous reports, fMLP alone is a poor inducer of $O_2^-\}$ production (Bergin et al., 2010). However, galectin-9 treated cells enhanced NADPH oxidase activity in a dose dependent manner. Of note, 50 nM dose of galectin-9, which did not trigger $O_2^-\}$ production alone (Figure 5.12), induced a marked release of $O_2^-\}$ following fMLP treatment indicating that galectin-9 in physiological doses can act as a neutrophil priming agent. Whether this effect is TIM-3 mediated or requires galectin-9 β-galactoside binding activity remains to be confirmed.

Collectively, these data indicate that extracellular galectin-9 can act as a neutrophil priming agent towards fMLP at physiological concentrations, revealing a novel role for galectin-9 in neutrophil function.
Figure 5.13 Galectin-9 primes neutrophils for fMLP induced $O_2^-$ production. Super $O_2^-$ production was analysed in neutrophils stimulated in the presence or absence of galectin-9 (0, 50, 100, 250 or 500 nM). Reduction of cytochrome C was measured at 550 nm every min. After 10 min, fMLP, (final concentration 1 μM) was added to cells to assess whether galectin-9 is capable of neutrophil priming. $O_2^-$ production was monitored every min for an additional 30 min. $O_2^-$ production in all galectin-9 treatments were significantly different compared to control neutrophils not pretreated with galectin-9. Differences among treatments were analysed by 2-way ANOVA comparec, * p<0.05.
5.3.2.4 Galectin-9 induces neutrophil degranulation via TIM-3 interaction

As galectin-9 was found to induce intracellular calcium mobilisation and tyrosine phosphorylation, in particular p-38 phosphorylation, processes involved in neutrophil degranulation, we next asked whether galectin-9 had an effect on neutrophil degranulation, the other key component of intravacuolar microbial killing in neutrophils. To this end, we adopted an *in vitro* approach where neutrophil treatment with stimulants would activate extracellular granule content degranulation to mimic the events that naturally occur within the phagocytic vacuole. Neutrophils isolated from peripheral blood from healthy volunteers were stimulated with galectin-9 (50 or 500 nM) or left untreated as a negative control for 15, 30 or 60 min at 37°C. Degranulation was monitored by western blot analysis of cell supernatants with antibodies against MPO, LL37 and MMP-9 as markers of primary, secondary and tertiary granules respectively.

Remarkably, galectin-9 stimulation triggered degranulation of all three granule subsets investigated in this study, azurophilic (primary), specific (secondary) and gelatinase (tertiary) granules (Figure 5.14). Of interest, galectin-9 was capable of inducing degranulation of primary granules in a dose response manner measured as an increase in MPO in supernatants. In addition to MPO, primary granules contain NE and other serine proteases such as Cathepsin G and PR3 which are all capable of inducing bacterial killing by different mechanisms (Korkmaz et al., 2008). Release of primary granule contents in resting neutrophils rarely occurs and it usually requires previous stimulation with a priming agent (i.e. TNFα) or a very potent stimulant (PMA) (Owen et al., 1997). Therefore, although physiological levels of galectin-9 were not capable of inducing O$_2^-$ production (Figure 5.12), 50 nM galectin-9 treatment was sufficient to induce MPO release, revealing galectin-9 as a potent stimulant for primary granule degranulation. In contrast, galectin-9 effect on secondary granules was less prominent, 50 nM galectin-9 induced a modest release of LL-37 at any time point compared with unstimulated supernatants.
Figure 5.14 Galectin-9 induces neutrophil degranulation in a dose and time dependent manner. Neutrophils were treated with galectin-9 (gal-9; 0, 50, 500 nM) and aliquots were taken at 15, 30 and 60 min. Cells were centrifuged and supernatants were collected. Supernatants (equivalent to $1 \times 10^5$ neutrophils) were resolved on 4-12% (w/v) SDS-PAGE gradient gels. Membranes were probed for (A) MPO, (B) LL37 or (C) MMP-9 as markers of primary, secondary and tertiary granules respectively. Blots are representative of at least three independent experiments carried out with different blood donors.
However, a supra-physiological dose of galectin-9 (500 nM) did trigger LL-37 release at all the studied time points in a time-dependent manner. Similarly, tertiary granule mobilisation to the plasma membrane was increased following treatment with a high dose of galectin-9 (500 nM) in a time-dependent manner; whilst a lower dose of galectin-9 did not induce a marked release of MMP-9.

In order to further elucidate the mechanism underlying galectin-9-evoked neutrophil degranulation, we asked whether this effect was mediated by TIM-3 receptor. Neutrophils were preincubated with a human TIM-3 antibody or relevant IgG control for 10 min at room temperature. Subsequently, galectin-9 (0, 50 or 500 nM) was added to induce degranulation for 15 min at 37°C. Neutrophils were treated with TNFα (30 ng) and fMLP (1 μM) as a positive control (Figure 5.15). TIM-3 blockade with an antibody significantly reduced galectin-9-evoked MPO release, whereas IgG control did not abrogate this effect. Similarly, TIM-3 antibody blocked degranulation of secondary and tertiary granules.

Taken together our results indicate that galectin-9 acts as a potent inducer of neutrophil degranulation via interaction with TIM-3. This effect is particularly evident for primary granule release.
Figure 5.15 Galectin-9 induces neutrophil degranulation via TIM-3 interaction. Neutrophils were incubated for 10 min at RT in the presence or absence of 10 μg/ml TIM-3 blocking antibody or mouse IgG control. Next, neutrophils were treated with TNF-α (30 ng/10^7 neutrophils), 1 μM fMLP or galectin-9 (0, 50, 500 nM) and aliquots were taken at 15 min. Cells were centrifuged and supernatants were collected. Supernatants (equivalent to 1 × 10^5 neutrophils) were resolved on 4-12% (w/v) SDS-PAGE gradient gels. Membranes were probed for (A) MPO, (B) LL37 or (C) MMP-9 as markers of primary, secondary and tertiary granules respectively. Blots are representative of at least three independent experiments carried out with different blood donors.
5.4 Discussion

The number of different types of cells expressing TIM-3 is ever expanding, and is not limited to Th cells anymore. Indeed, TIM-3 function appears to differ in cells from the adaptive and innate immune system (Anderson et al., 2007), or undoubtedly in non-immune cells as we (Vega-Carrascal et al., 2011) and others (Huang et al., 2010, Wu et al., 2010) have demonstrated. Nonetheless, TIM-3 expression in neutrophils has not been identified to date. Furthermore, specific studies on the role of galectin-9, a TIM-3 bona fide ligand, are rare within the literature. To the best of our knowledge, the only report on the effect of galectin-9 in human neutrophils is a study on the lack of neutrophil chemotactic activity as opposed to eosinophils (Matsumoto et al., 1998). Extrapolating from the most recognised effect of galectin-9 in other cell types, induction of apoptosis would be the likely effect in neutrophils. Although this point has not been specifically studied in human neutrophils, two recent studies report that galectin-9 induces apoptosis in myeloid leukemia derived cell lines (Kobayashi et al., 2010) and specifically in HL-60 cells (Kuroda et al., 2010). Other neutrophil functions that may be affected by TIM-3 include NADPH oxidase activation, neutrophil degranulation, bacterial killing and release of inflammatory modulators. Prior to embarking on an investigation into the role of TIM-3 within neutrophils, we set out to determine whether TIM-3 was expressed by this cell type.

Our data demonstrate TIM-3 expression in resting neutrophils by western blot analysis of whole neutrophil protein obtained by TCA precipitation. This technique preserves neutrophil proteins intact and protects them from rapid proteolytic degradation (Painter et al., 2006). These results were further confirmed by flow cytometric analysis of peripheral blood neutrophils, which along with immunoblots from biotinylated membranes, indicated that TIM-3 is present on the neutrophil membrane albeit there was some indication that TIM-3 may be cleaved from the membrane surface and released extracellularly. This latter finding raises the question of whether neutrophils release a soluble form of TIM-3. Indeed a soluble form of the TIM-3 receptor has been identified in murine splenocytes (Geng et al., 2006). This soluble receptor was found to be a splice form of the full length receptor lacking the cytosolic domain, that exhibited antitumor immunity in a mouse.
model via modulation of effector T cell function (Geng et al., 2006). Whether human soluble TIM-3 is also produced and if neutrophil-shed TIM-3 may have any effect in vivo remains unknown.

Surface expression of neutrophil receptors changes widely following recruitment into the lung. For instance, C5a receptor, CD11b and Fc gamma receptor II have been shown to be upregulated in human extravasated neutrophils whilst L-selectin was downregulated on the surface of neutrophils recruited into the airways (Fortunati et al., 2009). Therefore, in order to assess whether TIM-3 would still be present on neutrophils within the lung, TIM-3 expression was examined in extravasated peritoneal neutrophils obtained after thioglycollate injection. TIM-3 surface expression was confirmed in this context suggesting that TIM-3 may be present on the surface of neutrophils recruited to the airways. TIM-3 function may therefore be effective under normal lung inflammatory conditions but unlikely to occur in the CF setting due to proteolytic degradation of the receptor and possibly any other interacting protein (i.e. galectin-9) as previously illustrated in Chapter 4.

Next, we investigated whether TIM-3 expression was abnormal in CF neutrophils. In contrast with the observed upregulation of TIM-3 in CF bronchial epithelial cells, CF circulating neutrophils did not show significantly altered expression compared with healthy controls. This finding was corroborated by qRT-PCR, flow cytometry and western blot analysis of whole neutrophil protein obtained by TCA precipitation. These data support the notion of differential TIM-3 expression depending on cell type and activation stage, and raises questions about the mechanism underlying TIM-3 expression in immune and non-immune cells.

Prompted by the result demonstrating TIM-3 expression on the neutrophil surface, we next considered the role of TIM-3 in neutrophil function. TIM-3 has been implicated in modulation of macrophage and monocyte functions. For instance, in macrophages, TIM-3 was involved in modulating the expression of co-stimulatory receptors CD80 and CD86 (Frisancho-Kiss et al., 2007), in activation and release of inflammatory mediators.
including IL-1β (Jayaraman et al. 2010), NO (Zhao et al., 2009), TNFα (Anderson et al., 2007) and IL-12 (Zhang et al., 2011). Interestingly, TIM-3 has also been shown to be involved in bacterial killing (Zhao et al., 2009). Therefore we focused the study of TIM-3 mediated neutrophil activity on functions directly related with the prototypical neutrophil role as an antimicrobial mediator. Specifically degranulation and activation of the NADPH oxidase system were at the heart of this study.

Galectin-9 involvement in the neutrophil respiratory burst has not been reported to date. Galectins have been shown to be capable of directly inducing O$_2^-$ production in primed neutrophils (Almkvist et al., 2002; Karlsson et al., 1998). However, galectin-3 has been shown to induce O$_2^-$ release in unstimulated neutrophils, albeit at micromolar concentrations (Yamaoka et al., 1995). In our hands, galectin-9 could only promote O$_2^-$ generation at supra-physiological concentrations (<50 nM) in unstimulated neutrophils. However, a low dose of galectin-9 (50 nM) was shown to be sufficient to enhance fMLP-induced O$_2^-$ production. Collectively our data indicate that galectin-9 can act as a priming agent at physiological concentrations and may play a role in bacterial killing by promoting O$_2^-$ generation. Whether this is effect is TIM-3 mediated or not remains to be tested.

Additionally, our data show that galectin-9 at a physiologically relevant concentration is capable of inducing neutrophil degranulation and in particular release of primary granules via TIM-3. This interaction was confirmed by studies using a TIM-3 blocking antibody. This finding is of interest since primary granules are the most difficult to mobilise (Pham, 2006). Indeed serine proteases contained in primary granules are considered to be the true effectors of intravacuolar bacterial killing (Pham, 2006).

