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Expression and Regulation of The Immune Modulatory Enzyme Indoleamine 2,3-dioxygenase (IDO) in Human Epithelial Cells

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Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

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JULY 2016

Abstract

Epithelial cells (ECs) are key players in the modulation of immune responses in addition to its main role as a barrier against external stimuli. Upon stimulation with pathogens/allergens, airway ECs can produce a wide range of innate mediators that recruit and activate immune cells which play a key role in maintaining immune homeostasis in the lungs. One such mediator is indoleamine 2,3- dioxygenase (IDO), which is a rate-limiting enzyme involved in tryptophan (TRP) catabolism. IDO plays a role in the modulation of immune responses to antigenic challenges and also acts as an anti-microbial factor. Therefore, the main aim of this project was investigating IDO expression, immune regulatory function and regulation pattern in human airway ECs in response to clinically relevant toll-like receptor (TLR) ligands and allergen extracts. This was done in 2D cultures and in a 3D model using ECs from healthy and diseased donors.

The data clearly demonstrated that ECs constitutively produce IDO which is up-regulated in response to IFN-γ. However, I have shown for the first time, that IDO production and activity is down-regulated in response to TLR ligands and allergen extracts. Indeed, using gene silencing, I demonstrated that 'resting' ECs (i.e. with high IDO expression) can suppress T

cell activation in an IDO-dependent manner, but this regulatory function is lost in response to TLR agonists mimicking bacterial or viral infections. These data provide new insight into how ECs, as part of their function in maintaining immune homeostasis in airways, can influence downstream innate and adaptive immune responses through the production of IDO.

In this study, I also focused on developing an immunocompetent 3D model of human airway ECs in regards to produce certain *in vivo* characteristics. In addition, the effect of EC differentiation state and cross-talk with other cells on IDO activity was investigated. My data showed that EC lines and primary human bronchial ECs (pHBECs) could be differentiated at the air-liquid interface (ALI) and form ZO-1 tight junctions. Moreover, ECs had higher TEER values when co-cultured with fibroblasts than when cultured alone which indicates that fibroblasts facilitate EC differentiation, highlighting the importance of paracrine interactions and cross-talk between cells in maintaining EC barrier function. Moreover, the data showed differential regulation of IDO activity on the basis of culture conditions which suggest that differentiation and co-culture status might affect the orientation and expression pattern of TLRs. Furthermore, our data in regards to IDO regulation in physiological and

pathophysiological settings demonstrated the differential regulation of IDO and considerably different IDO basal levels in healthy cells compared with diseased cells, emphasizing the immunosuppressive role of IDO. In conclusion, these advances in the understanding of IDO expression and regulation in different tissue/cell types in health and disease could contribute to delineating the potential role of IDO in inflammatory responses in different pathologies and enable the development of novel IDO-targeted therapeutic strategies.

Publications arising from this thesis:

Wejdan A. Aldajani, Fabian Salazar, Herb F. Sewell, Alan Knox, and Amir M. Ghaemmaghami. (2016). Expression and regulation of immune-regulatory enzyme indoleamine 2,3-dioxygenase (IDO) by human airway epithelial cells and its effect on T cell activation. Oncotarget. doi: 10.18632/oncotarget.11586.

Conferences attended:

1. W. Aldajani, F. Salazar, A. Knox, A. Ghaemmaghami. (2016). The immune-regulatory role of indoleamine 2,3-dioxygenase (IDO)-producing human lung epithelial cells. 9th Saudi Students' Conference, 13-14 February, Birmingham, UK.
2. W. Aldajani, F. Salazar, A. Knox, A. Ghaemmaghami. (2016). Expression and regulation of the immune modulatory enzyme indoleamine 2, 3-dioxygenase (IDO) in human epithelial cells. Immunology Showcase 4th February, Nottingham. UK.
3. W. Aldajani, F. Salazar, A. Knox, A. Ghaemmaghami. (2015). Expression and regulation of the immune modulatory enzyme indoleamine 2,3- dioxygenase

(IDO) in human epithelial cells. 4th European Congress of Immunology, 6-9 September 2015, Vienna, Austria.

4. W. Aldajani, F. Salazar, H. Harrington, A. Ghaemmaghami. (2014). Development of an immunocompetent model of human upper airway epithelium. 9th World Congress on Alternatives and Animal Use in the Life Sciences. 24-28 August, Prague, Czech Republic.
5. Wejdan A. Aldajani, Fabian Salazar, Helen Harrington, and Amir M. Ghaemmaghami. (2013). Development of an immunocompetent model of human upper airway epithelium. Joint Respiratory Research Day. 4th September 2013, Loughborough, UK.

Acknowledgements

First and foremost I would like to thank the Almighty God for His strength and grace, and for seeing me through. I would like also to express my sincerest gratitude and deepest appreciation to my supervisors Professor Amir Ghaemmaghami for his invaluable guidance and continuous support throughout the thesis period and for his trust by giving me the opportunity to carry out this project, and Professor Alan Knox for his kind supervision and making this project worthwhile. It has been a privilege and a pleasure working with them. I wish to extend my sincere thanks to the rest of my thesis committee members for their insightful comments and encouragement that helped me to widen my research from various perspectives.

I also take this opportunity to record my thanks to all my lab-mates in Allergy and Tissue Engineering Research Group for their help, suggestions, and friendship during my studies at the University of Nottingham, and all the faculty members of the Department of Immunology for their support and encouragement. Thanks to Dr David Onion and Miss Nicola Croxall for their help with the Flow Cytometer, to School of Life Sciences Imaging (SLIM) members for their support with microscopy and histology, and many thanks to Dr David Tooth for his help with HPLC.

I am indebted and eternally grateful to my beloved parents and siblings for their unceasing encouragement and support. My sincerest appreciation also extends to my loyal husband Mohammed and my daughter Sadeem for their patience, encouragement, and for being there throughout my study period. They were always beside me, raising my belief and confidence in myself and they helped me to make my dreams come true.

I would like to thank King Abdul-Aziz University, the Ministry of Higher Education in Saudi Arabia who sponsored my scholarship. I also would like to acknowledge the School of Life Sciences at the University of Nottingham for supporting my travel to International Conferences.

Undoubtedly, this period in the United Kingdom has been a wonderful and unforgettable enriching experience, and I feel very fortunate and happy that I have completed my journey successfully.

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Abbreviations

2D	Two-dimensional
3D	Three-dimensional
3-HAA	3- hydroxanthenilic acid
3-HK	3-hydroxykynurenine
AHR	Aryl hydrocarbon receptor
AJs	Adherens junctions
ALI	Air-liquid interface
ANOVA	Analysis of variance
APCs	Antigen presenting cells
ASM	Airway smooth muscle
ATP	Adenosine triphosphate
BEBM	Bronchial epithelial basal medium
BEDM	Bronchial epithelial differentiation medium
BEGM	Bronchial epithelial growth medium
BGP	Bermuda grass pollen
BPE	Bovine pituitary extract
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CF	Cystic fibrosis
CF-ECs	Cystic fibrosis epithelial cells
CFTR	Cystic fibrosis transmembrane receptor
CLRs	C-type lectin receptors
COPD	Chronic obstructive pulmonary disease
DCM	Dichloromethane
DCs	Dendritic cells

DMEM-F12	Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 HAM
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
ECs	Epithelial cells
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMTU	Epithelial mesenchymal trophic unit
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FN	Fibronectin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAS	Gamma activation sequence
GC	German cockroach
GCN2	General control non-derepressible 2
GFR	Growth factor receptors
GM-CSF	Granulocyte-macrophage colony stimulating factor
GPCRs	G protein- coupled receptors
H&E	Haematoxylin and eosin
HDM	House dust mites
HPLC	High Performance Liquid Chromatography
ICAM-1	Intercellular adhesion molecule -1
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon- γ
IgE	Immunoglobulin-E
iHBECs	Immortalised HBECs

IL	Interleukin
IPF	Idiopathic pulmonary fibrosis
IRF-1	IFN regulatory factor-1
ISRE	Interferon stimulated response elements
KYN	Kynurenine
LBP	LPS-binding protein
LPS	Lipopolysaccharide
MACS	Magnetic cell separation system
MD-2	Myeloid differentiation protein-2
MFI	Median fluorescence intensity
MMP-9	Matrix metalloproteinase-9
Mo-DCs	Monocyte-derived dendritic cells
NF-κB	Nuclear factor kappa B
NLRs	NOD-like receptors
NO	Nitric oxide
PAMPs	Pathogen-associated molecular patterns
PARs	Protease activated receptors
PBA	Phosphate-buffered albumin
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PC5	Phycoerythrin cyanin 5.1
PE	Phycoerythrin
PEA	Peanut
pen/strep	Penicillin/streptomycin
PET	Poly-ethylene terephthalate
PFA	Paraformaldehyde

PGA	Poly glycolic acid
PGE2	Prostaglandin E2
pHBECs	Primary Human bronchial epithelial cells
PI	Propidium iodide
PLA	Poly lactic acid
PLGA	Poly (lactic-*co*-glycolic) acid
polyI:C	polyriboinosinic polyribocytidylic acid
PRRs	Pattern recognition receptors
PS	Phosphatidylserine
QA	Quinolinic acid
RANTES	Regulated on activation, normal T cell expressed and secreted
RLRs	RIG-I-like receptors
ROS	Reactive oxygen species
RT	Room temperature
RTK	Receptor tyrosine kinase
SEM	Scanning electron microscopy
SEM	Standard error of mean
siRNA	Small-interfering RNA
SLPI	Secretory leukocyte protease inhibitors
TCRs	T-cell receptors
TDO	Tryptophan 2,3-dioxygenase
TEER	Trans-epithelial electrical resistance
TFA	Trifluoroacetic acid
TGF-β	Transforming growth factor-beta
Th	T helper cells

TJs	Tight junctions
TLRs	Toll-like receptors
TNF-α	Tumour necrosis factor
Treg	Regulatory T cells
TRP	Tryptophan
TSLP	Thymic stromal lymphopoietin
TWI	Transwell inserts
VCAM1	Vascular cell adhesion molecule 1
ZO	Zonula occludens

Chapter 1: Introduction

1.1 The immune system

Our immune system is constantly exposed to a plethora of potentially harmful substances from chemicals to pathogens to allergens that threaten the body's homeostasis. To combat these, the host uses both innate and adaptive immune responses, which have the ability to discriminate non-self from self-antigens and to detect and then eliminate pathogens. However, under certain conditions exacerbated responses can be generated against innocuous substances (e.g. allergens) or normal tissues resulting in chronic conditions such as allergy and autoimmunity (1, 2). Allergic responses such as asthma are classified as type I hypersensitivity. This chronic inflammatory disease is typically triggered by allergen exposure, leading to the production of high levels of immunoglobulin (IgE), and is characterised by airway obstruction, hyper-responsiveness and other pathophysiological features (3-6). In atopic individuals, recognition, processing and presentation of allergens will lead to the polarisation of naïve T cells towards a Th2 phenotype and secretion of type 2 cytokines such as IL-4 and IL-13. These events of allergic cascade including diverse of infiltrating and structural cells with different mediators that responsible

for asthma pathogenesis (Figure 1.1) which ultimately culminate in the class-switching of B cells and production of IgE the dominant class of antibody in allergic responses (7-9).

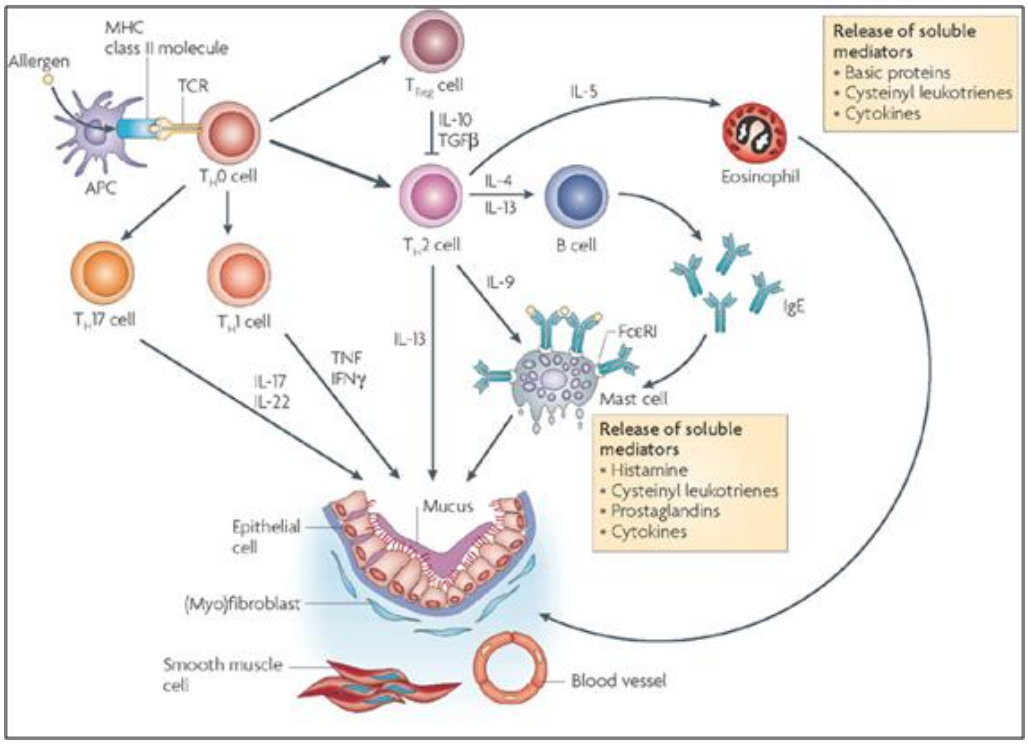


Figure 1.1: The allergic cascade. Predisposed individuals have a tendency to develop allergic sensitisation through Th2 activation and IgE synthesis in response to initial allergen exposure. Subsequent exposure to allergen causes inflammatory-cell recruitment and activation as well as release of wide range of pre-formed and newly synthesised mediator including histamine and cysteinyl leukotrienes and cytokines, which promote vascular permeability, smooth muscle contraction and mucus production. Moreover, chemokines produced by mast cells lead to eosinophils and Th2 cells recruitment and release of pro-inflammatory cytokines including IL-13, IL-5. Furthermore, different subsets of CD4 T cells have an impact on exacerbation of allergic diseases through production of various mediators. Adapted from (7).

The innate immune system including physical, chemical, or microbiological barriers represents the first line of defence mechanism, whereas adaptive immunity is the second, which involves antigen-specific reactions via T and B lymphocytes. This ultimately generates immunological memory with rapid response in the event of subsequent exposure and regulates host immune homeostasis (1, 10).

It has been generally realised that innate immune recognition depends on germline-encoded receptors that act rapidly against invading substances. Haematopoietic cellular elements of innate immunity include macrophages, dendritic cells (DCs), mast cell, neutrophils, eosinophils, and natural killer cells (1, 11, 12). Upon encountering the pathogen, a physical barrier such as epithelial cells (ECs) acts as the first sentinel via their tight junctions (TJs), mucus secretion, and cilia movement. Furthermore, the innate response also comprises secretion of cytokines, chemokines, lipid mediators, enzymes, and other anti-microbial peptides. The innate immune response also includes membrane-bound receptors that recognise pathogen-associated molecular patterns (PAMPs) as well as cytoplasmic proteins that bind these molecular patterns, which are expressed on the surfaces of invading microbes. All these mechanisms of innate immunity help to clear invading

pathogens from the host and maintain immune homeostasis (2, 11, 13).

1.2 Innate immune defence mechanisms in the lung

Large numbers of microbes, allergens, and particulate materials are brought into the respiratory tract through delivering oxygen to the systemic circulation. However, innate host defence mechanisms including physical and chemical systems limit or prevent these substances from their entry into lung tissue and spread to the circulation. The airway ECs are strategically positioned at the interface between the internal milieu of the host and the environment, and thus play a key role in the host defence system by clearing airborne antigens and allergens without inducing inflammation in order to maintain homeostasis (13, 14). ECs provide a physical barrier as well as actively contributing to the innate immune defence mechanism. Airway ECs employ multiple barriers including mucus, TJs, and adherens junctions (AJs), which play a crucial role in maintaining barrier function and the selective transport of different substances as well as a vast array of fluid and antimicrobial molecules that enable airborne pathogen clearance (14, 15).

1.3 Barrier function of the respiratory epithelium

There are three fundamental components of the airway epithelium barrier function. The first comprises secretory products and mucociliary clearance, which trap and remove inhaled allergens from the airways. The second is intercellular junctional proteins that regulate epithelial para-cellular permeability and the third is composed of secreted antimicrobial mediators that kill inhaled pathogens (14, 16).

1.3.1 Mucus and mucociliary function

Mucus production and cilia activity are major components that contribute to mucociliary function via respectively trapping inhaled allergens/pathogens and sweeping the trapped material from the lungs (16). The airway mucus consists of high molecular weight glycoproteins including the MUC5AC mucin, which is primarily produced by goblet cells, and MUC5B, produced by submucosal glands (14, 16, 17). In conditions of health, mucus secretion is normally regulated. However, abnormal goblet cell differentiation and mucus hyper-secretion are major features of chronic airway diseases such as chronic obstructive pulmonary disease (COPD),

asthma, and cystic fibrosis (CF) (16, 18). Diverse inflammatory mediators such as tumour necrosis factor (TNF)-α, IL-β, and IL-13 or environmental factors such as cigarette smoke and microbial pathogens also cause mucus overproduction (16). Therefore, airway obstruction might potentially be limited through remedies that help to restrict mucus hyper-production and by modulating mucociliary clearance in chronic airways diseases.

1.3.2 Intercellular junctional proteins

In the mucosal epithelium, airway para-cellular permeability is maintained via TJs and AJs. The formation and maintenance of barriers is an essential requirement of the physiology of the conductive airway tract. TJs play a key role in the regulation and selective transport of different substances through the para-cellular pathway, whereas AJs regulate cell-cell adhesion and support TJ formation (15, 16). In healthy individuals, these junctional proteins prevent inhaled pathogens and allergens from disrupting the normal regulation of gene expression, cell proliferation, and differentiation in the airways (19, 20). Thus, their disruption increases epithelium permeability and interferes with its normal repair and differentiation. For example, abnormally

proliferated/differentiated airway ECs with high permeability have been observed in patients with chronic airway disorders such as COPD and asthma (21, 22). Specifically, TJs consist of multiprotein complexes including transmembrane proteins such as claudins 1-5, occludins, E-cadherin, β- and α-catenin, and junctional adhesion molecules as well as cytoplasmic scaffolding proteins such as zonula occludens (ZO-1-3). Both transmembrane and cytoplasmic junctional proteins collaborate together to produce a selective barrier via connecting the membranes of adjacent cells and anchor transmembrane proteins to the cytoskeleton (Figure 1.2) (16, 23).

It has been previously shown that some respiratory viruses could increase permeability and decrease the trans-epithelial resistance of the airway epithelium through interacting with TJ proteins or by dissociating AJ complexes (16). For example, rhinovirus promotes barrier dysfunction through the increased generation of reactive oxygen species (ROS) that ultimately leads to the dissociation of ZO-1, caludin-4, and occludin from the TJs and enhances bacterial translocation across polarised airway ECs (24, 25). Furthermore, the dissociation of AJs via different factors such as smoking or allergens exhibiting protease activity has been observed to be linked with epidermal growth factor receptor (EGFR) activation and to

induce cell proliferation and goblet cell metaplasia/hyperplasia (26, 27).

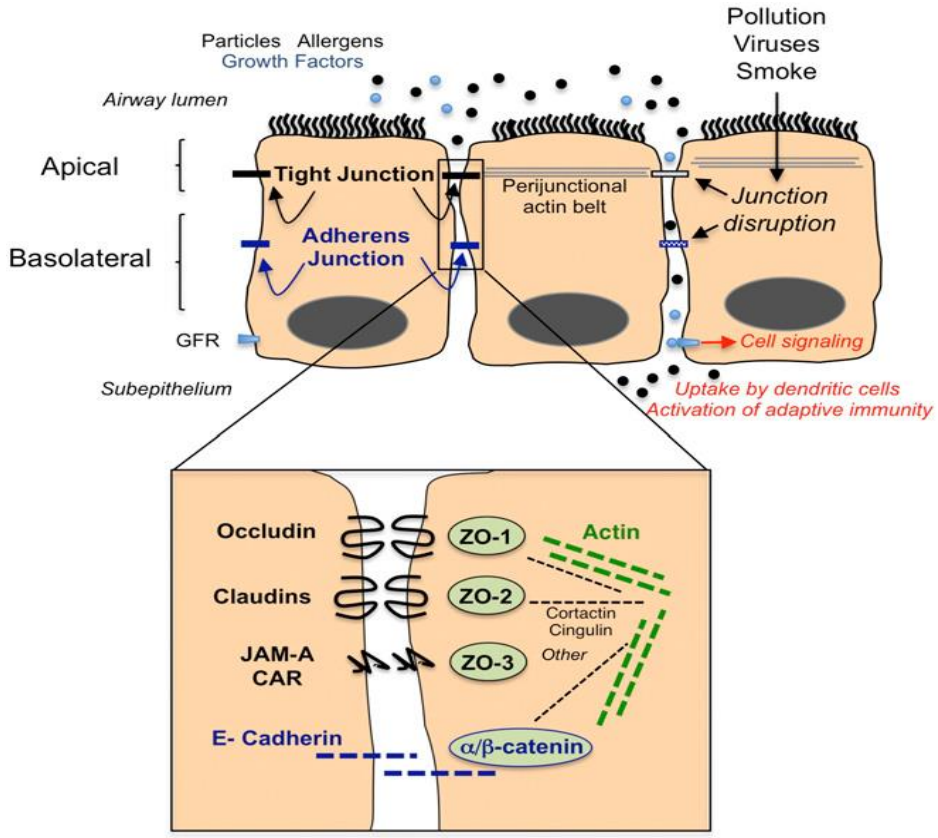


Figure 1.2: Diagram demonstrating the junctional structures of the airway epithelium including tight TJs and AJs. Disruption of the epithelial junction in response to airborne pathogens and allergens results in increased permeability. Barrier dysfunction thus leads to cell signalling through the production of cytokines and growth factors that interact with growth factor receptors (GFR), which recruit innate immune cells that initiate inflammation and immune reactions. The bottom panel shows specific TJs and AJs and indicates that junctional proteins are linked to the actin cytoskeleton via several potential adaptor proteins. Adapted from (28).

1.3.3 Antimicrobial mediators of the airway epithelium

It is well recognised that ECs not only provide a physical barrier but also can provide an efficient biochemical barrier to sense pathogen exposure and respond by increasing the production of a wide variety of antimicrobial peptides as well as cytokines, chemokines, and other mediators that initiate an inflammatory reaction through recruitment of immune cells to the site of infection. These antimicrobial substances include enzymes, protease inhibitors, oxidants, and peptides, which are all directly responsible for killing the invading microbes (13, 16).

Lysozyme represents one antimicrobial component of the airway host defence that displays microbicidal or microbiostatic activities against gram-negative or gram-positive bacteria (29). In addition, several antimicrobial peptides may also exhibit a synergistic effect for pathogen elimination. For example, lysozyme can inhibit the growth of gram-negative bacteria in the presence of lactoferrin, which causes disruption of the bacteria outer membrane and allows the lysozyme to enter the peptidoglycan layer (30). Lactoferrin exerts antimicrobial or antiviral activity through the sequestration of iron, which is important for microbial growth or by inhibiting virus RNA and DNA (29, 31). In addition,

protease inhibitors such as secretory leukocyte protease inhibitors (SLPI), elastase inhibitor, and α1-antiprotease are essential to immobilise and kill invading bacteria through reducing the effect of proteases expressed by pathogens which lead to diminished activation of innate and adaptive immune responses. Therefore, the balance between proteases and anti-proteases in airways during infections is essential in maintaining immune homeostasis (13, 16). Furthermore, defensins (e.g., α-defensins and human β-defensins class 1 to 5) and cathelicidins (e.g., LL37) comprise the most abundant antimicrobial peptides in the respiratory tract. Defensins exert their activity against bacteria and viruses through their C-terminal cationic peptide, which binds to the microbe and inhibits its biological function (13, 16, 32). Finally, airway ECs produce considerable amounts of oxidants, which provide innate defence mechanistic factors such as nitric oxide and hydrogen peroxide via nitric oxide synthase and dual oxidase, respectively. It has been shown that both enzymes exhibit excessive expression in chronic airway diseases, wherein they exert microbicidal activity. Generally, any defect in these defence mechanism substances has the potential to lead to impairments in microbial clearance (16, 33, 34).

1.4 Pathogen and allergen recognition by airway ECs

Although lung ECs were initially regarded simply as a barrier to inhibit or prevent pathogen penetration, it is currently well established that they can recognise airborne pathogens and allergens through a wide range of pattern recognition receptors (PRRs) such as protease activated receptors (PARs), toll-like receptors (TLRs), NOD-like receptors (NLRs), and C-type lectin receptors (CLRs), which ultimately mount innate immune responses. Thus, the activation of PRRs causes a cascade of events culminating in the production of cytokines, chemokines, and danger signals that recruit and activate immune cells, which in turn activate innate and adaptive immune responses (Figure 1.3) (35, 36).

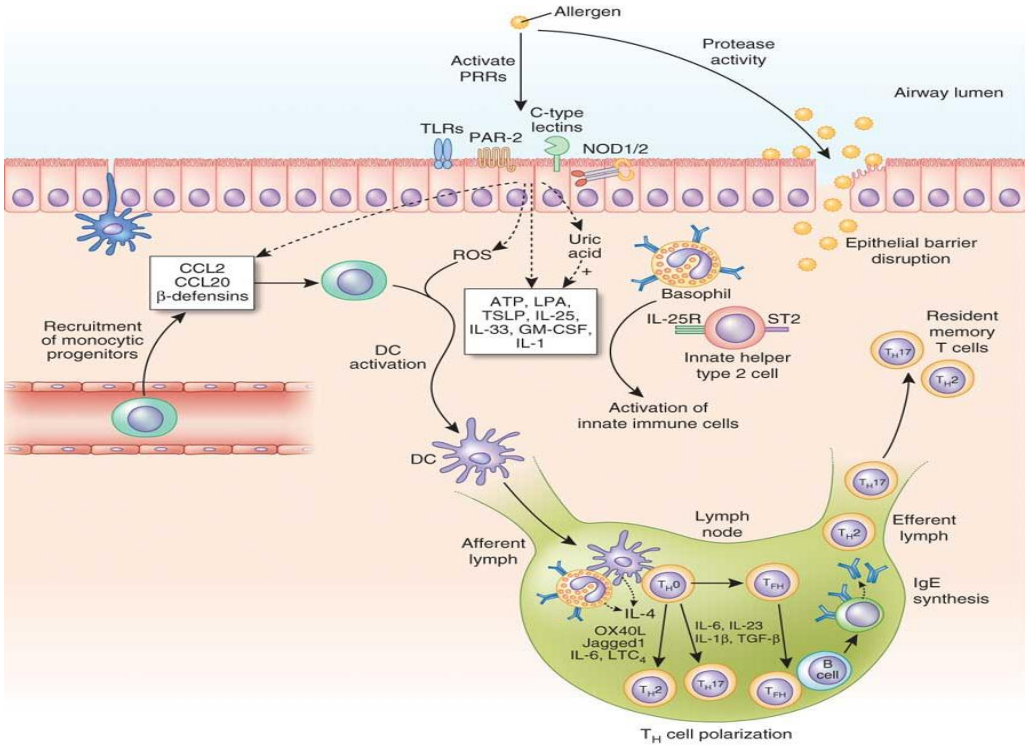


Figure 1.3: Cross-talk between ECs and immune cells. Some inhaled allergens can activate PRRs on ECs and DCs and breakdown TJs to provide access to DCs, however, other allergens can induce ROS production to activate DCs and ECs via nuclear factor kappa B (NF-κB) activation. Thus, activation of PRRS and ROS results in increased production of cytokines such as IL-1, IL-6, IL-25, IL33, and granulocyte-macrophage colony-stimulating factor (GM-CSF) as well as thymic stromal lymphopoietin (TSLP), a danger signal; adenosine triphosphate (ATP) and uric acid; as well as chemokines including CCL-2, CCL-17, and CCL-20. In turn, this leads to activation of the local network of DCs and coordinates the subsequent immune responses through the recruitment of other immune cells such as eosinophils and Th cells to the site of allergen exposure. Adapted from (37).