The exact mechanism behind galectin-9/TIM-3 promotion of NADPH oxidase activation and neutrophil degranulation would require extensive and thorough investigations. Nevertheless, within this chapter we presented evidence that galectin-9 induces intracellular calcium mobilisation and distinctive intracellular phosphorylation events that are usually associated with neutrophil function. In particular, we have shown that MAPK signalling pathways could be initiated by galectin-9 treatment and these effects are dose-
dependent. Additional studies using TIM-3 antibodies to clearly determine the role of TIM-3 in these events, supported by the use of specific kinase inhibitors, would enlighten our currently limited knowledge on the pathways underlying TIM-3/galectin-9 mediated neutrophil functions.

To summarize, in Chapter 5 TIM-3 expression on resting and primed neutrophil cell surfaces was demonstrated. Galectin-9 was shown to stimulate signal transduction in the neutrophil leading to MAPK signalling cascades and intracellular calcium mobilisation, indicating an active role in modulating neutrophil activity. We also provided evidence for a role of galectin-9 in neutrophil degranulation and NADPH oxidase activity, possibly via TIM-3 interactions. Taken together we have identified a novel role for TIM-3/galectin-9 in neutrophil function with potentially important consequences in neutrophil antimicrobial activity. Although abnormal TIM-3 expression was not detected in CF resting neutrophils compared to healthy controls, any TIM-3/galectin-9 mediated activity in the CF lung would be abrogated by proteolytic degradation. Thus, a better understanding of the TIM-3 related mechanisms in the neutrophil could yield some explanations on the defective killing ability of neutrophils in the CF airways. Chapter 6 will focus on investigating the potential neutrophil antimicrobial functions mediated by TIM-3, and in particular whether TIM-3 or galectin-9 have a direct effect on neutrophil-mediated bacterial killing.
CHAPTER 6: ROLE OF TIM-3 IN BACTERIAL KILLING

6.1 Introduction

Having established in Chapter 5 a role for galectin-9 in neutrophil activation in terms of intracellular calcium mobilisation, induction of intracellular signalling cascades, degranulation and priming of the neutrophil NADPH oxidase, we set out to investigate whether galectin-9 plays a role in neutrophil-mediated bacterial killing via TIM-3 interaction.

Galectin-9 expression is enhanced in host cells upon infection by a variety of pathogens. For instance, galectin-9 expression has been shown to be raised in different models of viral infection including human endothelial cells infected with Dengue virus (Warke et al., 2003), liver Kupffer cells infected with Hepatitis C (Mengshol et al., 2010) or lymphoid cells in a mouse model of Herpex simplex virus infection (Sehrawat et al., 2010). Indeed, double-stranded RNA which mimics viral infection, is sufficient to induce galectin-9 expression in vascular endothelial cells (Imaizumi et al., 2007, Ishikawa et al., 2004) highlighting the prominent role of galectin-9 in viral infections.

Bacterial components have also been reported to enhance galectin-9 expression. In particular, LPS a major component of the outer cell membrane in gram-negative bacteria, was shown to induce galectin-9 expression in vascular endothelial cells (Imaizumi et al., 2007) in line with our results in bronchial epithelial cells (Vega-Carrascal et al., 2011). Similarly, LPS treatment also induced galectin-9 intracellular production in a monocytic cell line, leading to the transcription of pro-inflammatory cytokines IL-1α and IL-1β (Maatsura et al., 2009). Moreover, LPS intraperitoneal administration in a mouse model of sepsis induced a marked increase in galectin-9 levels in peritoneal lavage fluid (Tsuboi et al., 2007). Of interest, galectin-9 deficient mice were more susceptible to LPS-induced inflammation, whereas galectin-9 transgenic mice or concomitant treatment with exogenous galectin-9 suppressed LPS-induced inflammatory effects (Tsuboi et al., 2007). Moreover, bacterial infections have been shown to directly induce expression of galectin-9, as evidenced in human periodontal ligament cells infected with Porphyromonas
*gingivalis* (Kasamatsu et al., 2005). However, galectin-9 enhanced expression following bacterial infection is not limited to gram-negative bacteria as a recent study has shown that lung macrophages increase galectin-9 expression following infection with *Mycobacterium tuberculosis* (Jayaraman et al., 2010). Of note, inflammatory cytokines can also modulate galectin-9 expression. In particular, IFN-γ, which is crucially involved in orchestrating the inflammatory response in microbial infections, has been shown to induce galectin-9 expression in several cell types, including endothelial cells (Imaizumi et al., 2002), macrophages (Mengshol et al., 2010) and fibroblasts (Asakura et al., 2002). Collectively, all these data suggest a role for galectin-9 in both bacterial and viral infections.

Galectin-9 may have several functions during microbial infections. Firstly, galectin-9 has been found to stimulate innate immune responses by recruitment of immune cells, notably neutrophils (Tsuboi et al., 2007) and eosinophils (Imaizumi et al., 2002). Secondly, galectin-9 appears to be involved in the inhibition of the adaptive immune response. There are numerous examples in the literature on the role of galectin-9 limiting T cells responses due to induction of T cell apoptosis, whilst expanding T regulatory cells (see (Wiersma et al., 2011) for a comprehensive review). Galectin-9 appears to exert this effect, at least in part, through the expansion of immunosuppressive granulocytes, also known as myeloid-derived suppressor cells (Dardalhon et al., 2010). Interestingly, pathogens can modulate the host immune response by expressing galectin-9 homologues. For instance, *Toxascaris leonine*, a dog parasite, expresses a galectin-9 homologue that downregulates the inflammatory response in the gut by inhibiting the production of cytokines by T cells in a mouse model of colitis (Kim et al., 2010b).

Thus, galectin-9 appears to play a role in triggering innate immunity and inhibiting the adaptive immune response during microbial infection to limit tissue damage. These functions most likely require TIM-3 interactions. However, a more direct role in bacterial killing has recently been revealed for TIM-3/galectin-9. TIM-3 expressed on T cells promoted host resistance to *Mycobacterium tuberculosis* by limiting intracellular replication in infected macrophages. Macrophage infection increased surface expression
of galectin-9 that could bind to TIM-3 on Th1 cells. This interaction, in which TIM-3 acted as a ligand and galectin-9 as a receptor, triggered caspase-1 production and IL-1β secretion in macrophages, leading to bacterial killing (Jayaraman et al., 2010). This reverse TIM-3/galectin-9 signalling mechanism has been only reported for Mycobacterium tuberculosis-infected murine macrophages. An additional study has shown that Escherichia coli (E. coli) killing by human peripheral monocyctic cells was TIM-3 dependent as phagocytosis and killing mechanisms were inhibited in the presence of soluble TIM-3 (Zhao et al., 2009)

6.2 Aims of this chapter

There is compelling evidence for galectin-9 overexpression following microbial infection. Additionally, TIM-3 has been involved in bacterial killing in peripheral blood monocyctic cells and macrophages. However, whether TIM-3 or galectin-9 play a role in neutrophil-mediated bacterial killing has not been investigated to date. We hypothesised that galectin-9 may bind to LPS on the surface of gram-negative bacteria facilitating interaction with TIM-3 expressed on neutrophils, resulting in enhanced neutrophil mediated killing. The purpose of this chapter was to investigate the role of TIM-3 in bacterial killing. To this end the following aims were set:

1. To establish whether TIM-3 is involved in neutrophil-mediated bacterial killing, in particular SA and PA, two archetypal infecting organisms associated with CF lung disease.
2. To determine whether galectin-9 is involved in neutrophil-mediated bacterial killing.
3. To elucidate whether TIM-3/gal-9 mediated neutrophil killing is bacterial species dependent.
6.3 Results

6.3.1 TIM-3 is involved in killing of gram-negative but not gram positive bacteria

Because TIM-3 has been shown to induce antagonistic effects on bacterial killing by different immune cells, we firstly investigated the role of TIM-3 in neutrophil killing of the most predominant pathogens in CF, namely PA and SA (LiPuma, 2010). To this end, rhTIM-3-Fc, a soluble version of TIM-3 was employed. This human recombinant chimeric protein was linked to human IgG1 Fc tail (Pro100-Lys330) to facilitate purification. rhTIM-3-Fc has been reported to enhance killing of *Mycobacterium tuberculosis* by infected murine macrophages (Jayaraman et al., 2010). On the other hand, rhTIM-3-Fc has been shown to block *E. coli* killing by purified human peripheral blood monocytic cells depleted of T and B cells (Zhao et al., 2009).

As shown in Fig. 6.1, the presence of rhTIM-3-Fc fusion protein (5 μg/ml) significantly (p<0.05) increased survival of serum opsonised PA compared to controls at the earlier time points of 2 min (44% versus 22% survival) and 4 min (22% versus 7 % survival). This result is in agreement with the previous study in human immune cells which demonstrated that blocking TIM-3 interaction with serum-opsonised bacteria resulted in decreased bacterial killing (Zhao et al., 2009). In contrast, rhTIM-3-Fc treatment did not alter SA neutrophil mediated killing significantly (Fig. 6.2). Bacterial killing of serum opsonised bacteria was similar to the IgG1-Fc tail (Pro100-Lys330) control (result not shown). These results suggest that TIM-3 expressed in neutrophils is directly involved in a killing mechanism specific for PA not present in SA.
Figure 6.1 Blockade of neutrophil TIM-3 reduces PA killing. Neutrophils were preincubated at RT for 10 min in the presence of 5 μg/ml rhTIM-3-Fc or control IgG1Fc (control IgG). Neutrophils (1 × 10⁶) were then exposed to PA (1 × 10⁷) opsonised with 50 % autologous serum. Aliquots were removed at 2, 4, 8 and 16 min. Serial dilutions of the bacterial suspensions were plated on triplicate tryptic soy agar plates and incubated at 37°C. Viable bacterial colony forming units were counted the following day. Data shown as mean ± SEM of n=3 independent experiments with neutrophils from different donors. Statistical significance analysed by t-test at each time point. * p< 0.05.

Figure 6.2 Blockade of neutrophil TIM-3 does not affect SA killing. Neutrophils were preincubated at RT for 10 min in the presence of 5 μg/ml rhTIM-3-Fc or control IgG (IgG1-Fc) (control IgG). Neutrophils (1 × 10⁶) were then exposed to SA (1 × 10⁷) opsonised with 50 % autologous serum. Aliquots were removed at 2, 4, 8 and 16 min. Serial dilutions of the bacterial suspensions were plated on triplicate tryptic soy agar plates and incubated at 37°C. Viable bacterial colony forming units were counted the following day. Data shown as mean ± SEM of n=3 independent experiments with neutrophils from different donors. Statistical significance analysed by t-test at each time point. Data were not statistically significant between treatments.
6.3.2 Galectin-9 binds to LPS

The previous experiment has demonstrated that TIM-3 on neutrophils is involved in bacterial killing. However, there are no known bacterial components that can directly bind to TIM-3. Thus, it is likely that the interaction blocked by soluble TIM-3 is galectin-9/TIM-3, since galectin-9 is the only bona fide ligand for TIM-3. Indeed galectin-9 has been found to be present in human serum of healthy individuals (Mengshol et al., 2010, Chagan-Yasutan et al., 2009), albeit in low concentrations (<50 pg/ml; 0.002 nM). Furthermore, galectin-9 has been reported to bind to Leishmania major through specific interaction with poly-β-galactosyl epitopes, promoting interactions with macrophages. Another member of the galectin family, galectin-3 has also been found to bind to glycans expressed on the surface of parasites such as Leishmania major (Pelletier and Sato, 2002) or Schistosoma mansoni (van den Berg et al., 2004) and fungus such as Candida albicans (Fradin et al., 2000). Additionally, galectin-3 can also bind to gram-negative bacteria including Klebsiella pneumonia (Mey et al., 1996) and an attenuated strain of Salmonella typhimurium, BRD509 (Li et al., 2008). Thus, galectins present in serum can potentially act as pathogen recognition molecules against a varied range of microorganisms.