1.4.1 Protease activated receptors (PARs)

PARs are G-protein coupled receptors that are activated via proteolytic cleavage of their extracellular N-terminal domain, thereby functioning as a sensor for extracellular proteases.

There are several types of PARs including PAR-1 to 4, which are primarily responsible for the regulation of homeostasis, thrombosis, and cardiovascular functions. However, PAR-2 is found abundantly in airways and is activated by trypsin-like serine proteases, which have an essential function in mediating the proliferative and inflammatory responses linked to tissue damage (35-37). It has been found that some proteolytic allergens including house dust mites (HDM), cockroaches, pollen, and papain can activate PAR-2 in ECs and lead to the secretion of cytokines and chemokines that are linked to the induction of Th2 immunity (36, 38, 39). These proteases lead to activation of TNF-α signalling pathway and induce matrix metalloproteinase-9 (MMP-9), which is associated with the pathogenesis of bronchial asthma (35, 40). Furthermore, it was shown that some allergens induce disruption of TJs and AJs through their protease activity, which thereby induces cytokine production, cell detachment, and morphological change in airway ECs (27, 41). For example, it was elucidated that IL-33 contributes to protease-dependent allergic airway inflammation (42). In addition, it was also found that activation of PAR-1,-2, and -3 through EGFR led to the recruitment of immune cells such as neutrophils and eosinophils to the site of infection (35).

1.4.2 Toll-like receptors (TLRs)

TLRs represent PRRs that have been extensively studied, especially on DCs; however, a wide range of innate immune cells also express TLRs. Airway ECs express diverse TLRs with different expression levels, which can recognise and respond to microbial exposure including bacterial, fungal, and viral products (13, 43). In particular, TLRs can detect a wide variety of PAMPs such as tri-acyllipopeptides as detected by TLR1, bacterial lipoprotein and peptidoglycan (TLR2 ligands), double-stranded RNA (TLR3), lipopolysaccharide (LPS; TLR4), flagellin (TLR5), bacterial and viral sites of un-methylated CpG DNA (TLR9), di-acyl lipopeptides (TLR6 ligand), and single stranded viral RNA (TLR7) (13, 44).

Upon microbial engagement to TLRs, NF-κB becomes activated and directs the production of pro-inflammatory cytokines, interferons, chemokines, and antimicrobial mediators (45, 46). TLR4 is widely expressed in ECs in addition to antigen presenting cells (APCs) and represents the most intensively studied among the members of the TLR family. TLR4 is a major recognition receptor for LPS, a component of gram-negative bacterial cell walls (13, 35). Triggering innate immune responses via the recognition of LPS by TLR4 includes several steps. Initially, LPS interacts with a serum protein termed LPS-binding protein (LBP), which helps to transfer LPS

to the membrane-anchored protein CD14; this interaction is enhanced through the participation of another accessory molecule, myeloid differentiation protein-2 (MD-2). CD14-LPS binding is followed by the recruitment and activation of the adaptor molecule MyD88 and ultimately the activation of NF-κB, leading to the increased production of pro-inflammatory cytokines (35, 47, 48). In comparison, TLR2, which recognises lipoproteins from gram-positive bacteria as well as yeast and mycobacteria and constitutes the other member of the TLR family that is expressed constitutively in ECs, has been found to be regulated *in vitro* in response to bacterial products and inflammatory mediators (13, 47, 48).

Triggering of TLR4 signalling by LPS, HDM, and other allergens has been shown to be crucial in the induction of allergic Th2 immune responses (35, 36). However, given that ECs express an array of TLRs, it is likely that TLRs other than TLR4 might participate in the induction of features of allergic asthma. For example, the mRNA expression of TSLP was induced in airway ECs via a TLR3 ligand (dsRNA) and in the presence of IL-4, suggesting its contribution to the Th2-mediated triggering of allergic airway inflammation (49). Furthermore, TLR2-expressing ECs might be involved in airway inflammation through their activation by GM-CSF and IL-6 cytokines (36). In addition, other allergens might act through different TLRs and

produce pro-inflammatory cytokines such as observed following the activation of TLR9 in ECs in response to bacterial DNA, which causes an excessive production of IL-8. Collectively, the innate recognition of PAMPs by TLRs leads to their activation and thus initiates inflammatory responses via increasing the production of cytokines and chemokines, which induce the recruitment of innate immune cells to the site of infection and ultimately mediate the control of adaptive immunity (36, 44).

1.4.3 NOD-like receptor (NLRs)

Although NLRs are widely expressed in leukocytes, human airway ECs have been found to express some subsets of NLRs such as NOD1, and NOD2 (50). Wong *et al.* found that bacterial infection mediated NOD1 and NOD2 activation via the stimulation of eosinophil interaction with bronchial ECs through the up-regulation of intercellular adhesion molecule (ICAM)-1 on both cell types and the production of CCL2 and CXCL8 in allergic asthma (51). In addition, NLRP3 has been shown to induce Th2 immunity in response to some adjuvants, which leads to the production of inflammatory cytokines (52). Furthermore, the activation of NLRP3 during inflammation leads to the predominant production of IL-17 (53). Therefore,

NLR ligand stimulation may provide new insights towards the development of novel treatment approaches for EC- and immune cell-associated airway allergic diseases.

1.4.4 C-type lectin receptor (CLRs)

CLRs comprise the main PRRs that orchestrate anti-fungal immunity via triggering intracellular signalling that initiates innate and adaptive immunity. These receptors recognise carbohydrate, protein, and lipid components of pathogens (54). CLRs have been intensively studied in myeloid cells; however, they are also expressed in non-myeloid cells such as pulmonary ECs and can be regulated in response to pathogen recognition. For instance, an *in vitro* study showed that *Aspergillus fumigatus* induces the protein and mRNA expression of CLRs (dectin-1) in airway ECs, which leads to the generation of antimicrobial and inflammatory responses (55). During HDM-mediated allergic asthma, it has been shown that dectin-2 is the main CLR involved in HDM recognition in DCs whereas the β-glucan receptor dectin-1 initiates allergic airway inflammation in response to HDM and other allergens in human airway ECs (56). Therefore, given that the majority of microbial PAMP-associated initiation and activation of innate immune responses occurs at the

epithelium in accordance with its location as the first line of defence, a better understanding of the functions of all PRR-expressing ECs would likely provide crucial insight into protective host responses in airway inflammatory diseases.

1.5 Lung ECs in health and disease

Healthy ECs with their numerous underlying specialised cells collaborate in the regulation, metabolism, and activation of inflammatory cells in response to injury to clear the inhaled pathogen and to achieve lung immune homeostasis. Under normal circumstances, the damaged epithelium tends to repair itself immediately through the stimulation of substantial repair pathways within 24 hours after injury during which time cells display enhanced mitotic activity (57, 58). This also occurs when damaging agents cause the disruption of the epithelial barrier, which leads to down-regulation of the junctional proteins and increases the expression of extracellular matrix (ECM) components such as collagen, MMPs, and fibronectin (FN). Furthermore, ECs release mediators such as transforming growth factor-β (TGF-β) and EGF, which assist with their migration to form a temporary barrier. This process also includes EC and goblet cell proliferation and differentiation as well as augmentation of fibroblast proliferation and their

differentiation into myofibroblasts, which ultimately restores the structure and function of the barrier (59, 60).

In contrast, in chronic inflammatory airway diseases such as asthma, COPD, bronchiectasis, and CF, the airway epithelium becomes more susceptible to damage and its normal repair processes are compromised. It is widely recognised that ECs are key contributors to immune function in the lungs and one of the essential components of pathogenesis of airway diseases (61, 62). The development of airway disease requires the activation of immune cells such as invariant natural killer T cells and macrophages that produce the IL-13 and drives airway ECs to produce inflammatory mediators which can significantly alter the normal mucociliary clearance (62). Respiratory viral infection leads to an expansion of airway progenitor ECs which are a subset of integrin α6 -expressing basal or secretory cells that are involved in increased IL-33 expression (62). It has been also shown that alveolar ECs play a key role in idiopathic pulmonary fibrosis (IPF) via activation of mesenchymal cells with the formation of fibroblastic foci and the exaggerated accumulation of ECM, mirroring abnormal wound repair (63). Moreover, ECs damaged as well as ECs apoptosis are essential feature with IPF through FAS-signalling pathway and as a result of induction of ROS and angiotensin peptides that released by myofibroblasts (64, 65).

In respect to airway apical junctional proteins, it has been shown that there is a dysfunction in the structure and functions of TJs and AJs in airway diseases (28). For example, a reduction in the expression of α-catenin, E-cadherin, and ZO-1 was identified in subjects with asthma compared with healthy individuals (22). Additionally, a sharp reduction in trans-epithelial electrical resistance (TEER) readings was noted in the airway ECs of patients with asthma, accompanied by an increased permeability (66). Furthermore, it was found that there is a dysfunction in the control of para-cellular ion trafficking in the lung epithelium of patients with CF patients exhibiting a defective CF transmembrane receptor (CFTR). This was characterised as a decrease in TEER measurements as a result of defective localisation of TJ proteins (67).

Structure and function alterations of airway ECs are strongly associated with asthma including cell dedifferentiation, migration, differentiation, and maturation as well as connective tissue deposition (68). ECs of asthmatics exhibit low viability with swollen of ciliated cells and loss of cilia (69). Activated ECs in asthma lead to greater production of prostaglandin E2, fibronectin, cytokines, growth factors and NO synthase (70, 71) with high expression of adhesion molecules (72). Moreover, eosinophilia is one of the major features of asthma which is associated with induction the

damage of bronchi as well as airway remodelling (73). A plethora of immune cells and T lymphocytes are found in the airways mucosa of patients with fatal asthma. Several studies showed a considerable association between the activation of alveolar macrophages and the severity of asthma (74, 75) and also involved in the regulation of the airway remodelling through the secretion of growth-promoting factors (76). Therefore, upon repeated injury and repair processes, airway remodelling will occur, which is defined as a chronic activation of the airways leading to airway wall structural changes as well as disruption of EC/fibroblast communication (57, 77, 78). The main features of airway remodelling are changes in cellular and molecular composition including the alteration of ECM architecture through enhancing the formation of its constituents, thereby thickening the reticular basement membrane and narrowing the airway epithelium (77, 79). This thickness of the sub-epithelial reticular layer is due to hyperplasia of the interstitial collagen type I, III, and V proteins as well as to FN and tenascin in response to TGF-β2. In comparison, the normal epithelial basement membrane is not as thick as that in subjects with asthma and serves pivotal functions such as facilitating the adhesion and migration of ECs and regulating their phenotype along with maintaining their polarity (57, 58, 79). In asthma, a relationship has been

identified between the increase in the TGF-β concentration and the change in the sub-epithelial matrix (78, 80, 81). Furthermore, the inhibition of metalloproteinase enzymes leads to a decreased rate of ECM degradation, which is associated with sub-epithelial fibrosis in conjunction with the acute thickness of the ECM (79, 82).

It is believed that the production of correct mucus levels with normal viscoelasticity is crucial for efficient mucociliary clearance (58). However, excessive mucus secretion is also one of the pathological features of the airway wall thickening associated with goblet cell hyperplasia in asthma as well as with mucus gland enlargement in CF (77-79). This overproduction of viscous mucin can cause breathlessness and the blocking of the airway in individuals with asthma. Various cytokines such as IL-3, IL-4, IL-9, and TGF-α have a direct or indirect effect on the excess secretion of mucus (77). Furthermore, it has been shown that IL-13 plays an important role in asthma pathogenesis and leads to enhanced mucus production and goblet cell hyperplasia in animal models (78, 83).

1.6 Epithelial- mesenchymal interactions

An epithelial mesenchymal trophic unit (EMTU) represents the interactions between pulmonary ECs and mesenchymal cell types including sub-epithelial fibroblasts, neural tissues, and ECM in controlling local microenvironments during lung development, regulation of inflammatory responses, and maintenance of tissue homeostasis (57, 59, 84). Several autocrine and paracrine factors orchestrate the reciprocal epithelial-mesenchymal interactions during EMTU homeostasis regulation, of which TGF-β serves as the master regulatory cytokine (84). The attenuated fibroblast sheath is located in close proximity to the epithelium; these cells have the ability to differentiate into myfibroblasts that produce ECM proteins and pro-inflammatory cytokines. Thus, fibroblasts comprise the major source of the collagen I and III that is produced in response to IL-1β and TGF-β, which induce the expression of pro-collagen. Additionally, TGF-β also induces the expression of collagen IV from ECs and of other ECM proteins such as FN and tenascin. Furthermore, TGF-β inhibits the synthesis and activity of MPPs and enhances the activity of MMP inhibitors. However, fibroblasts release IL-6, which activates MMPs such as MMP-2 (collagenase) and MMP-9 (gelatinase) and leads to enhanced ECM remodelling. Moreover, fibroblasts produce considerable amounts of prostaglandin E2 (PGE2), which

promotes the inhibition of surrounding fibroblast proliferation (57, 59).