Interestingly, blockade of TIM-3 interaction with serum-opsonised bacteria only reduced killing of PA and not SA. The main difference between these two pathogens is that they belong to a different class of bacteria. PA is a gram-negative bacterium, whereas SA is a gram-positive bacterium. One of the main characteristics of gram-negative bacteria is the presence of LPS on the outer surface which is greatly responsible for the pathogenicity of this class of bacteria. Of note, although there are no reports of galectin-9 directly binding to bacteria, galectin-3 has been shown to bind to LPS from different strains of gram-negative bacteria (Gupta et al., 1997, Li et al., 2008, Mey et al., 1996). Since galectin-9 binding to LPS has not been reported in the literature, we assessed whether galectin-9 was capable of binding to LPS from PA origin by a modified solid binding ELISA. Commercially available LPS purified from PA (50 μg/well), was employed to coat a 96-well plate and wells were incubated overnight with increasing levels of galectin-9 (0-1.5 μM, 0-50 μg/ml). Binding was determined by using a galectin-9 specific antibody. A
positive response could only be obtained if direct interaction between LPS and galectin-9 occurred. Fig. 6.3 shows that galectin-9 binds to PA LPS. Maximal binding was obtained at approximately 100 nM galectin-9.

Once galectin-9 was shown to be capable of binding PA-derived LPS, we next investigated whether galectin-9 could directly bind to bacteria. To this end, both SA and PA cultures were fixed with paraformaldehyde and incubated overnight with galectin-9 (500 nM). Importantly, galectin-9 did not bind to SA (Fig. 6.4A). In contrast, galectin-9 was detected on the surface of PA bacteria (Fig. 6.4B). Collectively these data suggest that galectin-9 binds to PA and may possible bind to other gram-negative bacteria via LPS interaction on bacterial surfaces. Conversely, since SA belongs to gram-positive bacteria and does not express LPS, it does not bind to galectin-9. Thus, galectin-9 is revealed as a novel pathogen recognition molecule, specific for gram-negative bacteria and in particular PA.
Figure 6.3 Galectin-9 binds to LPS. High-binding 96-well plates were coated with commercial PA LPS (50 μg/ml). Galectin-9 (0-1.5 μM, 0-50 μg/ml) was added and binding was assessed after incubation with galectin-9 antibody (10 μg/ml) followed by a HRP-conjugated antibody. After a final wash, ABTS was added and galectin-9/LPS binding was confirmed by measuring absorbance at 405 nm. Measurements were done in triplicate. Results are shown as mean ± SD and are representative of two independent experiments.
Figure 6.4 Galectin-9 binds to PA but not to SA. Galectin-9 binding to bacteria was measured by flow cytometry. Paraformaldehyde fixed PA and SA (1 × 10⁶) were incubated overnight at 4°C with PBS containing 500 nM galectin-9 or PBS alone. After washing, bacteria were incubated with 20 μl PE-labelled galectin-9 antibody or PE-labelled mouse IgG control for 30 min at RT. Galectin-9 binding to SA (A) or PA (B) was assessed by flow cytometry. (C) Quantification of galectin-9 binding to bacteria. Results are representative of three independent experiments. Graphs show mean fluorescence intensity (MFI) +/- SEM. Data analysed by t-test compared to PBS control, * p< 0.05.
6.3.3 Galectin-9 treatment does not exhibit direct bactericidal activity against gram-negative bacteria

As our data demonstrated that galectin-9 binds to LPS, we next investigated the antibacterial properties of galectin-9 alone. In this study we hypothesised that galectin-9 binds to bacteria via LPS, facilitating interaction with TIM-3 expressed on the neutrophil surface and promoting enhanced bacterial killing. Therefore, it was important to firstly determine if galectin-9 alone exhibited antibacterial effects. Direct antimicrobial activity of galectin-9 has not been reported in the literature, however galectin-3 has been shown to reduce growth of the gram-positive bacterium *Streptococcus pneumonia* (Farnworth et al., 2008) and to have antifungal activity against *Candida albicans* (Kohatsu et al., 2006). Therefore, we investigated if galectin-9 had any direct effect on PA viability. PA bacteria (1.5 × 10⁷ CFU) were incubated in PBS with or without galectin-9 (final concentration 50 or 500 nM) at 37°C for 30 min or 2 h. None of these concentrations had a detrimental effect on bacterial growth (Fig. 6.5A) suggesting that, unlike galectin-3, galectin-9 does not have direct bacteriostatic or bactericidal properties towards PA. Similar results were obtained with *B. cepacia* a group of gram-negative bacteria that have also been shown to colonise the airway of CF patients (LiPuma, 2010) (Fig. 6.5B).
Figure 6.5 Galectin-9 is not bactericidal towards gram-negative bacteria. PA (A) or B. cepacia (B) cultures were incubated with galectin-9 (50 and 500 nM) for 30 or 120 min, diluted and plated onto tryptic soy agar plates. Bacterial growth was assessed the following day (n = 3) Figure shown is representative of two independent experiments.
6.3.4 Galectin-9 opsonisation of PA induces bacterial killing

Having established that galectin-9 can bind to gram-negative bacteria via interaction with LPS on PA, we next asked if galectin-9 opsonisation would be sufficient to induce bacterial killing by neutrophils. To this end, PA cultures were opsonised with different doses of galectin-9 instead of serum and then subjected to a neutrophil killing assay. Interestingly, as shown in Figure 6.6, opsonisation with 50 nM galectin-9 was sufficient to induce an average of 25% more killing after 2 min compared to non-opsonised bacteria (70% versus 95% survival; p<0.05). This enhanced killing promoted by opsonisation with galectin-9 was sustained throughout the duration of the reaction with an average increase in PA killing of 19% at 16 min (54% versus 73% survival; p<0.05). Surprisingly, increasing the dose of galectin-9 during the opsonisation process to 100 nM or 500 nM did not increase bacterial killing, but abrogated the enhanced effect observed with 50 nM to levels similar to non-opsonised bacteria. These results were consistently obtained in independent experiments with neutrophils from at least three different donors. Survival percentages of serum-opsonised bacteria were included as a positive control. Therefore, these results indicate that galectin-9 opsonisation is sufficient to induce approximately 20% of neutrophil-mediated killing. However, this effect appears to be dose-specific (50 nM galectin-9) rather than dose-dependent.
Figure 6.6 Galectin-9 opsonisation of PA enhances bacterial killing. PA bacteria (1 × 10⁷) were opsonised in PBS containing 0, 50, 100 or 500 nM galectin-9 for 30 min at 37°C. After washing, PA were incubated with neutrophils (1:1) and aliquots removed at the indicated times. Serial dilutions were plated in tryptic soy agar plates in triplicate. Serum-opsonised PA killing is illustrated as a positive control (serum). Results shown are mean ± SEM of n=3 independent experiments with neutrophils from different donors. Percentage survival of PA bacteria opsonised with galectin-9 (50nM) was significantly lower compared to PBS controls. All other gal-9 concentrations were not statistically significant. Data analysed by 2-way ANOVA followed by Bonferroni post-test. * p< 0.05.
6.3.5 Galectin-9 opsonisation of PA induces bacterial killing via TIM-3 interaction

We have demonstrated in Chapter 5 that interaction of exogenous galectin-9 with TIM-3 promoted neutrophil intracellular signalling that activated neutrophil functions required for bacterial killing including reactive species production and mobilisation of granules containing proteolytic enzymes. To address whether the mechanism underlying galectin-9 mediated PA killing requires TIM-3 interaction, neutrophils were pretreated with TIM-3 blocking antibody (10 µg/ml) for 10 min. This antibody binds to the IgV domain of TIM-3 (Hastings et al., 2009) and has been shown to specifically block TIM-3/ligand interactions in other settings (Golden-Mason et al., 2009, Hastings et al., 2009). Treatment of neutrophils with TIM-3 blocking antibody did not alter bacterial killing of non-opsonised bacteria (data not shown). Conversely, blockade of TIM-3 abrogated the promotion of bacterial killing in PA opsonised with galectin-9 (50 nM) (Figure 6.7). This effect was not observed when neutrophils were pretreated with a relevant mouse IgG control. Therefore, our data demonstrate that a physiologically relevant dose of galectin-9 is sufficient to facilitate PA killing by neutrophils and that this effect is mediated through TIM-3 expressed on the neutrophil surface. This result contrasts with a recent report of enhanced bacterial clearance in infected macrophages due to interaction of galectin-9 on the surface of phagocytes with TIM-3 expressed on stimulatory T cells (Jayaraman et al., 2010). Instead, our results agree with previous reports of TIM-3 positively involved in killing of gram-negative bacteria (Zhao et al., 2009). Thus, galectin-9/TIM-3 interactions appear to be directly involved in bacterial killing by immune cells, although the mechanism may differ depending on cell type and be pathogen specific. In particular, we provide evidence for galectin-9 binding to LPS in gram-negative bacteria, promoting interaction with TIM-3 expressed on neutrophils and consequently enhancing bacterial killing.
Figure 6.7 Galectin-9-evoked enhanced PA killing is TIM-3 mediated. Neutrophils (1 \times 10^7) were incubated for 10 min at RT in the presence or absence of 10 \mu g/ml TIM-3 blocking antibody (TIM-3 Ab) or mouse IgG control (IgG). PA opsonised with 50 nM galectin-9 were incubated with neutrophils (1:1) and aliquots removed at the indicated time points. Serial dilutions were plated in tryptic soy agar plates in triplicate. Non-opsonised bacteria (non-opsonised) or bacteria opsonised with 50% (v/v) autologous serum (serum opsonised) were included as negative and positive controls respectively. Blockade of TIM-3 reversed the enhanced killing induced by PA opsonisation with galectin-9 (50 nM). Results shown are mean ± SEM of n=3 independent experiments with neutrophils from different donors. Percentage PA survival in samples treated with TIM-3 blocking antibody was not significantly different from non-opsonised bacteria. All other treatments were statistically significant. Data analysed compared to unopsonised bacteria control by 2-way ANOVA followed by Bonferroni post-test. * p< 0.05.
6.4 Discussion

Having established a role for galectin-9/TIM-3 interactions in neutrophil function, experiments within Chapter 6 focused on the bactericidal role of TIM-3 and galectin-9. TIM-3 has been previously reported to be implicated in bacterial killing although the proposed mechanisms of action are disparate. In a mouse model of tuberculosis infection, *Mycobacterium tuberculosis* infected macrophages increased intracellular killing following stimulation by TIM-3 expressing T cells (Jayaraman et al., 2010). In this paradigm galectin-9 served as a cell surface receptor in macrophages and TIM-3 was the ligand, reversing the traditional TIM-3/gal-9 signalling pathway. Although galectin-9 lacks intracellular phosphorylation motifs, galectin-9 activation by TIM-3 promoted caspase-1 processing and release of IL-1β which stimulated intracellular killing in an autocrine manner. In contrast, innate immune cells isolated from human blood peripheral monocyctic cells, markedly reduced *E. coli* killing in the presence of soluble TIM-3 (Zhao et al., 2009). In this model, TIM-3 expressed in phagocytes appeared to be directly involved in phagocytosis and intracellular production of reactive oxygen and nitrogen species.