ECM receptors including integrin contribute to cell behaviours and structure and are involved in the balance of synthesis and degradation (59). However, any imbalance caused by increased ECM deposition can lead to thickening of the basement membrane, sub-epithelial fibrosis, smooth muscle hyperplasia, and abnormal alterations, which are collectively termed epithelial structural remodelling (37, 59). Subsequently, mutual interactions between structural cells, mesenchymal cells, and immune cells then occur through the release of EGF from ECs and TGF-β from eosinophils and myofibroblasts to further increase the production of ECM components (37).

During airway inflammation, the continual activation of the EMTU as a result of exposure to biologically active pathogens and allergens leads to constant tissue remodelling and altered airway function. This is supported by the induction of multiple growth factors, providing the mucosal environment to sustain chronic inflammation (85). In particular, the observed up-regulation of EGF and impairments of EGFRs in the airway epithelium of patients with asthma is thought to most likely be the consequence of unregulated production of TGF-β. It has been shown that patients with asthma exhibit defects in the

production of protective interferons in response to viral infection such as by rhinovirus, which contributes to exacerbations of asthma (85). In addition, integrins such as β6 have been found to inhibit the inflammatory response, whereas other integrin subunits such as β1 may instead induce inflammation (86). For example, lymphocyte aggregation was identified in the conducting airways of β6 knockout mice. Similarly, activated lymphocytes were also found in tissue engineered to overexpress β1 integrin in a murine model (57, 87).

Airway smooth muscle (ASM) cell hyperplasia and hypertrophy reflect the major events of chronic airway remodelling and airway thickening of the asthma spectrum (77). The regulation of ASM mitogenesis occurs by intracellular pathways including the activation of receptor tyrosine kinase and G protein-coupled receptors. The synergy between these two receptors can induce ASM proliferation. In addition, in a primate model of asthma, HDM exposure led to ASM cell hypertrophy with a resultant increase in their mass and bundle size (88).

Recent studies suggest that ASM cells can regulate submucosal airway inflammation either directly or indirectly through the secretion of cytokines, growth factors, and other co-stimulatory molecules that might collectively promote airway remodelling (68, 88). For example, ASM cells secrete

GM-CSF in asthmatic airways, which play a major function in eosinophil recruitment and survival (89). In addition, during airway remodelling ASM cells are capable of migrating from the periphery of the smooth muscle bundle to the submucosa in response to PDGF, TGF-β and IL-1β (88, 90). Furthermore, a study by Brightling, *et al*. demonstrated that disrupted airways in subjects with asthma led to increased numbers of tryptase (granule-derived serine proteinase)- positive mast cells and ultimately resulted in ASM cell infiltration by mast cells, which is associated with the airway remodelling found in asthma (91). Additionally, ASM-produced-chemokines can also play an important role in airway remodelling. ASM cells are able to release regulated on activation, normal T cell expressed and secreted (RANTES; the C-C family chemokine CCL5) in response to IL-1β and TNF-α, particularly when incubated with a combination of T helper (Th1)-derived cytokines. The RANTES chemokine has a potent chemoattractant activity that helps to recruit eosinophils, macrophages, and other inflammatory cells and enhance airways inflammation (68, 92). Taken together, these findings indicate that cellular processes in health and disease including cell proliferation, differentiation, migration, and control of apoptosis or the sometimes dramatic change in airway architecture are regulated via interactions between ECs

and mesenchymal cells involving ECM components as well as cooperative integrin and growth factor signalling pathways (57, 84).

1.7 Cross-talk between the lung epithelium and immune cells

The paradigm that the epithelium provides an essential function in host immunity for maintaining the homeostasis of innate and adaptive immune responses or for the development of pathology is becoming increasingly clear. The induction of adaptive immunity in pathological settings is characterised by development of the Th2 immune response starting with the infiltration of mast cells and eosinophils to the injured epithelium, followed by the excessive production of cytokines and chemokines such as IL-25, IL-33, TSLP, and CCL-20 to activate DCs that help to prime CD4+ T cells towards Th2-biased immune responses (93). Airway ECs and DCs interact with each other via the production of cytokines, chemokines, and other mediators or via cell-cell cross-talk, which leads ultimately to DC activation and the regulation of immune homeostasis (93, 94). Therefore, communication between DCs and ECs is critical for driving the Th2 allergic response (6, 95, 96). For example, ECs can respond to enzymatically active

allergens through their PARs, which leads to the activation of NF-κB and induces the production of cytokines such as IL-1, IL-6, IL-25, IL33, and GM-CSF as well as TSLP, a danger signal; ATP, and uric acid, which in turn leads to the recruitment and maturation of DCs. Allergens can also bind to TLRs or CTLRs of the epithelium, stimulating the production of chemokines such as CCL-2, CCL-17, and CCL-20 via NF-κB signalling, which attract DCs and recruits other immune cells such as eosinophils and Th2 cells to the site of allergen exposure (3, 93, 97). In addition, it was found that ECs also release CCL-20 chemokines, which interact with CCR6 in DCs and induce their migration to the epithelium (98). Furthermore, allergens, especially those with serine and cysteine protease enzymatic activity, can break down the EC TJs and access intraepithelial DCs in the basolateral side of epithelium and activate them directly (3, 99).

The activation of airway DCs is important for the induction of B cell, T cell, and NK cell-mediated immune responses. In airway inflammatory responses, the TSLP produced by ECs can induce the expression of MHC class I and II molecules on DCs, which enhances the induction of CD4+ T cell differentiation into Th2 cells (3, 93). IL-25 and IL-33 as well as other cytokines produced by ECs play a crucial role in airway inflammation by inducing the production of IL-4, IL-5, and IL-13 via driving

Th2-mediated inflammation (100, 101). In addition, EC production of GM-CSF in response to the Derp1 allergen of HDM or cigarette smoke induces DC-driven Th2 responses (3). It also important to note that, because the type of immunity elicited by the host is highly based on the nature of the response as defined by ECs; thus, the innate immune recognition by airway ECs determines the functional properties of airway DCs (94). Collectively, these data indicate that all of the mediators that are produced by airway ECs in response to airborne pathogens and allergens cooperated together as well as with immune cells to regulate mucosal haemostasis, initiate immune responses, and orchestrate airway remodelling and inflammation.

1.8 Induction of adaptive T cell immunity by DCs

After activation in the periphery, DCs start their maturation process and migrate to the local draining lymph nodes where, through the production of cytokines and the expression of co-stimulatory molecules, they stimulate the activation and differentiation of naïve T cells to different subsets of $CD4^+$ Th or $CD8^+$ T cytotoxic cells (6). Under physiological conditions this will lead to optimal clearance of pathogens; however, under pathological conditions (e.g. allergic diseases) such

activation of T cells could lead to chronic inflammation and tissue damage (8). Generally speaking, mature DCs that have been exposed to bacterial products secrete high levels of IL-12, which stimulates the differentiation of Th cells to Th1. Conversely, DCs that have been stimulated by allergens typically support the differentiation of Th cells to Th2 (6, 96). Th1-type cytokines (mainly IFN-γ) tend to produce the pro-inflammatory responses responsible for killing intracellular parasites and for perpetuating autoimmune responses (102). Immoderate pro-inflammatory responses can lead to uncontrolled tissue damage. The Th2 subset produces cytokines including IL-4, 5, and 13 which help B cells to proliferate and differentiate and produce humoral-type immune responses such as induction of IgE and eosinophilic responses in atopy. IL-10 is also produced by Th2 cells and associated with anti-inflammatory response (102, 103). Th2 responses could counteract the Th1 mediated microbicidal action. Therefore, the optimal scenario would be that humans should produce a well-balanced Th1 and Th2 response, suited to the immune challenge (102). DCs can become activated via the IL-25 produced by basophils, which leads to sustained Th2 responses through the production of Th2-derived cytokines (95). In addition, DCs can induce regulatory T cells (Treg) and Th17 responses through the production of a range of cytokines

determined by the nature of the stimulants encountered (6, 96).

Differentiated Th cell subsets have numerous functions in immunity. Some studies have suggested that healthy individuals express Th1 cells that may have a protective role against asthma. One explanation for this effect is based upon the existence of counterbalancing T_{reg} cells, which actively suppress allergen responses in healthy subjects as compared to allergic individuals (3, 104). Other studies have indicated that Th1 cells are responsible for the early stages of airway inflammation in allergic murine models (105, 106). Further, some evidence has shown that Th17 cells produce IL-17, which is associated with neutrophilic inflammatory events that occur during tissue remodelling in asthma (7). However, Th2 cells and its cytokines are instead considered as a predominant player in the induction and maintenance of inflammation in asthmatic individuals (3).

Chronic Th2 immune responses are associated with allergic asthma. This is due to infiltration of Th2 cells as well as of other immune cells such as mast cells, basophils, and eosinophils. This infiltration of inflammatory cells is one of the main characteristics of asthma and is responsible for the range pathologies from damage to ECs to airway remodelling with

concomitant changes in airway structure and function (6). Th2 cells produce an array of cytokines (e.g., IL-4, IL-5, IL-9, IL-13, and TNF-α) that contribute to asthma pathogenesis in different ways (3). For example, IL-13 producing Th2 cells promote goblet cell hyperplasia with enhanced mucus production and bronchial hyper-reactivity (107). For example, in a study performed in mice model by Cohn L. *et al.* it was shown that the IL-4 produced by Th2 cells can induce asthma pathogenesis through mucus production (108). In addition, IL-4 and IL-13 stimulate the up-regulation of ICAM1 and vascular cell adhesion molecule 1 (VCAM1) on blood vessels, which promote the recruitment of inflammatory cells (3, 107). Furthermore, the TNF-α and IL-4 produced by Th2 cells can increase the expression of VCAM1, which helps to recruit eosinophils to the airways and enhance their activation (3, 106). Th2 cells also have the ability to stimulate B cells to produce IgE in susceptible individuals through their secretion of IL-4 and IL-13 (3).

1.9 *In vitro* three-dimensional (3D) models of human lung epithelium

Most studies of the human airway are carried out using cells grown in two-dimensional (2D) monolayers. 2D cell culture models grow in non-physiological environments that fail to

mimic the phenotypes and histological features of cells *in vivo* (109). Cells grown in plastic dishes and in 2D lack the main criterion of cell-cell communication that is supported by ECM (110). To provide a better understanding of cell communication *in vitro*, it is important to develop 3D cell culture models that mimic different aspects of living tissue (109, 111). Towards this end, *in vitro* cell culture models and lung tissue engineering have been extensively studied. This process was initiated by culturing the cells on a collagen gel layer that supports the production of surfactant proteins. Subsequently, models had focused on the formation of a capillary barrier to support gas exchange in the lung. However, whereas these models had provided some information within regard to the physiological functions of airways, they lacked cell-cell or cell-ECM interactions (112).

The use of *in vitro* 3D tissue models for the provision of more complex physiological properties is characterised by the combination of cell culture with scaffolds or devices that support cell growth, organisation, and differentiation (113). One of the main requirements is to grow cells on scaffolds constituted with ECM proteins such as collagen, elastin and laminin, which support cell differentiation functions and facilitate cross-talk between cells (109, 110). Amongst other effects, ECM proteins provide differentiation signals through

the binding of cell surface receptors such as integrins to the ECM, which also assists with the transfer of biochemical signals between cells (114). Therefore, culturing cells in 3D versus 2D conditions provides the third dimension for mechanical inputs and is crucial for effecting the cell adhesion that critically impacts integrin ligation, cell contraction, and intracellular signalling (113).

Accordingly, there are considerable differences between 2D and 3D cell culture models. In terms of migration, cells in 2D are dependent on the strength of cell surface adhesion to the polymer to migrate, which is vastly different from the migration of cells in 3D matrices, which is dependent on the mechanical properties (113, 115). For example, it has been shown that the migration of fibroblasts in a 3D collagen model occurs by an extension of the fibroblasts which react with collagen fibres based on fibroblast myosin; this results in contraction of cellular actin and ultimately in cell movement. However, this mechanism of motility is lacking in 2D models, which are not affected by myosin. Thus, the establishment of a 3D model is important to mimic *in vivo* situations in terms of collagen modelling by fibroblasts, which is crucial for cell motility, wound repair, development, and the regulation of cell growth (116). Furthermore, distinct polarizing chemical cues are observed in 3D in contrast to 2D models. For example, the

ECM in 3D cultures of ECs supports the spread of biomolecules inside cells, leading to a change in polarity, by aiding the formation of TJs and AJs and thus impacts on intracellular signalling and phenotypic fate. However, this phenomenon is absent in undifferentiated cells in 2D culture (117). In addition, it is also believed that 3D cell cultures have different cell behaviour partly because of their ability to up and down-regulate different genes unlike cells cultured in 2D (115). In particular, cells in 3D show decreased proliferation associated with high differentiation, which reflects changes in the transcription levels of adhesion molecules (114).

A wide range of studies have established the utility of ECs in 3D culture to provide a more *in vivo*-like system that exhibits a greater resemblance to physiological conditions. Hermanns *et al.* have shown that co-cultivation of ECs with endothelial cells on collagen-coated transwell inserts (TWI) provides cell-cell cross-talk that affects cellular morphology, differentiation, orientation and polarization (118). Another study has established the importance of the collagen scaffold-3D co-culture model of human bronchial ECs with fibroblasts on ECM remodelling. This was validated by the observation of increased production of the ECM proteins MMP-2 &-9 and of mucus production along with collagen III up-regulation and myofibroblast differentiation (119). Furthermore, a study from

our group has demonstrated that co-cultivation of ECs with fibroblasts in a 3D model of an electro-spun poly-ethylene terephthalate (PET)-based scaffold results in the accelerated differentiation of airway ECs relative to that observed in mono-cultures. In addition, the recovery of the barrier after allergen exposure was found to be more efficient and rapid in the 3D co-culture model than in 2D and mono-cultured ECs (120).