In order to clarify if soluble TIM-3 enhanced or blocked bacterial killing by neutrophils, neutrophils isolated from whole blood were incubated with 5 μg/ml recombinant TIM-3 fusion protein at a dose within the range employed in previous studies (Jayaraman et al., 2010, Zhao et al., 2009). In our hands, TIM-3 treatment inhibited PA neutrophil mediated killing whereas it did not have any effect on SA compared with controls. These data also rule out galectin-9 acting as a receptor in neutrophils during PA or SA bacterial killing as previously proposed for *Mycobacterium tuberculosis* infected monocytes (Jayaraman et al., 2010), although a role in killing of other pathogens cannot be disregarded. Our data suggest instead that TIM-3 expressed in neutrophils is directly involved in bacterial killing in agreement with previous results linking TIM-3 function on phagocytes with *E. coli* killing (Zhao et al., 2009). However, how serum-opsonised bacteria stimulate bacterial killing in this context was not explained. We hypothesised that galectin-9 present in serum can actually bind to bacteria itself promoting interaction with neutrophils and possibly stimulating bacterial killing (Figure 6.8).
Figure 6.8. Schematic representation of role of TIM-3/galectin-9 in neutrophil-PA interactions. Galectin-9 binds to LPS expressed on the surface of gram-negative bacteria (i.e. PA). Galectin-9 opsonised bacteria interact with neutrophils via TIM-3 expressed on the plasma membrane.
Indeed, galectins can act as pathogen recognition molecules against a wide range of microorganisms, although different affinities for specific glycan epitopes may determine the immune response in the host. For instance, both galectin-3 and galectin-9 bind to *Leishmania major* poly-β-galactosyl epitopes, but only galectin-9 induced uptake of the parasite by macrophages (Farnworth et al., 2008). Moreover, LPS from different bacterial species has been shown to bind to galectin-3, including *Klebsiella pneumonia* (Mey et al., 1996), *E. coli, Salmonella typhimurium* (Li et al., 2008) and notably, PA (Gupta et al., 1997). LPS interaction with galectin-3 appeared to occur at two independent sites, through specific recognition of the lipid A/inner core region or the o-polysaccharide chain (Mey et al., 1996). The N-terminal part of galectin-3 has been shown to bind specifically to the rough form of LPS (LPS devoid of side chains). In contrast, the C-terminal part of galectin-3 specifically interacted with β-galactoside present on side-chains of smooth LPS (LPS containing the o-polysaccharide chain). N-terminal binding to LPS lipid A/inner core region explains why galectin-3 can bind to LPS from different strains of gram-negative bacteria since this area is highly conserved. On the other hand, the β-galactoside binding by the C-terminal region of galectin-3 would be more species specific as this area is highly variable even within species. To the best of our knowledge, galectin-9 has not been reported to bind to LPS, therefore, whether any of the carbohydrate recognition domains can bind to LPS and the nature of this interaction was previously unknown. Our data demonstrate that galectin-9 bound to PA but not SA, corroborating the selective role of galectin-9 in PA killing and potentially other gram-negative bacteria expressing LPS.

The observed increased bactericidal activity prompted us to confirm whether galectin-9 itself exhibited bactericidal or bacteriostatic properties. Although galectin-9 itself has not been found to have bactericidal properties, galectin-3 has been reported to reduce bacterial counts of the gram-positive bacterium *Streptococcus pneumonia* (Farnworth et al., 2008). Similarly, galectin-3 also exhibited antifungal properties against *Candida albicans* (Kohatsu et al., 2006). Our results showed that galectin-9 at physiological (50 nM) or a high dose (500 nM) did not display bactericidal properties against either PA or *B. cepacia*, indicating that galectin-9 was not directly involved in bacterial killing and the bactericidal properties required the activity of phagocytes.
Subsequently, we asked if galectin-9 was sufficient to induce PA killing by neutrophils. It is noteworthy that bone marrow-derived neutrophils from the galectin-3 knock-out mouse showed similar phagocytic levels compared to wild type controls. However, treatment with exogenous recombinant galectin-3 increased phagocytic ability of serum opsonised *Streptococcus pneumonia* (Farnworth et al., 2008), suggesting that exogenous galectins are involved in induction of bacterial phagocytosis by neutrophils. The data from this set of experiments indicated that galectin-9 opsonisation alone could promote bacterial killing by up to 25%. This effect was only achieved with 50 nM opsonisation, surprisingly, incubation with higher levels of galectin-9 did not increase bacterial killing and actually abrogated the bactericidal effect. We calculated that the levels of galectin-9 can reach approximately 1 nM in the airways using as a guidance the values obtained in non-CF bronchiectasis BAL, and accounting for dilution factors (Rennard et al., 1986). Using the same dilution factor, a value of up to 18 nM can be extrapolated from a different study that measured galectin-9 levels in BAL of patients with eosinophilic pneumonia (Kato et al., 2010). This value is within the range employed in our studies (50 nM). The reason why higher doses of galectin-9 proved ineffective in promoting bacterial killing remains unexplained. Observation of bacteria under the microscope after opsonisation with low (50 nM) and high dose of galectin-9 (500 nM) did not reveal any evidence of lack of bacterial motility or bacterial clumping that could explain a reduction in neutrophil killing due to the hindering of phagocytosis of large bacterial aggregates. It would be of interest to measure levels of galectin-9 on the surface of PA to determine whether bacteria respond by altering components of the cell surface with the higher doses of galectin-9. Alternatively, "hyperopsonised" PA may also overstimulate the neutrophils and induce different intracellular responses that could negatively impact upon bacterial killing.

Because we demonstrated that galectin-9 was capable of inducing bacterial killing by neutrophils, the role of TIM-3 was investigated using TIM-3 blocking antibodies directly targeted against the epitope responsible for galectin-9/TIM-3 interaction. Blockade of TIM-3 abrogated the neutrophil mediated killing of PA promoted by galectin-9 opsonisation confirming the implication of TIM-3 in the observed bactericidal effects. Our data suggest that TIM-3 is involved in killing of gram-negative bacteria, in particular PA
via interaction with galectin-9 that binds to bacterial LPS. This novel finding underscores the role of TIM-3 in infection and reveals another potential mechanism that contributes to the high bacterial burden in the CF airways.

We have previously shown in Chapter 4 that both TIM-3 and galectin-9 are completely absent in the adult CF lung due to proteolytic degradation by neutrophil derived proteases. The lack of bacterial killing via this novel mechanism may have a negative impact on the eradication of gram-negative bacterial infections, which may in part explain why PA is so successful in colonisation of the CF lower airways. CF infants are initially infected with SA and H. influenzae, but soon become infected with PA (Cystic Fibrosis Foundation Patient Registry, 2010). Initially these infections may be sporadic and alternate different strains, usually presenting a non-mucoid phenotype and responsive to antibiotic therapy (Burns et al., 2001). Eventually, chronic colonisation ensues, generally by a single infecting strain with a mucoid phenotype (reviewed in (Cramer et al., 2010, Govan and Deretic, 1996)). The median age for the onset of PA colonisation has been traditionally established at around 10 years of age (Cramer et al., 2010), however, PA presence in the lungs of pre-school children has been reported in several studies (Burns et al., 2001, Dakin et al., 2002, Pittman et al., 2011). Interestingly, the onset of PA infection coincides in time with the appearance of elevated levels of NE and a concomitant decline in galectin-9 presence in BAL. Our results have shown that TIM-3 is inactivated in airway epithelial cells by proteolytic degradation and hypothesise a similar inactivation occurring to airway neutrophils. The absence of galectin-9 or TIM-3 may result in impaired PA clearance by neutrophils and promote PA colonisation. Therefore, early intervention with aerosolised proteolytic resistant galectin-9 could be beneficial in preventing bacterial colonisation in the CF airways, preferably in conjunction with NE inhibitors such as A1AT.

In this chapter we have examined the role of galectin-9/TIM-3 bactericidal activity. We have demonstrated that TIM-3 expressed on neutrophils plays a direct role in bacterial killing. Opsonisation of PA with a physiologically relevant dose of galectin-9 promoted significant bacterial killing that could be reversed by blockade of neutrophil TIM-3.
receptors. This effect appeared to be gram-negative bacteria specific as galectin-9 did not bind to SA, a gram-positive bacterium. Indeed, the interaction between galectin-9 and PA may occur via LPS binding. This previously unidentified mechanism for neutrophil killing of PA is likely to be disrupted in the CF lung due to protease action with important consequences for bacterial clearance.
CHAPTER 7: GENERAL DISCUSSION

There is a growing body of evidence supporting the critical role of TIMs as modulators of the immune response in infection, autoimmunity, cancer, transplant tolerance and kidney and liver aseptic injury (reviewed in (DeKruyff et al., 2010). However, despite the initial discovery of the TIM gene family presence in a chromosomal region linked to airway hyperreactivity (McIntire et al., 2001), the role of TIM receptors in airway inflammation is poorly understood. TIMs have been implicated in asthma (Encinas et al., 2005, Hu et al., 2009, Kearley et al., 2007), sarcoidosis (Idali et al., 2009), and in mouse models of pulmonary fibrosis (Kim et al., 2010a), pneumonia (Wang et al., 2011a) and tuberculosis (Jayaraman et al., 2010), but their role in CF related lung inflammation has not been investigated. It has been reported that airway epithelial proinflammatory cytokine dysregulation and neutrophil-dominated inflammation are the main causes of morbidity in CF (Davis, 2006). Therefore, the aim of this study was to determine if TIMs signalling mechanisms on bronchial epithelium and neutrophils may also play a role in CF lung disease.

In line with the aims and objectives set for this study, Chapter 3 and Chapter 5 focused on the characterisation of TIM expression in bronchial epithelial cells and neutrophils respectively. Both cell types are believed to play a critical role in inflammatory processes in the CF lung. Chapter 4 examined the role of TIM-3 function in epithelial cells in the context of the CF airways, whereas work presented in Chapter 5 and Chapter 6 described the effects of TIM-3 and galectin-9 in neutrophil function.

Within Chapter 3, the expression of TIMs, in particular TIM-3, was characterised in bronchial epithelial cells. Our data demonstrate for the first time that TIMs are present and constitutively upregulated in CF bronchial epithelial cells. These results were confirmed in patient samples. A detailed characterisation of TIM-1 and TIM-3 expression in bronchial epithelial cells revealed that both receptors are expressed in CF airway epithelial cells. TIM-3 expression has been previously reported in lung tissue (van de Weyer et al., 2006) and in murine bronchial epithelial cells (DeKruyff et al., 2010) a result
supporting our data in human bronchial epithelial cells. TIM-1 expression is believed to be expressed only in damaged epithelium (Ichimura et al., 1998), although we have reported for the first time that TIM-1 is constitutively expressed in CF bronchial cells but not in healthy epithelium (Vega-Carrascal et al., 2011). Of note, a recent study has reported the expression of TIM-1 in human tracheal cells (Kondratowicz et al., 2011) although bronchial epithelium was not included in this latter study. Therefore it would be of interest if TIM-1 expression was confirmed to be confined to CF but not healthy bronchial epithelium, which could contribute to the endobronchial infections and pro-inflammatory status observed in the CF lung.

Additionally, we demonstrated the membrane localisation of a functional TIM-3 receptor, responsive to galectin-9 stimulation, which substantiates the role of this protein as a receptor in the human bronchial epithelium. We also initiated studies of TIM expression in the CF lung context, demonstrating that proinflammatory mediators highly present in CF BAL, such as LPS, can modulate TIM expression. In addition, we have established that TIM-3 may play a potential role in the inflammatory response within the CF lung as it is constitutively upregulated in the CF bronchial epithelium and LPS can further increase its expression. Furthermore, we have provided evidence for an association between CF and TIM expression, which constitutes the first report of a specific genetic mutation resulting in altered TIM-3 expression. The link between CFTR function and TIM expression was demonstrated by pharmacological inhibition of CFTR induced in normal cells which induced upregulation of TIM-3 and its ligand galectin-9. Although the exact mechanism by which the CFTR mutation results in up-regulation of TIM-3 remains unknown and has yet to be explored, it is likely that the constitutive upregulation of TIM-3/galectin-9 is a consequence of the described pro-inflammatory status of CF airway epithelium (Hunter et al., 2010, Vij et al., 2009).

Because TIM-1 was downregulated by LPS while TIM-3 was highly upregulated in vitro, we sought to confirm this point in patient samples. All the patient samples were colonised with PA which is an important source of LPS in the CF lung. In these bronchial brushings TIM-3 was overexpressed in CF patients whereas TIM-1 was not detected.
Subsequently, we focused only on TIM-3 function in bronchial epithelial cells as it will likely be primarily expressed once chronic bacterial colonisation with PA takes place. The physiological relevance of TIM-3 overexpression in CF bronchial epithelium was studied in detail in Chapter 4. TIM function in epithelial cells has not been reported to date, therefore we sought to characterise in detail the expression of galectin-9, the bona fide TIM-3 ligand (Zhu et al., 2005). Our data show that galectin-9 was upregulated in CF bronchial epithelial cells. However, galectin-9 was not detected in any adult CF sample tested although it was found to be present in BAL samples from patients with other respiratory diseases such as COPD and non-CF bronchiectasis, which display neutrophilic infiltration to a lesser extent and therefore lower levels of NE. Interestingly, galectin-9 was detected in BAL from infants (0-2 years old) and the levels decreased with age in a NE dependent manner. Since galectin-9 is known to be susceptible to proteolytic cleavage by NE and other proteases (Nishi et al., 2005) the decrease in galectin-9 levels in CF BAL is likely due to proteolytic degradation by neutrophil derived proteases. In addition to galectin-9, TIM-3 was also found to be readily degraded by serine proteases, in particular NE and PR3. Indeed, exposure of CF bronchial epithelial cells to a combination of both PR3 and NE completely abolished TIM-3 cell surface expression indicating that TIM-3 would be quickly inactivated in cells within the adult CF lung.

The unopposed action of neutrophil proteases has been shown to inactivate a variety of cell surface receptors in the CF lung, including the phosphatidylinerse receptor in macrophages (Vandivier et al., 2002), the CXCR1 chemokine receptor in neutrophils (Hartl et al., 2007) and proteinase-activated receptor-2 in bronchial epithelial cells (Dulon et al., 2003) with detrimental effects in clearance of bacteria and apoptotic cells and amplifying the pro-inflammatory response. Of major importance, the extraordinarily high protease burden in the CF lung also causes the degradation of the natural protease inhibitors A1AT, elafin and SLPI (reviewed in (Greene and McElvaney, 2009)) exacerbating the deleterious effect of NE and other proteases.
The second part of the study focused on the role of TIM-3 in the CF lung pathology, armed with the knowledge that any TIM-3/galectin-9 mediated effects would not be operational in the CF lung due to proteolytic inactivation. Although TIM-3 has been shown to bind to phosphatidyl serine receptors and mediate clearance of apoptotic bodies (Nakayama et al., 2009, Kobayashi et al.), we focused on the immunomodulatory properties of TIM-3. Given that TIM-3 signalling has never been reported in epithelial cells, no downstream targets could be monitored to assess TIM-3 function. Our data demonstrated that TIM-3 expressed on epithelial cells was phosphorylated upon galectin-9 engagement leading to production of cytokines. Specifically, galectin-9 treatment (50 nM) induced production of the potent neutrophil chemoattractant IL-8. Interestingly, IL-8 was mainly released apically in polarised monolayers, suggesting that TIM-3 would show predominantly apical localisation in the airways in line with the luminal TIM-3 expression reported in human and porcine tracheal explants (Kondratowicz et al., 2011). In addition to IL-8, IL-17 production was also induced by stimulation with galectin-9 (50 nM). This galectin-9 dose is physiologically relevant and in range with our own estimated levels of galectin-9 in non-CF BAL (1 nM) or reported by others in the lungs of patients with eosinophilic pneumonia (18 nM) (Katoh et al., 2010). Thus, our results indicated that TIM-3 in epithelial cells can modulate the inflammatory response and induce recruitment of neutrophils into the airways by producing cytokines following stimulation with galectin-9. Although this mechanism would primarily occur only during an infection episode, it is constitutively upregulated in CF and would therefore explain, at least partially, the reported high levels of neutrophils in the lung of CF infants (Tan et al., 2011).

TIM-3 is expressed on the surface of several immune cells (Su et al., 2010, Freeman et al., 2010); however, TIM-3 expression in neutrophils has not been reported to date. Therefore the expression of TIM-3 and its putative role in neutrophil function was the focus of Chapter 5. TIM-3 was found to be expressed on resting peripheral blood neutrophils. Cell surface analysis by western blot analysis of membrane samples obtained by biotinylation revealed that in addition to full size TIM-3, a smaller fragment (approx. 16 kDa) was also detected. This result indicated that TIM-3 expression on the surface may be dynamic and TIM-3 shedding by proteolytic action may occur, raising
questions about the putative role of a soluble TIM-3 released by neutrophils. Fluctuations of TIM-3 cell surface expression have been reported in human monocytes and TIM-3 shedding or receptor internalisation has been put forward as plausible explanations (Zhang et al., 2011). Indeed, a soluble form of TIM-3 produced by splice variation has been reported to have antitumor immunity properties in a mouse model of cancer (Geng et al., 2006). The storage of receptors in neutrophil cytosolic vesicles has been reported for galectin-3 (Feuk-Lagerstedt et al., 1999) and translocation of these receptors was required for galectin-3 to modulate O$_2^-$ production (Karlsson et al., 1998). Therefore, we examined whether TIM-3 expression was present in extravasated neutrophils using a mouse model of intraperitoneal thioglycollate injection. Our results indicate that TIM-3 would be present on the surface of neutrophils recruited to the airways, as thioglycollate elicited neutrophils also showed TIM-3 cell surface expression. Neutrophil treatment with galectin-9 showed that physiological concentrations of the TIM-3 ligand were capable of inducing intracellular calcium phosphorylation and activation of MAPK cascades. The demonstrated effects of galectin-9 (50 nM) in neutrophils described in Chapter 5 include neutrophil priming for enhanced fMLP-induced O$_2^-$ production and promotion of degranulation, in particular of primary granules. Taken together these results suggest that galectin-9 can activate the neutrophil and possibly enhance antimicrobial activity.

Additionally, we have also uncovered a novel TIM-3 mediated mechanism in neutrophil antimicrobial function within Chapter 6 (Figure 7.1). In the normal lung, galectin-9 expression would be increased following bacterial infection. Galectin-9 could opsonise gram-negative bacteria via LPS binding. This interaction would facilitate interactions with neutrophils via TIM-3 expressed on the surface, leading to enhanced neutrophil function in terms of promotion of O$_2^-$ production and degranulation within the phagocytic vacuole resulting in an increase in bacterial killing. Importantly, this mechanism would be limited to gram-negative bacteria as galectin-9 was not found to bind to gram-positive SA. This mechanism would necessarily be inactivated in the adult CF lung as the high burden of neutrophil derived proteases would result in degradation of both TIM-3 on the surface of neutrophils and soluble galectin-9. Interestingly, CF infants are initially infected with SA and H. influenzae, but soon become infected with PA (Cystic Fibrosis Foundation Patient
Registry, 2010). The median age for the onset of PA colonisation has been traditionally established at around 10 years of age (Cramer et al., 2010). Absence of galectin-9 and high levels of NE around that age, along with proteolytic degradation of TIM-3 in airway neutrophils, could jointly result in impaired clearance of gram-negative bacteria via the described mechanism. Indeed data from the US CF patient registry indicate that around that period infections with PA and other gram-negative bacteria such as *B cepacia* are more predominant. Thus restoration of galectin-9 and TIM-3 expression in the CF lung may assist in the clearance of PA, the archetypical CF pathogen.
Figure 7.1. Schematic representation of TIM-3/galectin-9 role in neutrophil-mediated bacterial killing mechanism. (1) In the normal lung bacterial infection would increase levels of galectin-9 that would bind to LPS on gram-negative bacteria (i.e. PA) resulting in bacterial opsonisation. (2) Galectin-9 opsonised bacteria would interact with neutrophil via TIM-3. (3) Galectin-9/TIM-3 interaction would trigger a series of signalling cascades including MAPKs and PI-3K leading to neutrophil activation. (4) Within the phagocytic vacuole TIM-3/galectin-9 interactions would trigger further neutrophil activation in the form of enhanced NADPH oxidase activation and $O_2^-$ production and increased intravacuolar degranulation, in particular primary granule release. As a result TIM-3/galectin-9 would enhance antimicrobial clearance of gram-negative bacteria. This paradigm would not be applicable for gram-positive bacteria (i.e. SA) due to a lack of galectin-9/LPS interaction.
Collectively, our data indicate that galectin-9/TIM-3 interaction in the airway epithelium promotes neutrophil recruitment due to stimulation of cytokine production by bronchial epithelial cells. In an acute infection context in the normal lung, this initial response would have a protective effect as neutrophils could be recruited into the lung to resolve the infection (Figure 7.2). Indeed galectin-9 conferred protection against LPS-mediated inflammation in mice, as galectin-9 raised in response to LPS intraperitoneal injection induced neutrophil recruitment into the peritoneal cavity (Tsuboi et al., 2007).

Subsequently, galectin-9 could also induce the termination of the local inflammatory response by selectively modulating apoptosis of these immune cells. Indeed, TIM-3 has been shown to be a negative modulator of Th-1 type immune responses (Zhu et al., 2005, Dardalhon et al., 2010).

This dual effect of TIM-3/galectin-9 on different cell types has previously been reported in dendritic cells versus T cells (Anderson et al., 2007), endothelial cells versus T cells (Huang et al., 2010) but not in epithelial cells or neutrophils. However, the effect of TIM-3 on resolution of airway inflammation by selective modulation of neutrophil apoptosis through galectin-9 interactions has yet to be evaluated. Whether galectin-9 can induce apoptosis in human neutrophils has not been reported, although galectin-9 triggered apoptosis of neutrophil related cell lines derived from chronic myelogenous leukemia patients (Kashio et al., 2003, Kobayashi et al., 2010, Kuroda et al., 2010).
In a chronic inflammatory scenario as observed in the CF lung, dysregulation of TIM-3 signalling may have a detrimental effect. The biological relevance of this observation would imply that a dysregulation of TIM-3 function in CF bronchial epithelial cells may contribute to the persistent neutrophil recruitment and acute inflammatory status of the CF lung. Initially, constitutively activated TIM-3/galectin-9 would result in cytokine production and neutrophil infiltration in the airways of CF newborns, even in the absence of bacterial infection. Indeed, elevated IL-8 and neutrophil airway infiltration prior to bacterial colonisation has been previously documented (Rosenfeld et al., 2001, Khan et al., 1995). As disease progresses, the presence of bacteria and LPS may amplify TIM-3 mediated neutrophil recruitment. Subsequently, upon chronic inflammation and infection, the lack of TIM-3 and galectin-9 protein due to proteolytic cleavage may result in a dysregulation of this signalling axis, which would then fail to terminate the inflammatory response due to lack of selective apoptosis of inflammatory immune cells. Indeed, disruption of this mechanism using TIM-3 or galectin-9 blocking antibodies has been reported to result in increased neutrophil infiltration and tissue damage in a model of liver ischemia/reperfusion (Uchida et al., 2010), experimental adhesion formation (Tzianabos et al., 2008) and multiple sclerosis (Zhu et al., 2005). Conversely, administration of recombinant galectin-9 ameliorated symptoms in a model of arthritis (Seki et al., 2007, Arikawa et al., 2009), diabetes (Chou et al., 2009) and multiple sclerosis (Seki et al., 2008). This beneficial effect, although still unclear, is believed to be caused by selective induction of apoptosis of pro-inflammatory cells and expansion of regulatory cells. For instance, galectin-9 has also been shown to exhibit a protective effect in lung infection in a mouse model of hypersensitivity pneumonitis induced by Trichosporon asahii (Arikawa et al., 2010) and in a model of acute lung injury (Kojima et al., 2011) through the expansion of immunosuppressive macrophages.