It is therefore reasonable to suggest that the development of *in vitro* 3D co-culture models of lung epithelium with relevant structural (e.g. fibroblasts or ASM cells) and effector cells (e.g., DCs and mast cells) might better mimic *in vivo* conditions and bridge the gap in our knowledge by providing a platform to better understand aspects of lung development, physiology and pathology. Such models would facilitate the use of different cell types in the context of airway diseases such as allergic inflammation as well as for disease modelling and testing new drug leads.

1.10 Indoleamine 2,3-dioxygenase (IDO) and the regulation of immune responses

IDO (also referred to as IDO1) is the rate limiting enzyme that contributes to metabolic immune regulation (immune suppression and immune tolerance) through catalysing the

essential amino acid tryptophan (TRP) along the kynurenine (KYN) pathway (Figure 1.4). TRP is required in several physiological processes including cell growth and functioning in addition to its requirement for protein synthesis (121, 122). Depending on the cell type and the various expression levels of other Kyn pathway enzymes, a cascade of reactions occur, producing diverse biologically active Kyn pathway metabolites including L-Kyn, kynurenic acid, anthranilic acid, 3-hydroxykynurenine (3-HK), 3-hydroxyanthranilic acid (3-HAA), quinolinic acid (QA) and picolinic acid (123). Kyn and other downstream metabolites can act on the aryl hydrocarbon receptor (AHR) by binding to it in the cytosol which represents a paracrine mode of cell signalling, affecting the metabolism of xenobiotics and promoting carcinogenesis (123, 124). Moreover, kynurenic acid can mediate the transport of organic anions in the brain and kidney via organic anion transporters-1 and -3 (125) as well as it represents an efficient activator of IL6 production (126). QA plays a crucial role in cellular homeostasis, function and survival through generation of NAD+ (127). 3-HK and 3-HAA have pronounced redox activity and regulate T cell function (124). Collectively, IDO-expressing cells can influence the function and phenotype of surrounding cells that do not necessarily express IDO themselves such as T cells (123).

The first step in the KYN pathway of TRP catabolism is the conversion of TRP to N-formyl-kynurenine. In mammals, this reaction is carried out independently by tryptophan 2,3-dioxygenase (TDO; mostly expressed in the liver) or by IDO1 and IDO2, which are expressed by different cell types (121, 122, 128). IDO1 and its isozyme IDO2 have similar structures and are situated adjacent to each other on human and mouse chromosome 8. However, although both proteins have similar enzymatic activity, they exhibit different expression patterns and molecular regulation under certain pathological conditions (128, 129). IDO is highly expressed in inflammatory states along with IFN-γ and consequent to certain infectious agents such as influenza as well as following LPS exposure (130, 131). In normal physiological settings, IDO is pivotal in modulating immune activation to antigenic challenges and it contributes towards balancing inflammation and tolerance in various physiological functions such as pregnancy, infectious disease, allergic response, autoimmune disorders, transplantation, and cancer (132, 133). For example, IDO acts in immune defence by providing maternal tolerance to foetal tissues or transplanted organs, suppresses local tissue inflammation and autoimmunity, and inhibits the immunity to cancer and chronic infection (121).

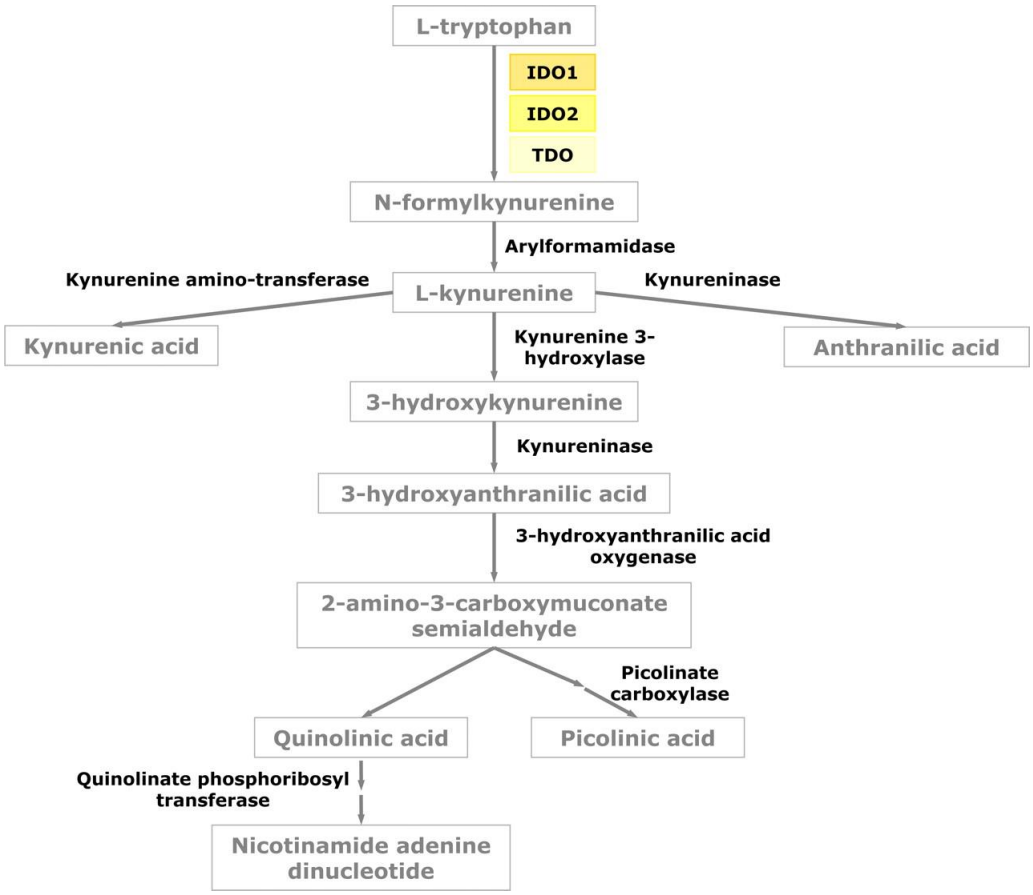


Figure 1.4: The kynurenine (KYN) pathway. The dietary TRP that is not used in protein synthesis, serotonin and melatonin synthesis, or tryptamine and kynuramine synthesis will metabolise via the KYN pathway that is controlled by the rate limiting enzyme IDO or by TDO. Adapted from (134).

IDO are highly expressed by dedicated APCs such as macrophages and DCs; however, several different cell types can express IDO constitutively such as tumour cells and non-haematopoietic cells including ECs, endothelial cells and fibroblasts. It was found that IDO is constitutively expressed mainly in mucosal tissues, whereas it is induced during

inflammation in most tissues in response to pro-inflammatory cytokines (e.g. interferon-γ; IFN-γ) associated with immune activation (121). It has been suggested that the induction of TRP degradation via IDO in response to (IFN-γ) represents a significant antimicrobial and immune-regulatory mechanism in lung cells (135). Furthermore, it was demonstrated that IFN-γ deficient mice exhibit very low expression of IDO in lung ECs and endothelial cells during chronic tuberculosis, and that this was associated with increased IL-17 production and the substantial recruitment of neutrophils to the lung (136). It has also been suggested that the induction of IDO-mediated TRP starvation in response to IFN-γ suppresses the replication of various intracellular pathogens such as *Toxoplasma gondii*, *Chlamydia pneumonia*, and *Trichuris muris*, which ultimately impacts the host resistance to early infection (137-140). Some pathogens develop strategies drawn from the IDO pathway to counter host immune responses. For example, *Escherichia coli* attenuates innate immunity through TLR-mediated up-regulation of IDO and IL-10 expression (141, 142). Furthermore, evidence has revealed that IDO serves an important immunosuppressive role during viral infections, such as by HIV, influenza, and hepatitis B and C (143-145).

Initially, authors proposed an immune regulatory role of IDO through its ability to mediate foetal-maternal tolerance by

providing a natural immunosuppressive mechanism that inhibits maternal T cell-mediated rejection of murine allogeneic foetuses (146). Several studies revealed different mechanisms under the concept of an immune-regulatory role of IDO-mediated TRP starvation. For example, an *in vitro* study has demonstrated that downstream metabolites of TRP degradation provide an immune-regulatory effect via the induction of murine thymocyte and T cells apoptosis (147). Another study showed that T cell proliferation was inhibited via reducing the availability of TRP in the local tissue microenvironment (133). A further *in vivo* murine model as well as *in vitro* human ECs model found that both TRP starvation and KYN metabolites resulted in the activation of general control non-derepressible 2 (GCN2), a stress-responsive kinase dependent on the down-regulation of the T cell receptor (TCR), which drives T cell differentiation towards Treg generation (148, 149). It has also been shown that the KYN-dependent generation of Treg cells rather than of Th17 cells occurs via activation of the AHR-that is expressed by naïve T cells, in which TGF-β is required for optimal Treg generation (150, 151). Although it is widely accepted that IDO represents a potent inhibitory signal for Th1 responses, the implications of IDO for Th2 responses appears more complex in the context of inhibitory and stimulatory functions and

depend on the experimental model used (123). For example, it was elucidated that absence of IDO in murine model lead to attenuated Th2 cytokine production, airway hyper-responsiveness and IgE level (152). However, another study showed that 3-HAA as one of the main KYN metabolites induces apoptosis of Th1 but not Th2 lymphocytes in murine model (147). Moreover, IDO was found to effect its immunosuppressive actions through inhibition of the $CD8^{+}$T cell-mediated cytotoxic function, which provides protection against allograft injury (153). Finally, further study also demonstrated the involvement of IDO in modulating inflammatory responses, in particular in B cell-mediated autoimmunity (154).

As has been previously mentioned, IDO plays a fundamental role in various forms of immune dysregulation including allergy. IDO has also been shown to exert a protective effect in different models of experimental asthma (46, 155, 156). Notably, Th2 cytokines (i.e. IL4 and IL-13) have been shown to inhibit IDO activity (157). Furthermore, KYN metabolites such as 3-HAA can induce PDK1 activation and lead to the inhibition of NF-κB activation, which ultimately leads to the death of activated Th2 cells and the inhibition of experimental asthma (156, 158). Collectively, there has been a substantial increase our understanding of the fundamental biological role

of IDO and its regulation in diverse physiological and pathological processes, which pinpoints IDO as an attractive target for therapeutic manipulation (121).

1.11 Hypothesis and aims

ECs have always been considered as part of the innate immune system. However, the full extent of their contribution in protecting the host, not only through providing a dynamic physical barrier but also by producing a plethora of immune regulatory and anti-bacterial mediators, has been only recognised in recent years. Both the barrier and effector functions of ECs are thought to be influenced by their communication with stromal (e.g. fibroblasts) and immune (e.g. DCs) cells. These interactions can collectively initiate and regulate inflammatory responses to different stimuli and maintain lung immune homeostasis.

Furthermore, much work has been carried out over the years to understand the immune-regulatory effect and antimicrobial activity mediated by IDO in DCs; however, both of these properties have remained only minimally defined in airway ECs. Here, it was hypothesised that IDO from ECs plays a role in mediating their immune regulatory activities through

engagement of different allergen extracts and bacterial pathogens and that this IDO activity of ECs can be modulated by fibroblasts and/or DCs. Therefore, the main aim of the project was to investigate the expression and regulation of IDO in human ECs in isolation and in a 3D co-culture with immune and stromal cells.

The main objectives of the study were:

1- To investigate the regulation of IDO in ECs in response to different TLR ligands and allergen extracts;
2- To evaluate the effect of IDO production by ECs on T cell activation;
3- To study the effect of ECs differentiation and cross-talk with other cells on their IDO activity; and
4- To elucidate the influence of disease status (e.g. asthma, COPD, and CF) on ECs IDO regulation.

Chapter 2: Investigating IDO activity and expression in human epithelial cells

2.1- Introduction

IDO is a rate-limiting enzyme in the KYN pathway that breaks down the essential amino acid TRP, which has been implicated in diverse immune-regulatory responses in health and disease (121, 133, 159, 160). It is well recognised that IDO plays a crucial role in various biological processes such as inflammation, microbial infection, and cancer. IDO is also known to promote immune tolerance during pregnancy and transplantation. Therefore, IDO is a potential therapeutic target for treating a wide range of chronic diseases (133, 140, 161, 162). Moreover, a mechanistic study of IDO revealed that it serves a role in controlling allergic airway inflammation, in which plasmacytoid dendritic cells could have a protective function via their effect on T cells and other immune cells (161). Furthermore, it has been demonstrated that IDO is expressed in the colon and that it provides a protective effect through its upregulation during inflammation (163, 164). For example, IDO inhibition during experimental colitis of murine model resulted in increased mortality and augmented normal inflammatory responses. It is therefore suggested that IDO

plays an important function in the inhibition of Th1 responses within the gastrointestinal tract (164).

The role of IDO in immune defence mechanisms may be to inhibit T cell growth/responses (immune-regulatory effects) or restrict microbial growth. Depletion of TRP and increased production of downstream TRP catabolites impact T cell activation by inducing apoptosis through activation of the stress-response kinase GCN2, or by inducing tolerance through activation of the AHR (151, 165). Other studies have provided evidence that IDO-mediated tryptophan depletion is a potent antimicrobial mechanism (135, 166, 167). It was found that IFN-γ-activated lung ECs showed antimicrobial activity against *Toxoplasma gondii*, *staphylococcus aureus*, and Herpes simplex virus in an IDO- dependent manner. Therefore, it is suggested that TRP depletion via IDO induction is a major mediator of antibacterial, antiparasitic, antiviral, and immune-regulatory effects in lung epithelium (135). Interestingly, it has been shown that IDO produced by goblet cells plays an important role in the regulation of epithelial cell turnover post-infection challenge (140).