Clinical implications

Our findings have important therapeutic implications. The data presented suggest that manipulation of the TIM-3 signalling pathway may be of therapeutic value in CF and other lung diseases with a neutrophilic component. The hallmark of CF lung pathology is
persistent bacterial colonisation accompanied by a chronic cycle of neutrophil-driven inflammation. Therefore therapies aimed at controlling the aberrant immune response and reducing the NE burden would be tremendously beneficial. Hence, any therapeutic intervention targeting TIM-3 and galectin-9 in the CF lung should consider including antiprotease supplementation. Indeed, several studies have demonstrated restoration of anti-NE capacity in the CF airways following treatment with aerosolised antiproteases (reviewed in (Griese et al., 2008, Reeves et al., 2010a). In particular, inhaled A1AT has proved very promising. NE levels in the lungs of CF patients were reduced (Hartl et al., 2007, Hubbard et al., 1989) and PA killing rate improved (Griese et al., 2007, McElvaney et al., 1991) following aerosolised A1AT treatment. Aerosolisation of recombinant SLPI to CF patients has also been shown to increase anti-NE protection in the lung epithelium surface (McElvaney et al., 1992), although the beneficial effects disappeared after 12 h administration as opposed to the longer anti-NE protection afforded by aerosolised A1AT treatment (Smith et al., 1989). A number of synthetic protease inhibitors such as sivelestat and midostaurin have also been tested but have not reached clinical practice yet, mainly due to undesired side effects (Griese et al., 2008). In addition, semi-synthetic inhibitors obtained by chemical modification of natural antiproteases are currently being developed.

In light of the data reported in this thesis, it would be interesting to test if supplementation of galectin-9 can ameliorate CF lung symptoms in vivo providing a novel therapeutic treatment for CF patients. Treatment could include aerosolised galectin-9 as TIM-3 is expressed apically in the lung and also to avoid undesired systemic effects, in particular downregulation of Th1 and Th17 cell functions that are required for the effective recruitment of neutrophils into the lung. Indeed systemic administration of galectin-9 has proved deleterious in a model of Klebsiella pneumoniae lung infection. In this model of systemic administration of galectin-9 resulted in a reduction in Th cells and diminished neutrophil recruitment into the lung with the consequent lack of microbial killing (Wang et al., 2011a).

Moreover, restoration of TIM-3/galectin-9 levels in the CF lung may have other effects in addition to modulation of the immune response or enhancement of antibacterial activity.
For instance, galectin-9 can also act as a urate transporter (Lipkowitz et al., 2004, Graessler et al., 2000). Urate, along with glutathione, is an important antioxidant produced in the lung to counteract PA–induced oxidative damage to lung tissue (Day et al. 2004) and they are reduced in CFTR knockout mice (Velsor et al., 2001) and adult CF BAL (Roum et al., 1993). Thus, degradation of galectin-9 by neutrophil derived proteases may contribute to the harmful oxidative burden in the CF lung.

The data presented warrant further investigations and postulate TIM signalling mechanisms as exciting research niches. Characterisation of the proteins and the pathways underlying these mechanisms will allow design of personalised therapies and the development of new diagnostic tools for CF disease progression.
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“Dysregulation of TIM-3/Galectin-9 pathway in the cystic fibrosis airways”

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Abbreviations used in this paper: A1AT, alpha-1-antitrypsin; BAL, bronchoalveolar lavage; Cath G, cathepsin G; CF, cystic fibrosis; CFTR CF transmembrane conductance regulator; CFTRinh172, CFTR Inhibitor-172; LSC, laser scanning cytometry; NE, neutrophil elastase; PR3, proteinase-3; rhTIM-3, recombinant human TIM-3; TIM, T-cell Ig and mucin domain containing molecule; SLPI, serine leukoprotease inhibitor.
Introduction

Cystic fibrosis (CF) is the most common lethal genetic disease in Caucasians that is caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR) chloride channel (1, 2). CF patients suffer from persistent pulmonary infections accompanied by chronic neutrophil-dominated inflammation that results in severe lung injury and ultimately death. Several mechanisms have been proposed to explain how CFTR mutations lead to chronic lung disease in CF including altered ion transport across the airway epithelium and dehydration of the airway surface layer (3) and increased production of proinflammatory cytokines in the CF airway, arguably due to constitutive NFκB activation (4). Regardless of the initial cause, hyperinflammation in the CF lung occurs early and continues throughout life (5). This inflammatory state is further amplified by bacterial infections, in particular *Pseudomonas aeruginosa* (6, 7). The presence of bacteria promotes the airway epithelium to release proinflammatory cytokines such as the potent neutrophil chemoattractant IL-8 (8). This sustained inflammatory response recruits neutrophils in an attempt to resolve bacterial infection, yet paradoxically, chronic neutrophil stimulation also leads to neutrophil necrosis (9). As a consequence, neutrophils are not cleared effectively and neutrophil contents, including proteases, are released into the lung perpetuating the inflammatory cycle (10). Thus, a better understanding of the mechanisms involved in inducing and controlling inflammation is required for the successful design of novel intervention strategies in CF.

There is a growing body of evidence supporting the critical role of T-cell immunoglobulin and mucin-domain-containing molecules (TIMs) as modulators of the immune response in infection, autoimmunity, cancer, transplant tolerance and kidney and liver aseptic injury (reviewed in (11, 12)). Despite the initial discovery of the TIM gene family presence in a chromosomal region linked to airway hyperreactivity (13), the role of TIM receptors in airway inflammation is poorly understood. TIMs have been implicated in asthma (14-16), sarcoidosis (17) and pulmonary fibrosis (18), but their role in CF related lung inflammation has not been investigated. TIM-3 is expressed in a variety of immune cells, including Th-1 (19), Th-17 (20), dendritic cells (21), NK cells (22), NKT cells (23), monocytes (23), macrophages (24, 25), and mast cells (26). There is mounting evidence that TIM-3 is a potent regulator of both the adaptive and innate immune response, however, the mechanism depends on both cell type and specific disease. Indeed, TIM-3 has exhibited modulatory properties involved in tumour cell proliferation and immune evasion in non-immune cells including endothelial cells (27, 28) and epidermal melanocytes (29). TIM-3 function has also been implicated in neutrophil recruitment and tissue injury in several *in vivo* models (30-32). As neutrophil-dominated inflammation is one of the main causes of morbidity in CF (33), in the present study we investigated the expression of TIM-3 in bronchial epithelial cells and explored its role in the pathogenesis of CF lung disease.

Materials and Methods

Reagents

Unless stated otherwise, cell culture reagents were obtained from Gibco BRL (Karlsruhe, Germany). All other chemical reagents were purchased from Sigma-Aldrich (Dublin, Ireland) and were of the highest purity available.

Antibodies and recombinant proteins

Goat polyclonal anti-human TIM-3 was obtained from R&D Systems (Abingdon, UK). Rabbit polyclonal anti-human TIM-3 antibody (JM-308-100) was purchased from MBL International Corporation (Woburn, MA, USA). Mouse monoclonal anti-X-actin and anti-phosphotyrosine, clone 4G10 antibodies were purchased from Millipore (MA, USA). Secondary antibodies: donkey HRP-conjugated anti-goat, donkey anti-goat-FITC labelled antibodies were purchased from Santa Cruz Biotechnology (CA, USA). Anti-mouse HRP-conjugated antibodies were purchased from Pierce Biotechnology (IL, USA). Goat anti-rabbit HRP-conjugated (97074) was obtained from Cell Signaling Technology (Danvers, MA, USA). Human recombinant TIM-3 (rhTIM-3) was purchased from R&D Systems (MN, USA). Proteolytically stable human galectin-9 was a kind gift from GalPharma (Kagawa, Japan). Expression and purification of stable human galectin-9 was performed as previously described (34, 35). Briefly, galectins were expressed using the pET expression system (Novagen, Madison, WI) in *Escherichia coli* BL21 (DE3) and purified with a lactose-agarose column (Seikagaku Kogyo, Tokyo, Japan) and dialyzed against PBS. Endotoxin was eliminated with Celluline ETclean-L, a poly-e-lysine-conjugated resin (Chisso, Tokyo, Japan). Galectin preparations used in the present study were >95% pure as determined by SDS-PAGE, with <0.3 endotoxin units/ml (<0.03 ng/ml) detected by *Limulus* turbidimetric kinetic assays using a Toximeter ET-2000 instrument (Wako, Osaka, Japan). Protein concentration was determined using a bicinchoninic acid (BCA) assay reagent (Pierce Biotechnology) with BSA as a standard.

Cell culture
with primary antibody (1:20 in PBS) for 30 min. Then slides were washed and incubated with FITC-labeled secondary antibody (1:40 in PBS) for 30 min at 4°C in the dark. Control chambers were probed with secondary antibody only. Slides were washed with PBS, then cells were permeabilized and the nuclei stained in a single step using a 1:1 ratio of permeabilizing buffer (0.1% (w/v) sodium citrate, 0.1% (w/v) Triton X-100 in PBS) and 0.1 µg/ml solution of propidium iodide (PI) (Molecular Probes, Leiden, Netherlands) in PBS. Slides were washed with PBS and TIM-3 expression was quantified on a CompuCyte laser scanning cytometer (CompuCyte, MA, USA). Cell nuclei were identified by PI fluorescence (588 ± 10 nm) and TIM-3 surface expression was detected by FITC fluorescence (530 ± 20 nm) and quantified. At least 3×10^3 cells were counted in triplicate in each well.

**Immunocytochemistry and confocal microscopy**

HBE and CFBE cells (1×10^5) were grown in complete media in an eight-well chamber slide or on 13 mm diameter glass coverslips (YVR International Ltd, Dublin, Ireland). After removing media and washing with PBS, cells were fixed with either methanol or 4% (w/v) paraformaldehyde at room temperature for 20 min. Cells were washed again with PBS (×3) and blocked with 2% (w/v) BSA in PBS for 15 min at room temperature. Then cells were probed with the anti-TIM-3 antibody (1:50 in PBS) for 30 min at 4°C in the dark. After washing, cells were probed again with the corresponding FITC-labeled secondary antibody (1:100 in PBS). Cells were washed (×3) and mounted on a glass slide using Vectashield fluorescence mounting media (Vector Laboratories LTD, UK). Controls for this experiment included secondary antibody only. Cells were visualized by confocal microscopy using a LSM510 confocal microscope (Zeiss, Welwyn Garden City, UK). Images were captured at 40× magnifications under oil immersion.

**Cell surface biotinylation analysis**

Cell surface proteins from HBE and CFBE cells were isolated using a cell surface biotinylation kit (Pierce Biotechnology) according to the manufacturer’s instruction. Four 90% confluent 75-cm² flasks were quickly washed twice with 8 ml of ice-cold PBS per flask. 10 ml of ice-cold PBS containing 250 µg/ml EZ-Link sulfo-NHS-LC-biotin was added, and the cells were incubated with gently agitation at 4°C for 30 min. The biotinylation reaction was stopped by addition of 500 µl of quenching solution per flask, all contents were pooled and flasks were rinsed with a single 10 ml volume of TBS. The cells were pelleted after centrifugation at 500 × g for 3 min at 4°C. Cells were resuspended in 500 µl of the kit lysis buffer supplemented with protease inhibitors (100 µg/ml PMSF, 1mM NaVO₄, 1 µg/ml aprotinin) and incubated for 30 min on ice and sonicated using five 1-sec burst every 10 min using a Vibra-Cell VC 130 PB ultrasonic processor (Sonic & Materials, CT, USA). To improve solubilization efficiency cells were vortexed every 5 min for 5 sec. The cell lysate was clarified by centrifugation at 10,000 × g for 2 min at 4°C. The solubilized biotinylated proteins were isolated on immobilized NeutrAvidin agarose columns after 1 h incubation at room temperature with end-over-end mixing by rotation. The column was washed with 500 µl of the kit wash buffer supplemented with protease inhibitors followed by centrifugation (1000 × g for 1 min) and the flow-through was discarded (×4 times). The bound biotinylated proteins were eluted from the column by incubating at 95°C for 5 min with 400 µl sample buffer containing 50 mM DTT followed by a 2000 × g for 2 min centrifugation. The isolated proteins were stored at -20°C.

**Gel electrophoresis and western blot analysis**

Proteins were separated by SDS-PAGE on 10 or 12.5% (w/v) polyacrylamide gels. Proteins were transferred to a nitrocellulose membrane by semidy transfer at 150 mA for 90 min. Membranes were blocked with 5% (w/v) non-fat powdered milk in PBS containing 0.1% (v/v) Tween-20 (PBST) for 1 h at room temperature. Blots were incubated overnight at 4°C in blocking buffer containing antibodies against TIM-3 (1:1000), phospho-tyrosine (1:1000) or X-actin (1:1000) as a loading control marker where required. Subsequently, nitrocellulose membranes were washed for 30 min in PBST buffer (×2), probed with corresponding HRP-conjugated secondary antibody (1:1000) in PBST for 1 h and then washed again. Blots were developed with Immobilon western chemiluminescent HRP substrate (Millipore, Ma, USA) and visualised on the Syngene G:Box chemi XL gel documentation system (Synoptics, Cambridge, UK). Protein band size was determined by loading SeeBlue Plus2 Prestained molecular mass marker (Invitrogen, Bioscience, Ireland) on each gel.

**Inhibition of CFTR function**

HBE cells (1×10⁵) were serum starved overnight and then treated with 10 µM CFTRinh 172 (Calbiochem, Merck Chemicals Ltd, Nottingham, UK) in low serum medium for 48 h. Control cells were treated with vehicle 0.1% (v/v) DMSO. Cells were then collected in 0.5 ml of TRI reagent for subsequent RT-PCR analysis.

**TIM-3 expression under inflammatory conditions**
density was recorded using a microplate spectrophotometer (Bio-Rad, Tokyo, Japan). The galectin-9 ELISA limit of detection was 15.6 pg/ml.

Polarized cell culture.

CFBE cells were grown as polarized cultures at a liquid-liquid interface as previously described (42). Briefly, cells (7 × 10⁵) were seeded onto 1.0μm polystyrene hanging cell culture inserts (Millipore) and maintained in full media. Transepithelial electrical resistance (TEER) measurements were taken every 48h using the EVOM epithelial voltmeter (World Precision Instruments, Stevenage, UK) according to manufacturer’s instructions. Cells with TEER>1000Ω.cm² were used for experiments. Cells were serum starved overnight before treatment with galectin-9 (50 nM) in low serum media for 24 h. IL-8 levels in both apical (0.2 ml) and basolateral (1.2 ml) supernatants were measured by IL-8 ELISA.

Proteolytic degradation of recombinant TIM-3

Pooled CF or pooled non-CF bronchiectasis BAL (10 μl) was incubated with rhTIM-3 at 37°C. Non-CF BAL with non detectable NE activity or PBS was used as a negative control. Samples were collected at specific time points (0-30 min, 1-24 h). The reaction was immediately stopped by the addition of sample loading buffer (2 ×) followed by 10 min incubation at 95°C. Samples were stored at -20°C for subsequent western blot analysis. In some experiments, CF BAL samples were preincubated for 1 h at 4°C with 1 μl of 1.1mg/ml aprotinin, 5mg/ml soya bean trypsin inhibitor (SBTI), 0.2 M Pefabloc SC, 5 mg/ml E-64 (Calbiochem), 10 mg/ml pepstatin A, 0.5 M EDTA, 1mg/ml alpha-1-antichymotrypsin (A1CT, Calbiochem), 2.5 mM GM6001 (Millipore), 10 mg/ml benzamidine, 20 mg/ml O-phenanthroline, 10 mg/ml tumor necrosis factor-alpha protease inhibitor-1 (TAPI-1) (Calbiochem), 2mg/ml phosphoramide (Calbiochem), 3 mM N-(methoxyssuccinyl)-Ala-Ala-Pro-Val-chloromethyl ketone (CMK), 2mg/ml N-p-tosyl-l-phenylalanine chloromethyl ketone (TPCK), 10 mg/ml N-seryl-l-lysine chloromethyl ketone hydrochloride (TLCK), 1mg/ml alpha-1-antitrypsin (A1AT) (Athens Research and Technology, GA, USA), 1mg/ml elafin (ELA) (Proteo Biotech, Kiel, Germany) or 5mg/ml serine leukoprotease inhibitor (SLPI) (R&D Systems).

Cleavage of recombinant TIM-3 by serine proteases and determination of N-terminal sequence of proteolytic fragments

rhTIM-3 (100 ng) was incubated with 10⁻⁷ M, 5×10⁻⁸ M or 10⁻⁹ M of NE, Cath G (Elastin Products Co., Inc. (Owensville, MO, USA)) or PR3 (Athens Research and Technology) for 2 h at 37°C in 10 μl of 0.1 M HEPES buffer pH 7.5, containing 0.5 M NaCl, 0.05% (v/v) Brij-35. All reactions were stopped by addition of 2× loading sample buffer and boiling at 95°C for 10 min. Degradation of rhTIM-3 was analyzed by western blotting. For N-terminal-sequence analysis of proteolytic fragments, samples were separated by SDS-PAGE using a 10% (v/v) polyacrylamide gel and transferred to PVDF membrane. The membrane was rinsed in methanol for 5 sec and fixed in Ponceau staining buffer (40% (v/v) isopropanol, 10% (v/v) acetic acid, 50% (v/v) water and 0.1% (w/v) Ponceau S) for 20 min at room temperature. Proteolytic fragments were visualized after three 5 min consecutive washings in destaining buffer (45% (v/v) isopropanol, 45% (v/v) acetic acid, 10% (v/v) water). Bands corresponding to proteolytic fragments were excised from the membrane and analysed by N-terminal-sequence Edman degradation by Altabioscience (Birmingham, UK).

Cleavage of native TIM-3 by NE and PR3 on CFBE cell outer surface

CFBE cells (1×10⁷/well) were cultured in complete media in Lab-Tek chamber glass slides overnight. The media was then replaced by 1 ml low serum media containing NE (10⁻⁷ M), PR3 (10⁻⁷ M), or a combination of both (10⁻⁷ M each). Cells were incubated for 2 h at 37°C and then washed and fixed for TIM-3 detection on the cell outer surface by LSC as described above.

Neutrophil elastase activity assay

NE activity was determined using the chromogenic substrate N-(methoxyssuccinyl)-Ala-Ala-Pro-Val-p-nitroanilide specific for human NE. Samples (10 μl) were mixed with 90 μl of 3 mM substrate in assay buffer (0.5 M NaCl, 0.1% (v/v) Brij-35, 0.1 M HEPES, pH 7.5). Optical density was recorded at 405 nm for 5 min at 1 min intervals at 37°C using a Wallac 1420 Victor2 multilabel counter (PerkinElmer, MA, USA). NE activity in samples was calculated using an extinction coefficient of 9500 l×mole⁻¹×cm⁻¹. NE activity was expressed as milliunits/ml BAL (mU/ml BAL) defined as micromoles of peptide hydrolyzed/min/ml BAL. Samples were analyzed in duplicate.

Statistical analysis

Data were analyzed with GraphPad Prism 4.0 software package (GraphPad Software, CA, USA). Unless specified otherwise, data are expressed as mean ± SD of at least three independent experiments in triplicate. Differences were calculated by two-tailed Mann-Whitney test (2 data sets) or Kruskal-Wallis test with post-hoc Dunn’s multiple comparison test (≥2 data sets). The correlation between
expression (30). Therefore, we examined IL-8 production in CFBE cells upon galectin-9 stimulation. Galectin-9 (50 nM) promoted IL-8 production in CFBE cells as measured in cell culture supernatants after 24 h (Fig 4A). To determine whether IL-8 production was due to a TIM-3 signaling upon galectin-9 ligation, cells were exposed to galectin-9 pretreated with lactose (30 mM) or rhTIM-3 (100 ng/ml) which have been shown to prevent galectin-9 binding to TIM-3 (46). Blockade of the TIM-3/galectin-9 interaction by lactose or rhTIM-3 suppressed IL-8 production in CFBE cells (Fig 4B). These results suggest that galectin-9 is a natural agonist for TIM-3 in bronchial epithelial cells, inducing production of IL-8. Subsequently, we set out to establish the localization of TIM-3 in polarized cells. Apical treatment of CFBE cells with galectin-9 resulted in a 2-fold increase in IL-8 release demonstrating that TIM-3 is localized apically in CFBE cells (Fig 4C). Basolateral treatment also induced IL-8 release but to a lesser extent (1.5-fold increase), suggesting TIM-3 expression is also localized basolaterally.

**Galectin-9 is degraded in CF BAL**

Prompted by the results of in vitro experiments suggesting an upregulation of galectin-9 in CF (Fig 1C), we next measured the levels of galectin-9 in CF BAL by ELISA to determine whether galectin-9 function has relevance in vivo. Galectin-9 was not detected in any of the CF BAL samples tested, whereas galectin-9 was found in healthy controls (Fig. 5A). COPD and non-CF Bronchiectasis BAL samples served as inflammatory controls and showed varied levels of galectin-9 ranging from 0-1000 pg/ml (Fig. 5A). As BAL fluid is considered to be 25 to 100-fold diluted compared to epithelial lining fluid (39, 44), these values indicate that galectin-9 can be present in the nanomolar range on the lung surface in vivo in conditions with a marked neutrophilic component such as COPD or non-CF bronchiectasis, but not in adult CF.

Galectin-9 has been shown to be rapidly degraded by NE (47). Since this protease is abundantly present in CF BAL (48), we measured galectin-9 levels in CF infants and children’s BAL with low levels of neutrophil infiltration and therefore, low levels of neutrophil derived proteases. We found that galectin-9 levels decline with age (Fig. 5B) and correlate inversely with NE (Fig. 5C). These data collectively suggest that galectin-9 in the lung undergoes degradation by proteases, mainly neutrophil derived elastase.

**TIM-3 is degraded in CF BAL**

Since TIM-3 was not detected in CF BAL by western blot analysis (data not shown), we next investigated the effect of the proteolytic burden of the CF lung on the integrity of the galectin-9 receptor. rhTIM-3 (300 ng) was completely degraded after 24 h incubation at 37°C in CF BAL compared to the PBS control, as evidenced by the disappearance of the 64 kDa band on western blot analysis (Fig. 6A). In contrast, TIM-3 was only partially degraded in non-CF bronchiectasis BAL (Fig. 6A), possibly due to lower abundance of proteases compared to CF BAL (Table 1). A time course of TIM-3 proteolytic degradation showed that after only 10 min incubation, proteolytic degradation of the 64 kDa band commenced and TIM-3 was rapidly cleaved into smaller fragments of approximately 60 and 36 kDa (Fig. 6B). Longer incubation times showed a further fragmentation of TIM-3, possibly suggesting the involvement of various proteases at different stages of degradation. The 64 kDa band corresponding to the fully glycosylated form of TIM-3 was almost completely degraded after 2 h. After 24 h virtually no TIM-3 fragments were detected by western blot. In contrast rhTIM-3 was stable for 24 h in control non-CF BAL where NE activity was not detected and in PBS controls (data not shown).