It is well known that IDO expression is induced in several tissues by pro-inflammatory mediators, such as IFN-γ, interleukin—1 (IL-1), TNF-α, and endotoxins (168-170). IDO is

highly expressed in APCs. DCs express the highest levels of IDO in response to different stimuli, including cytokines such as type-I and type-II IFNs, costimulatory molecules and TLR ligands, among others. However, other non-immune cells such as endothelial cells, fibroblasts and ECs have been shown to express functional IDO (133, 171). It was demonstrated that diverse epithelial carcinoma cell lines and primary ECs are capable of expressing IDO after IFN-γ treatment (140, 149, 167, 172, 173). Previous studies have established that an increase in IDO activity and mRNA expression occurs in human cervical ECs (HeLa) after IFN-γ stimulation (170, 174). This effect was synergistically enhanced in the presence of IL-1 or TNF-α, but no synergistic increase occurred in response to IFN-γ with lipopolysaccharide (LPS). Moreover, it was proposed that this upregulation of IDO activity could be due to increased IFN-γ receptor expression and driven by NF-κB induction, which also plays a role in STAT1 and IFN regulatory factor-1 (IRF-1) signalling (167). Furthermore, LPS-induced IDO expression is mediated by an IFN-γ-independent mechanism in murine DCs (168). More recently, it was found that *Aspergillus fumigatus* spores induced IDO upregulation in human corneal ECs, suggesting an involvement of IDO from ECs in the immune responses against fungal infections (175). Data from another study revealed the importance of IDO expressed in

airway ECs in allergic airway disease by demonstrating IDO overexpression in airway ECs in response to *Aspergillus fumigatus* could limit the numbers and activities of $CD4^+$ effector T cells and reduce the levels of IL-4, IL-5, IL-13, and IFN-γ production (176). In summary, IDO serves various biological functions. For example, IDO participates in innate and adaptive immune responses, generates immunosuppression, and promotes systemic tolerance by activating Tregs.

In the current chapter the aim is to investigate the expression pattern of IDO in human airway ECs, as well as IDO regulation in ECs by diverse TLR ligands and allergen extracts.

2.2- Materials and Methods

2.2.1- Lung epithelial cells

Calu-3 cells (ATCC® HTB55™, human lung epithelial adenocarcinoma cell line) were maintained in Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 HAM (DMEM-F12; Sigma-Aldrich) containing 10% v/v heat-inactivated foetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 1% v/v non-essential amino acid solution (Sigma-Aldrich). Calu-3 cells were used between passage numbers 22 to 29 and cultured in T-75 culture flasks (Corning Life Sciences). Cell lines were incubated at 37ºC in a humidified atmosphere with 5% CO_2. Cells were harvested for use once they reached 90% confluence. For sub-culturing, cells were washed with Phosphate buffered saline (PBS; Sigma-Aldrich) and then incubated with trypsin-EDTA solution (Sigma-Aldrich) for 15–20 minutes until they detached. All experiments were performed under aseptic conditions in a laminar airflow cabinet.

2.2.2- Primary cell cultures

Primary Human bronchial epithelial cells (pHBECs), separated from bronchial brushings of healthy volunteers attending Nottingham University Hospital Respiratory Clinic who

provided informed written consent. The ethics committee and reference for AZA donors was Nottingham 2 (11/EM/0062) and for MMP-1 donors was Derby (12/EM/0199) respectively. Cells were maintained in bronchial epithelial growth medium (BEGM). BEGM was prepared from bronchial epithelial basal medium with SingleQuots™ kit including 13 mg/ml BPE, 0.5 mg/ml hydrocortisone, 0.5 µg/ml human recombinant epidermal growth factor, 0.5 mg/ml epinephrine, 10 mg/ml transferrin, 5 mg/ml insulin, 0.1 µg/ml retinoic acid, 6.5 µg/ml triiodothyronine, and 0.1% v/v gentamicin/amphotericin-B (all from Clonetics™ Airway Epithelial Cell Systems, Lonza). Cells were cultured in T-75 culture flasks (TPP90076) and incubated at 37ºC in a humidified atmosphere with 5% CO_2. For sub-culturing, cells were washed in 1 ml trypsin-EDTA in phenol red (Sigma-Aldrich), aspirated immediately, and incubated with 4 ml trypsin-EDTA. When all cells were detached, 4 ml of trypsin inhibitor (Invitrogen) was added to neutralise the trypsin.

2.2.3- IDO enzymatic activity assay

Calu-3 Cells, and pHBECs, (2×10^5 cells/ml) were seeded in 24-well plate and allowed to grow to 90-100% confluency in complete media supplemented with 100 µM L-TRP (Sigma-Aldrich) for 24 hours. Cells were stimulated with different TLR

agonists and allergen extracts including LPS, polyriboinosinic polyribocytidylic acid (polyI:C) (both from Sigma-Aldrich); synthetic lipoprotein of *Mycoplasma salivarium* (FSL-1), CpG-ODN2216 (5′-ggGGGACGA:TCGTCgggggg-3′) (both from Invivogen); HDM extract from *Dermatophagoides pteronyssinus*, peanut (PEA), Bermuda grass pollen (BGP) extract from *Cynodon dactylon*, , and German cockroach (GC) extract from *Blattella germanica* (all from Greer laboratories). Endotoxin content of HDM preparations was 615 EU/vial. IDO activity was measured by quantifying L-KYN production in culture supernatants using a colorimetric assay as has described previously (177). Briefly, 100 µl of 30% trichloroacetic acid (Fisher Scientific, UK) was added to 200 µl of culture supernatant. The mixture was vortexed and centrifuged at 10,000 rpm for 5 minutes. Then, 250 µl of the soluble phase was added to 250 µl of Ehrlich's reagent (100 mg of 4-(Dimethylamino)-benzaldehyde; Sigma-Aldrich) in 5 ml of glacial acetic acid (Fisher Scientific). Finally, 150 µl of the resulting solution was transferred into a microtiter 96-well plate (Greiner Bio-One), and absorbance was read at 492 nm. KYN concentrations were calculated using a standard curve (Sigma-Aldrich).

2.2.4- Staining for intracellular IDO1 expression

Calu-3 cells, and pHBECs (2×10^5 cells/ml) were stained for intracellular IDO1 expression and analysed by flow cytometry. Briefly, cells were added to a fluorescence-activated cell sorting tube (Elkay Laboratory Products Ltd, UK) and washed sequentially with cold PBS and phosphate-buffered albumin (PBA; PBS, 0.5% v/v bovine serum albumin (BSA), and 0.1% w/v sodium azide). Cells were incubated at room temperature (RT) for 10 minutes with 0.5 mL of cold fixation buffer (4% formaldehyde in PBS). Following fixation, cells were washed twice in flow cytometry permeabilisation/wash buffer (0.1% saponin [Sigma-Aldrich] in PBA). After permeabilisation, the cell pellet was dissociated by gently flicking the tube, and the cells were incubated with 5 µl of an Alexa Fluor® 488-labeled mouse IgG1 anti-IDO1 antibody (R&D Systems) for 30 minutes at 4°C in the dark. Subsequently, the cells were washed twice with permeabilisation/wash buffer and re-suspended in 0.5% formaldehyde. All flow cytometry analyses were performed using an FC 500 Flow Cytometer (Beckman Coulter). Data analysis was performed using Weasel software. An isotype-matched, Alexa Fluor® 488-labeled mouse IgG1 control antibody (R&D Systems) was used to determine the specificity of IDO1 staining.

2.2.5- mRNA isolation, cDNA synthesis and PCR

After washing the cells twice in ice-cold PBS, mRNA isolation and cDNA synthesis were performed using the μMACS One-Step cDNA Kit (Miltenyi Biotech), following the manufacturer's instructions. The cDNA purity was assessed using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). PCR was carried out in a TC-312 PCR Thermocycler (Bibby Scientific, Ltd.) using Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific). IDO1-specific mRNA expression was quantified relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the following primers (Eurofins): GAPDH Forward (5′-GAGTCAACGGATTTGGTCGT-3′), GAPDH Reverse (5′-GACAAGCTTCCCGTTCTCAG-3′), IDO1 Forward (5′-ACAGACCACAAGTCACAGCG-3′), and IDO1 Reverse (5′-AACTGAGCAGCATGTCCTCC-3′). After initial heating at 98℃ for 10 seconds, PCR was performed for 30 cycles of 98℃ for 1 second, 64℃ for 5 seconds and 72℃ for 15 seconds, with a final extension at 72℃ for 1 minute. PCR products were analysed on an E-gel pre-cast 2% agarose gel electrophoresis system (Invitrogen, UK), visualized under UV light (Alpha Innotech Corporation) and the molecular weight of the bands were estimated with the 100-bp DirectLoad DNA Ladder (Sigma-Aldrich).

2.2.6- Viability assay

Cells viability was estimated by using the LIVE/DEAD Viability/Cytotoxicity Kit and the alamarBlue Cell Viability Assay (both from Invitrogen). Briefly, Calu-3 cells (6 x 10^5 cells/ml) were seeded in a black, clear-bottom 96-well plate (Scientific Laboratory Supplies Ltd), with complete media. After reaching confluence, cells were treated with increasing concentrations of LPS for 24 hours. After washing 3 times with PBS, cells were incubated with reagents from the LIVE/DEAD Viability/Cytotoxicity Kit (1 μM Calcein AM, 10 μM EthD-1) or PBS for 1 hour before analysis. Cells were then washed with PBS and analysed by fluorescence microscopy, using a Leica DMIRE2 Fluorescence Time-lapse Microscope (Leica Biosystems). In a separate experiment, Calu-3 cells (6 x 10^5 cells/ml) were seeded in a 96-well plate with complete media. After reaching confluence, the cells were stimulated with different concentrations of LPS (Sigma-Aldrich) for 24 hours. Subsequently, 10 μl of alamarBlue reagent was added to the cells, and the cells were incubated for 4 hours at 37ºC. Fluorescence was measured using an excitation wavelength of 570 nm and an emission wavelength of 585 nm. Formaldehyde 4% (Sigma-Aldrich) was used as a positive control for cytotoxicity.

2.2.7- Western Blotting

Cells were washed with ice-cold PBS and then lysed using ice-cold radio-immunoprecipitation assay lysis buffer with protease and phosphatase inhibitor (Sigma-Aldrich, St. Louis, MO, USA) on a shaker for 30 min at 4°C. Subsequently, cell lysates were centrifuged at 13,000 rpm for 30 min at 4°C. Supernatants were collected and stored at −20°C. Protein concentration was determined using the DC Protein Assay (Biorad) following the manufacturer's protocol. The cell lysate was electrophoresed under reducing and denaturating conditions on a pre-cast 4–15% SDS-polyacrylamide gel (Biorad), and proteins were transferred to a nitrocellulose membrane (GE Healthcare). After a 2 h incubation in blocking buffer (5% non-fat milk in Tris-glycine buffer and 0.1% Tween 20), membranes were incubated with a primary rabbit monoclonal antibody [EPR1230Y] to IDO (Abcam, Cambridge, UK) overnight. After washing, the secondary peroxidase-conjugated antibody (goat anti-rabbit HRP) was added for 1 h. IDO was visualized using Amersham ECL prime Western Blotting Detection Reagents (GE Healthcare). GAPDH was used as a loading control with the rabbit monoclonal antibody (Abcam, Cambridge, UK).

2.2.8- Statistical analysis

Data are expressed as the mean ± standard error of mean (SEM). Statistical differences were determined using Student's *t* test to compare 2 groups, and one-way analysis of variance (ANOVA) was used to compare 3 or more groups. Tukey's post-test was applied, and $*p \leq 0.05$ was considered statistically significant. GraphPad Prism 6 software (GraphPad Software, La Jolla, Calif) was used for all statistical analysis.

2.3- Results

2.3.1- TLR4 stimulation down-regulated IDO in human airway ECs

To evaluate the immune-regulatory role of IDO in ECs, the basal levels of IDO expression in human lung ECs were initially investigated, with or without LPS (a prototype TLR4 ligand) stimulation. Calu-3 cells and primary ECs were cultured to confluency and incubated with increasing concentrations of *Escherichia coli* LPS for 24 hours, after which the IDO activity was assessed. Significant inhibition of IDO activity, based on L-KYN production, was observed in both Calu-3 cells and the primary cells in response to 1 or 10 μg/ml LPS (Figure 2.1). Moreover, there was a reduction in IDO1 protein expression in response to 2 μg/ml of LPS in both Calu-3 and primary ECs as well as a decreased IDO1 mRNA expression was more observed in calu-3 cells (Figure 2.2 A & B). These results justified using Calu-3 cells in subsequent experiments, as they behaved similarly to primary human bronchial ECs in terms of IDO expression and function.

To determine whether cell death contributed to IDO inhibition, cytotoxicity was assessed with alamarBlue and LIVE/DEAD staining. Only marginal cell death was observed following incubation with LPS (10 μg/ml; the highest LPS concentration

tested in the inhibition experiments described above), with 4% formaldehyde serving as a positive control for cytotoxicity (Figure 2.3 A & B). Furthermore, the alamarBlue data showed a range of LPS concentrations that did not affect cell viability (Figure 2.3 C). Collectively, these data indicated that IDO inhibition was not associated with cell death.

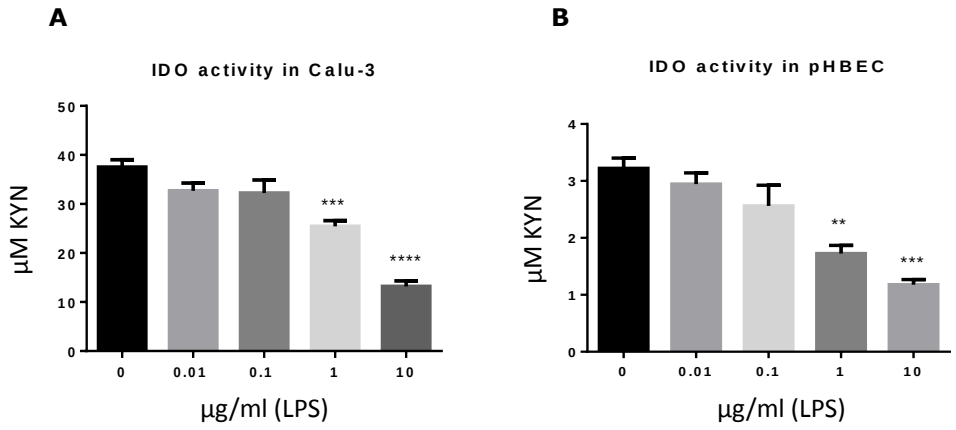


Figure 2.1: Down-regulation of IDO activity in airway ECs in response to the TLR4 ligand LPS. (A) Human airway epithelial cells (Calu-3) and (B) pHBECs were cultured to confluency and incubated with increasing concentrations of *E. coli* LPS for 24 hours, after which the supernatants were collected for IDO activity measurements. The data shown are representative of 3 independent experiments. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$.

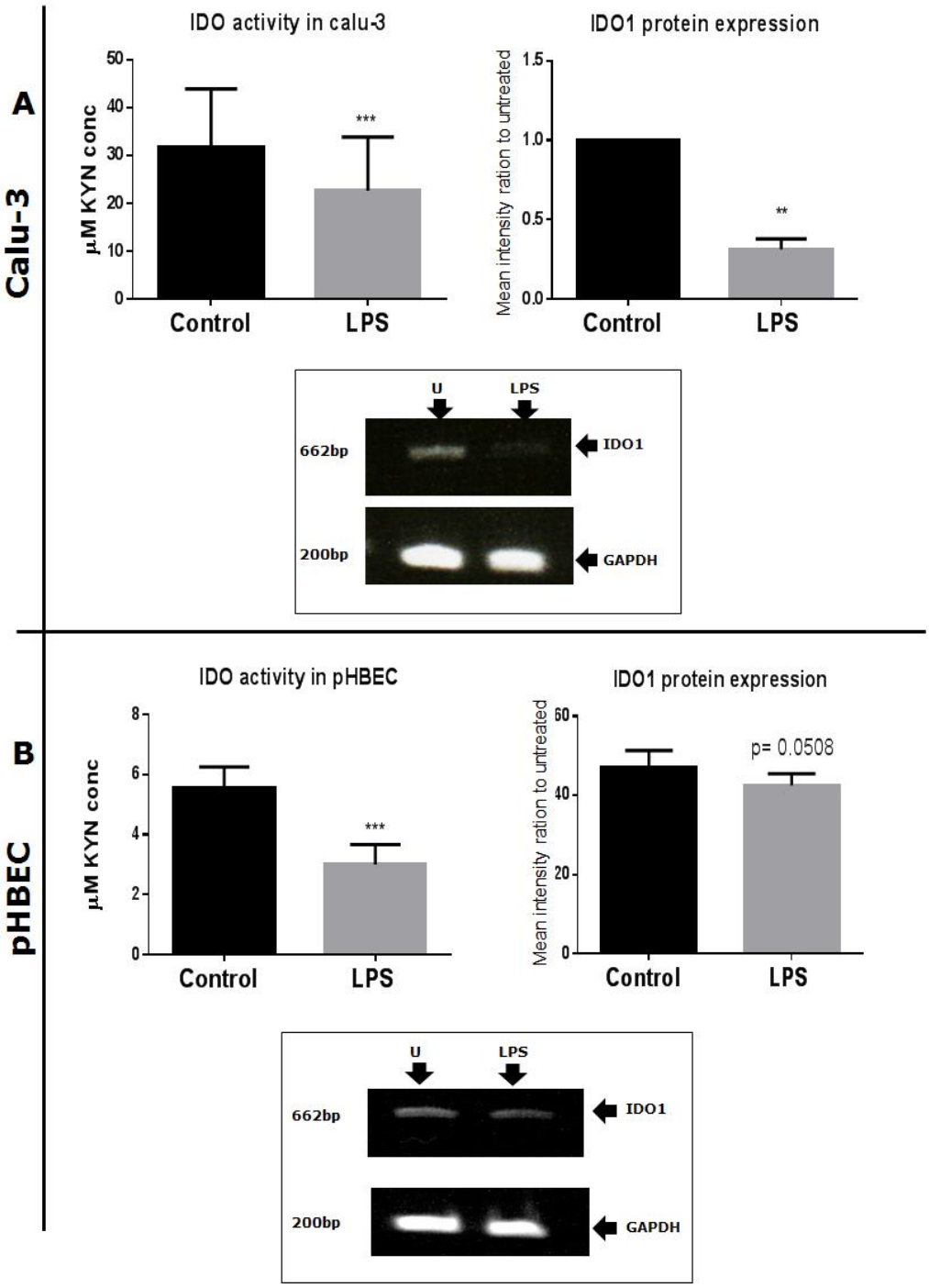


Figure 2.2: Down-regulation of IDO activity and expression in lung ECs in response to LPS. (A) Calu-3 cells, and (B) pHBECs, were cultured to confluency and incubated with 2 µg/ml LPS for 24 hours. Subsequently, IDO activity and expression levels (i.e. protein (via flow cytometry) and mRNA expression) were assessed. GAPDH was used as a reference gene. The data shown are representative of 3 independent experiments. U, untreated. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

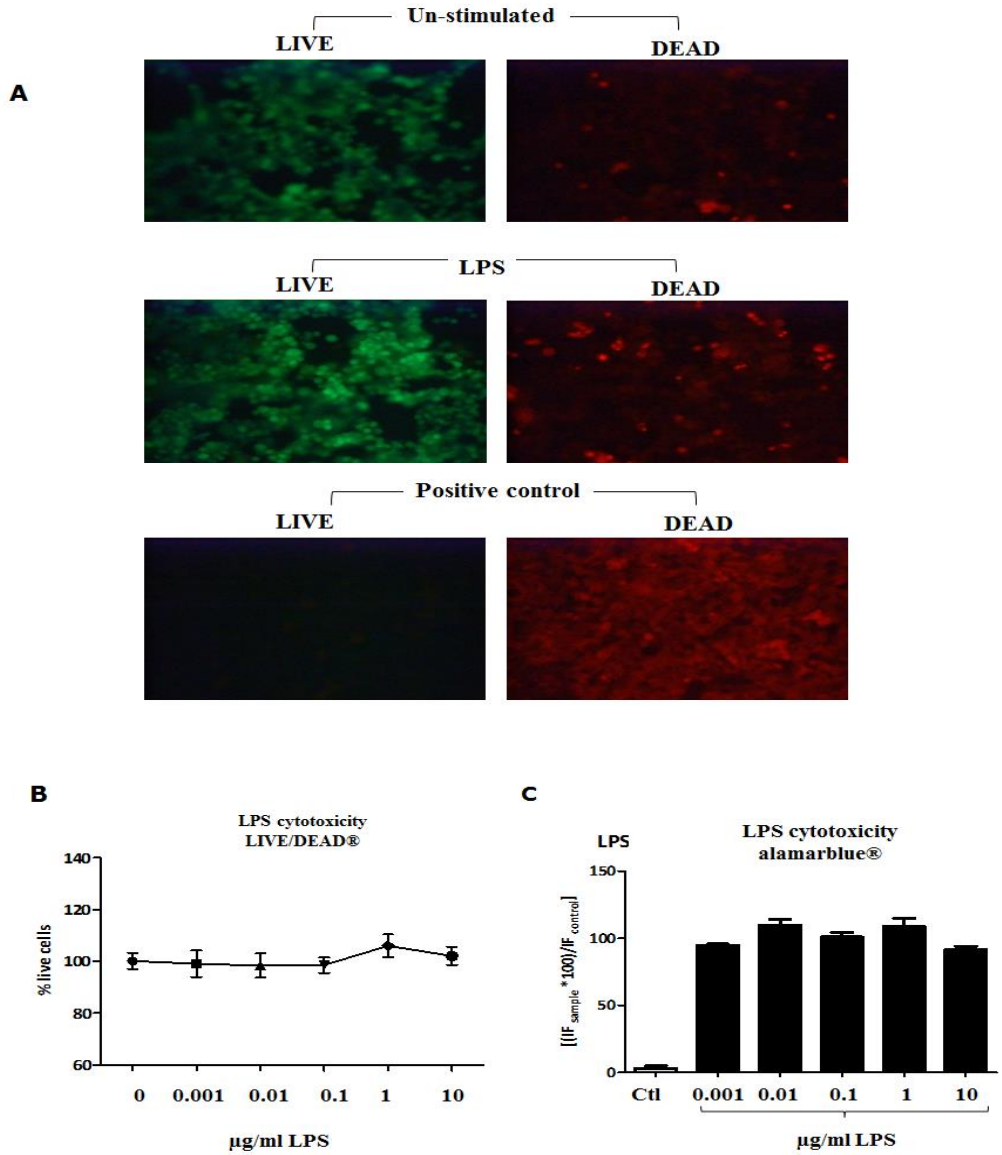


Figure 2.3: Cytotoxic effect of LPS in Calu-3 ECs. Calu-3 cells were cultured to confluency and incubated with LPS for 24 hours, after which cytotoxicity was analysed with alamarBlue® and LIVE/DEAD® assays. (A) LIVE/DEAD staining of Calu-3 cells stimulated with LPS (10 µg/ml) for 24 hours. Formaldehyde (4%) was used as a positive control for cytotoxicity. (B) Fluorescence quantification of LIVE/DEAD staining results (n = 2), expressed in % live cells. (C) AlamarBlue fluorescence readouts of Calu-3 cells stimulated with increasing concentrations of LPS for 24 hours. Formaldehyde (4%) was used as a control for cytotoxicity (n = 4).

2.3.2- Different TLR ligands and allergen extracts decrease IDO activity in human airway ECs

To determine whether the effect of IDO down-regulation was specific to LPS treatment, we next studied the effects of diverse allergen extracts and microbial products on IDO activity in human airway ECs after 24-hour incubation. Dose response study of increasing concentration of TLR ligands including poly I:C, FS:-1 and CpG as well as of allergen extracts (HDM, peanut, pollen, cockroach) were done as preliminary experiments (Appendix 1). As shown in Figure 2.4, TLR3 (polyI:C), TLR2/6 (FSL-1), and TLR9 (CpG) engagement markedly inhibited IDO activity. Furthermore, IDO activity significantly decreased in response to different allergen extracts, including BGP, PEA, GC, and HDM. We also quantified IDO1 protein expression levels in ECs before and after TLR ligation using western blotting. These experiments showed a marked reduction in IDO1 protein expression in TLR-stimulated ECs compared to unstimulated cells (Figure 2.4 C).

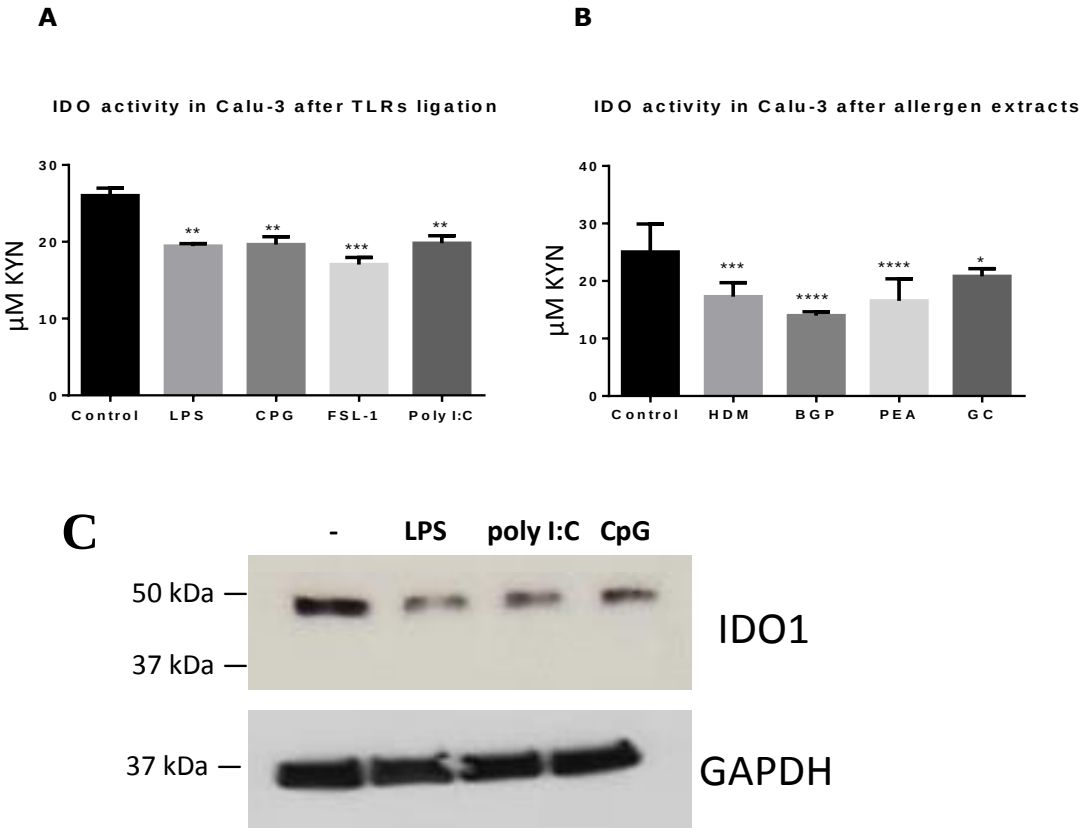


Figure 2.4: IDO activity and expression in Calu-3 ECs in response to diverse TLR ligands and allergen extracts. (A) Calu-3 cells were cultured to confluency and incubated for 24 hours with TLR ligands, including 2 µg/ml TLR4:LPS (n = 4), 10 µg/ml TLR9:CpG (n = 3), 10 µg/ml TLR2/6:FSL-1 (n = 3) or TLR3:polyI:C (n = 4). (B) Calu-3 cells were incubated for 24 hours with 10 µg/ml allergen extracts from HDM, Bermuda BGP, PEA, and GC, (n = 3). (C) Calu-3 cells were treated with 10 µg/ml LPS, polyI:C or CpG for 24 h, and IDO1 protein levels were assessed in cell lysates by western blot analysis; GAPDH was used as a control (n = 3). Observed band sizes were 45 kDa for IDO1 and 36 kDa for GAPDH, as predicted. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

To determine whether there is a difference in IDO activity following concomitant or sequential LPS exposure, Calu-3 cells were incubated with TLR2/6, TLR3, and TLR9 ligands in the presence or absence of LPS for 24 hours. In separate

experiments, cells were incubated with allergens for 24 hours, and some of these cells were further incubated with LPS for additional 24 hours to mimic subsequent infection following allergen exposure. Significant IDO down-regulation was observed following the single stimulation, and further reduction was observed in response to co-stimulation (Figure 2.5). These data suggested that co-stimulation with TLR ligands and allergen extracts could have an additive inhibition on IDO activity.