Our next aim was to identify the proteases implicated in TIM-3 degradation. Since proteolytic degradation of TIM-3 has never been reported, a systematic approach was adopted to categorize the proteases involved in TIM-3 fragmentation. CF pooled BAL aliquots were preincubated with specific protease inhibitors for each class of enzyme for 1 h at 4°C before adding rhTIM-3. After 2 h incubation at 37°C, samples were analyzed by western blot to identify which protease inhibitor could prevent degradation of TIM-3. Firstly, non specific protease inhibitors targeting the main protease families were employed: E64 is an inhibitor of cysteine proteases, Pepstatin A (Pep A) an aspartic protease inhibitor, GM6001 a general metalloprotease inhibitor and Pefabloc (PFEA) a potent serine protease inhibitor. Only Pefabloc, and to a lesser extent Pepstatin A, prevented the degradation of full length TIM-3 implicating a serine and an aspartic protease in TIM-3 cleavage (Fig. 6C). Aprotinin (Apro) and soya bean trypsin inhibitor (SBTI) are inhibitors of the serine protease chymotrypsin and trypsin families respectively, but only SBTI appeared to have a moderate inhibitory effect on TIM-3 degradation. Since a non-identified metalloprotease has been implicated in TIM-1 shedding (49) a wider range of metalloprotease inhibitors were tested in comparison with GM6001. The metalloprotease inhibitors used included o-phenanthroline (OP), tumor necrosis factor-alpha protease inhibitor-1 (TAPI-1), EDTA and phosphoramidon (Phos). However, none of the metalloprotease inhibitors were capable of inhibiting the rapid degradation of the 64 kDa band corresponding to full size TIM-3 (Fig. 6D). In addition to Pefabloc, several serine protease inhibitors were used to classify more precisely the serine protease involved in TIM-3 cleavage (Fig. 6E). MeOSuc-AAPV-CMK
Discussion

We report for the first time that TIM-3 and its ligand, galectin-9, are constitutively overexpressed in CF airway epithelial cell surface, an observation further confirmed in CF patient samples. This finding implies a novel role for CFTR in TIM-3 expression as pharmacological inhibition of CFTR in normal cells induced an upregulation of TIM-3 and its ligand galectin-9. We also established that TIM-3 is a functional receptor capable of modulating the inflammatory response in bronchial epithelial cells. Cells stimulated with physiological relevant levels of galectin-9 induced IL-8 production by CF bronchial epithelial cells, indicating that TIM-3 may initiate the neutrophilic dominated inflammation in the CF lung. Additionally, we demonstrated that the expression of TIM-3 can be modulated by LPS which underscores the importance of TIM-3 under inflammatory conditions. Interestingly, both galectin-9 and TIM-3 undergo rapid proteolytic degradation in the CF lung due to serine protease activity. These data suggest that the dysregulation of TIM-3/galectin-9 signaling may play an important role in the pathogenesis of lung disease (Fig. 8). Furthermore, constitutive upregulation of this receptor and its ligand may reflect a proinflammatory state in CF bronchial epithelial cells (50, 51). Increased TIM-3 and galectin-9 expression in the lung may explain the early neutrophilic airway infiltration observed in CF newborns (52) and in aseptic CF animal models (53). Of clinical relevance, we demonstrated that both TIM-3 and galectin-9 undergo rapid proteolytic degradation by serine proteases in CF BAL which could impact upon the previously described role of galectin-9, inducing resolution of inflammation via apoptosis of immune cells (32, 55).

TIM-3 expression has been previously reported in lung tissue (45) and in murine bronchial epithelial cells (11), data which is in line with our results on the expression of TIM-3 in human bronchial epithelial cells and bronchial brushings. In addition, we also provide evidence for the first time that both TIM-3 and its ligand galectin-9 are constitutively upregulated in unstimulated CF bronchial epithelial cells. Of interest, this also constitutes the first report of TIM-3 overexpression linked to a specific genetic mutation. Having established a link between lack of CFTR and TIM-3 upregulation in vitro and ex vivo, we demonstrated a direct association between CFTR function and TIM-3 expression. Pharmacological inhibition of CFTR with CFTRinh172 resulted in an upregulation of TIM-3 and its ligand galectin-9 expression. It has been proposed that wild type CFTR cell membrane expression suppresses NFκB mediated inflammation (51), and that functional CFTR is required for such an inhibitory effect (50, 51). Inhibition of CFTR activity post CFTRinh172 treatment caused an increase in NFκB activity in HBE cells whilst activation of CFTR activity following forskolin treatment reversed the effect (51). Given that NFκB has been predicted to have several binding sites in the TIM-3 promoter region (54), the mechanism behind the upregulation of TIM-3 after CFTR pharmacological inhibition could involve modulation of NFκB activity.

The constitutive overexpression of TIM-3 and galectin-9 prompted us to investigate the role of TIM-3 in the CF lung. TIM-3 function in epithelial cells has not been studied to date, therefore we sought to characterize in detail TIM-3 expression in bronchial epithelial cells prior to investigating possible TIM-3/galectin-9 signaling mechanisms. Firstly, we demonstrated that TIM-3 was present on the surface of epithelial cells which would enable the binding of galectin-9. Confocal imaging revealed a clear membrane localization of TIM-3 in CFBE and HBE cells. Similar cellular distribution was observed in HEK293T cells transiently transfected with an expression vector for mouse TIM-3 (24). We also analyzed TIM-3 expression under pro-inflammatory conditions. Cells were treated with LPS (10 μg/ml) from Pseudomonas aeruginosa, a well-characterized component of CF BAL capable of inducing an inflammatory response in HBE and CFBE cells (40). This elevated concentration of LPS has physiological relevance as it has been estimated that CF lung surface can contain LPS levels as high as 40-70 μg/ml (40) LPS treatment stimulated TIM-3 expression in HBE cells and particularly increased TIM-3 levels in CFBE cells. In addition, TIM receptor expression has been shown to be modulated by other bacterial toxins, for instance TIM-4 which has been reported to be upregulated by Staphylococcal enterotoxin B (55) and cholera toxin (56). Interestingly, LPS treatment downregulated TIM-3 expression in dendritic cells (57), whereas LPS exposure upregulated TIM-3 expression in endothelial cells (28) indicating a cell-type specific response. Of note, another TLR-4 agonist, the alarmin high mobility group box 1 (HMGB1) also upregulated TIM-3 expression in endothelial cells in an NFκB and AP-1 dependent fashion (28). We have also presented for the first time a functional role for TIM-3 in bronchial epithelial cells. We have demonstrated that galectin-9 signaling via TIM-3 receptor on CFBE cells induced IL-8 production in vitro. Furthermore, the prominent apical distribution of functional TIM-3 suggests that galectin-9 present within the airway lumen can initiate pro-inflammatory responses via interaction with TIM-3. This latter result could impact upon early recruitment of neutrophils in vivo and the described exuberant neutrophil infiltration that perpetuates the inflammatory response in the CF lung (58, 59).
such as aerosolized AIAT augmentation therapy (68, 69).
References


Figure Legends

Figure 1. TIM-3 and galectin-9 mRNA are upregulated in CF. A, TIM-3 mRNA expression was measured in the bronchial epithelial cell line CFB140 (CFBE) compared to its non-CF counterpart 16BE140 (CFBE). B, Differential TIM-3 mRNA expression in bronchial brushings from healthy controls (n=7) and CF patients (n=5). C, Galectin-9 mRNA expression was measured in CFBE cells compared to HBE cells. D, Differential galectin-9 expression in bronchial brushings from healthy controls (n=7) and CF patients (n=5). HBE cells were untreated (ctrl) or treated with 10 μM CFTRinh 172 for 48 h and TIM-3 (E) or Galectin-9 (F) expression determined. The differential expression levels were analyzed by qRT-PCR relative to non-CF controls and normalized to GAPDH expression. Bars show mean ± SD, n=3, statistical significance analyzed by Mann-Whitney test. * p<0.05, ns= non significant. Results are representative of three independent experiments.

Figure 2. TIM-3 protein is overexpressed in CF and upregulated by LPS. A, Quantification of TIM-3 surface expression in unstimulated HBE and CFBE cells by laser scanning cytometry. A minimum of 3000 cells were counted. Bar graph shows mean fluorescence intensity (MFI) peak values for each point. Data shown as mean ± SEM, n=3. B, Representative immunoblot of TIM-3 protein levels in HBE and CFBE whole cell lysates. Glycosylated TIM-3 was detected as diffuse bands at 50 and 64 kDa. An immunoblot for actin served as a protein loading control. C, HBE (white bars) and CFBE (black bars) cells (1x10^5) were treated with LPS (10μg/ml) for 24 h or low serum media as control and TIM-3 cell surface expression analyzed by laser scanning cytometry. Statistical significance calculated by Mann-Whitney test. * p<0.05. All results are representative of a minimum of three independent experiments.

Figure 3. TIM-3 expressed on bronchial epithelial outer cell membrane is a functional receptor. A, HBE or CFBE cells (1x10^5/well) were grown on cover slips and probed with anti TIM-3 followed by FITC labeled secondary antibody (left panel). Controls included secondary antibody only (right panel). Cell nuclei were visualized by DAPI (blue) and expression of TIM was detected by green fluorescence. White arrows indicate cell surface localization. Images for each antibody treatment were captured using identical image capture parameters at 40 x magnification under oil immersion. Images are representative of three independent experiments. B, Localization of TIM-3 in the outer cell membrane of bronchial epithelial cells. Cell surface proteins were isolated using a cell surface biotinylation kit. Recombinant human TIM-3 (rhTIM-3) was used as a positive control in the immunoblot. C, Phosphorylation of TIM-3 upon galectin-9 stimulation. HBE or CFBE cells were either left untreated or treated with 500 nM galectin-9 (Gal-9) for 15 min. TIM-3 was immunoprecipitated (IP) from cell membranes with goat polyclonal anti-TIM-3 antibody. Normal goat-IgG (IgG) was used as a control of non-specific binding. Activation of TIM-3 was analyzed by western blotting (WB) employing a mouse monoclonal anti-phospho-tyrosine antibody (p-tyrosine) (upper panel). Blots were stripped and reprobed with rabbit anti-TIM-3 antibody to confirm equal levels of immunoprecipitated TIM-3 (lower panel). The image shown is representative of two independent experiments.

Figure 4. Galectin-9 induces IL-8 production via TIM-3 in CFBE cells. A, CFBE cells (1x10^5) were treated with 50 nM galectin-9 (gal-9) or left untreated (ctrl). After 24 h IL-8 levels were determined in cell culture supernatant by ELISA. Statistical significance analyzed by Mann-Whitney test. * p<0.05. B, Blockade of TIM-3/galectin-9 interaction abrogates IL-8 production. CFBE cells (1x10^5) were treated with 50 nM galectin-9 (gal-9) alone or in combination with 30 mM lactose (gal-9 + lact), or 100 ng/ml recombinant humanTIM-3 (gal-9 + rhTIM-3). IL-8 levels were determined in cell culture supernatants by ELISA and expressed as percentage increase compared to control. Results are expressed as mean ± SD, n=3. Statistical significance analyzed by Kruskal-Wallis test. * p<0.05. C, CFBE cells were grown at a liquid/liquid interface as polarized monolayers. Cells were treated with 50 nM galectin-9 (black bars) or left untreated (white bars). After 24 h IL-8 levels were determined in the apical and basolateral compartment by ELISA. Statistical significance analyzed by Mann-Whitney test. * p<0.05. Results are representative of at least three independent experiments for monolayers or two experiments for polarized cells.

Figure 5. Galectin-9 is degraded in CF BAL. A, Galectin-9 levels were measured by ELISA in BAL from non-CF Bronchiectasis (Bron) (n=10), adult CF (n=14), COPD (n=5) and healthy controls (HC) (n=5). Statistical significance calculated by Kruskal-Wallis test. * p<0.05 compared to adult cystic fibrosis BAL. B, galectin-9 levels were measured in CF patients aged 0-2 years (n=4), 2-8 years (n=5) and >18 years old (n=14). Statistical significance calculated by Kruskal-Wallis test. * p<0.05 compared to 0-2 years old group. C, Correlation of galectin-9 levels and NE activity in BAL analysed by one-tailed Spearman correlation (r=-0.9384, p=0.0002). Horizontal bars represent median values.