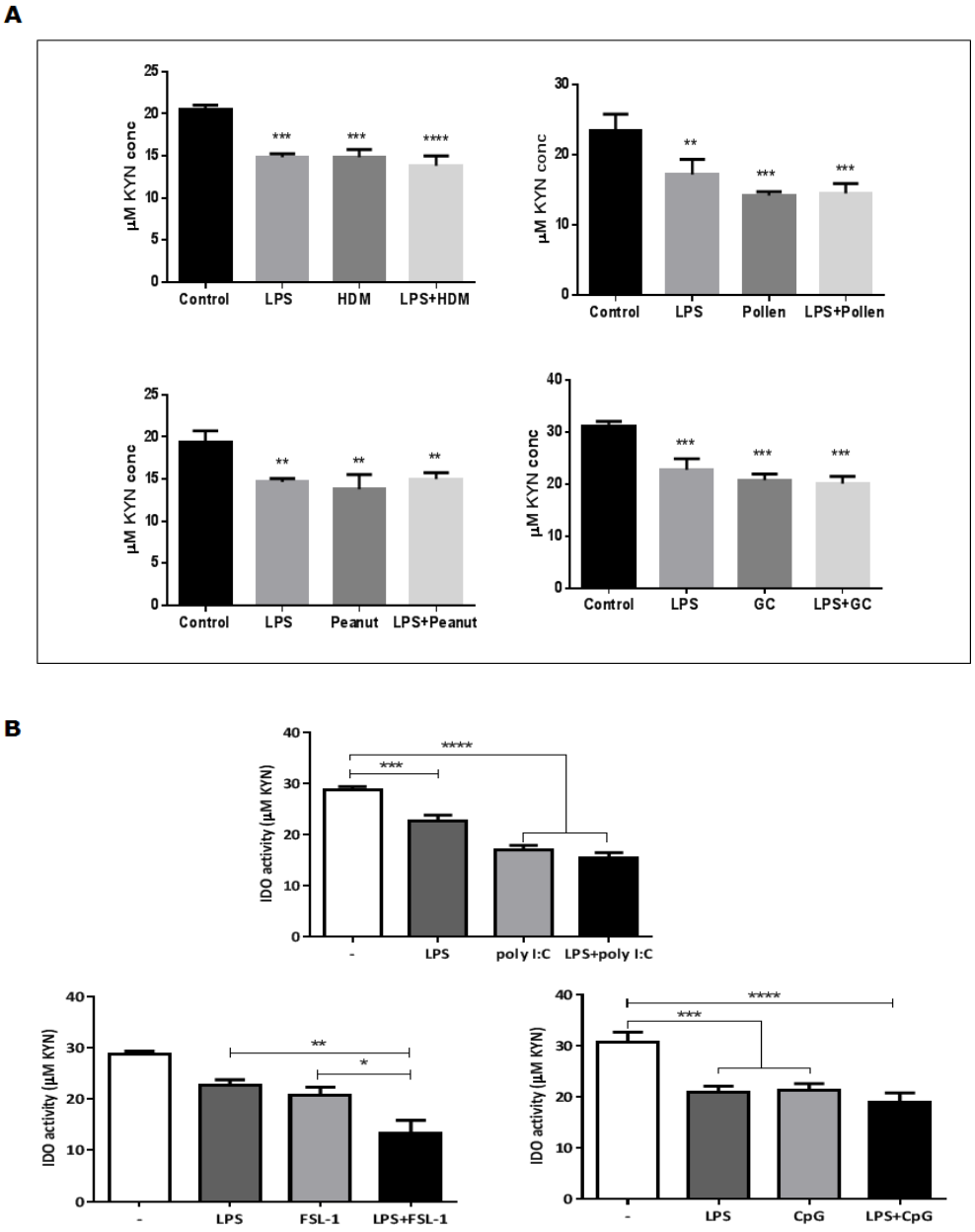


Figure 2.5: IDO activity in Calu-3 ECs in response to co-stimulation with TLR ligands and allergen extracts. (A) Calu-3 cells were cultured in a 24-well plate until they were almost confluent and incubated sequentially with 10 µg/ml allergen extract (HDM, pollen, peanut, or GC) for 24 hours and LPS for a further 24 hours. (B) Calu-3 cells were cultured to confluency and incubated with TLR ligands (10 µg/ml CpG, 10 µg/ml FSL-1, or polyI:C) in the presence or absence of 2 µg/ml LPS for 24 hours. The data shown are representative of 3 independent experiments. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

In separate experiments, pHBECs were cultured to confluency and incubated with TLR4, TLR9, HDM, or GC for 24 hours. Although compared with the Calu-3 cell line (~20–40 μM), the basal level of KYN production in pHBECs supernatants is low (~3–6 μM), a down-regulation of IDO activity was observed in response to a wide range of TLR ligands and allergen extracts used in both primary ECs (Figure 2.6) and the Calu-3 cell line, highlighting the conserved nature of IDO regulation in response to TLR ligands and allergen extracts.

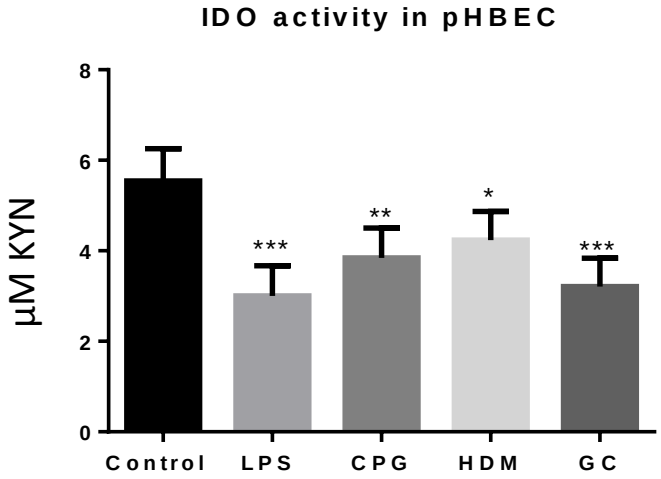


Figure 2.6: IDO activity in primary ECs in response to exposure to diverse TLR ligands and allergen extracts. pHBECs were cultured to confluency and incubated for 24 hours with 2 μg/ml LPS, 10 μg/ml CpG, 10 μg/ml HDM, or 10 μg/ml GC, after which the supernatants were collected for IDO activity measurements. A significant reduction was observed in response to the TLR ligands and allergen extracts tested, compared with the IDO activity observed in control cells. The data shown are representative of 3 independent experiments. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

2.3.3- IFN-γ stimulation up-regulates IDO activity in human ECs

Interferons, in particular IFN-γ, are stimulators of IDO induction during inflammation in several cell types (167-169). Accordingly, the regulation of IDO activity in respiratory EC lines and primary cells in response to IFN-γ or co-stimulation with LPS and HDM was investigated. Calu-3 cells and pHBECs were cultured to confluency and incubated with 10 ng/ml IFN-γ (as a single treatment), or subsequently with 2 μg/ml LPS, and 10 μg/ml HDM for 24 hours. As shown in Figure 2.7, IFN-γ markedly increased IDO activity in both Calu-3 cells and primary cells. However, in primary ECs, co-stimulation with IFN-γ and LPS inhibited IDO activity significantly compared with single stimulation with IFN-γ. Taken together, these results demonstrated a diverse pattern of change in IDO activity in airway ECs following stimulation with different immune regulatory stimuli such cytokines and TLR ligands which highlight the potential role of IDO produced by ECs in controlling immunity and maintaining lung epithelium homeostasis.

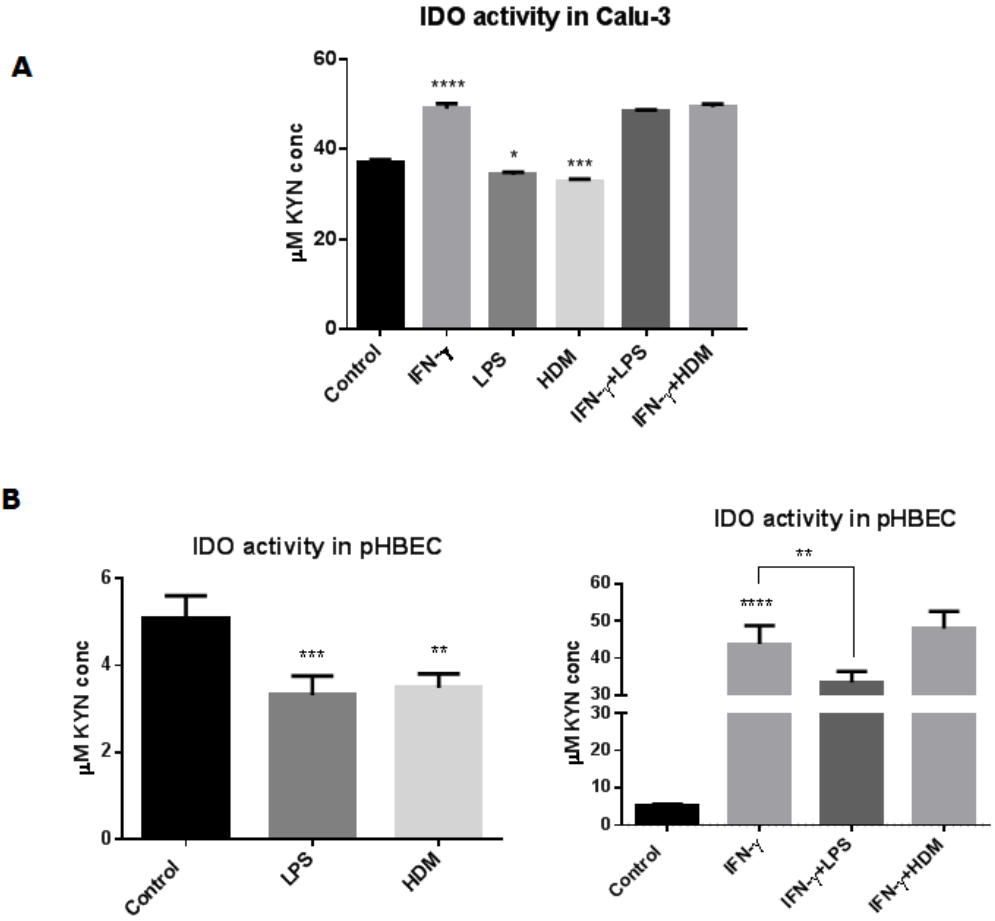


Figure 2.7: IDO activity in human lung ECs in response to IFN-γ, LPS, and HDM. (A) Calu-3 cells and (B) pHBECs were cultured to confluency and incubated for 24 hours with 10 ng/ml IFN-γ, 2 µg/ml LPS, 10µg/ml HDM, or a combination of IFN-γ with LPS or HDM. The data shown are representative of 3 independent experiments. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

2.4- Discussion

IDO is a tryptophan-depleting enzyme in the KYN pathway that is extensively involved in modulating immune responses. It is well known that among immune cells, DCs express high levels of IDO and they are one of the main producers of IDO-derived metabolites in the immune system (121, 133, 160). It has been shown that both IDO1 and IDO2 may activate the GCN2 pathway; however, this effector mechanism can be reversed by TRP restoration only in the case of IDO1, suggesting that IDO2 generates a unique TRP-independent signal (178). Moreover, it has been shown that IDO2 promoter is itself a target for AhR activation, which suggest the possibility that IDO2 may act in a feed-forward loop to reinforce the expression of GM-CSF and other cytokines that promote DC maturation. Furthermore, a unique presence in the IDO2 promoter of a binding site for the transcription factor IRF-7 of DCs, suggests a distinct role in these APC (179). However, the area of IDO2 regulation requires further study.

Over the last decade, ECs have become recognized as key players in modulating airway immune responses. Upon stimulation with allergens, airway ECs are able to produce a wide range of cytokines, chemokines and danger signals that can activate other immune cells, including DCs, basophils and

innate lymphoid cells among others (46, 176). Previously, it was shown that ECs can express functional IDO after stimulation with pro-inflammatory cytokines such as IFN-γ and TNF-α (140, 149, 170, 172, 174). However, the role of TLR ligands and allergens in modulating IDO in airway ECs and how this could impact on immune responses have remained elusive. The primary focus of this chapter is to establish the baseline IDO activity in airway ECs and to understand the patterns of IDO expression in airway ECs and primary bronchial ECs upon encountering diverse allergens as well as the role of TLR engagement in IDO modulation. This background information would help to evaluate how IDO regulation could impact other immunological events (e.g. T cell responses) in the airway epithelium. Findings from this study show that airway ECs express IDO constitutively under resting conditions and down-regulation of IDO activity was observed in response to a wide range of TLR ligands and allergen extracts used in both primary ECs and the Calu-3 cell line. Down-regulation of IDO activity upon TLR4 engagement was also reflected in IDO mRNA and protein-expression levels, but it was not associated with impairment in cell viability. In this study, viability testing was performed on the Calu-3 cells but not in primary ECs. Given that the majority of the data was generated based on Calu-3 cell lines and in the same time we

had some difficulties in culturing primary ECs successfully, it is essential to mention that this is one of the limitation in this study. However, this could be an important experiment to consider in the future.

Herein, the results also showed that the synthetic lipoprotein derived from *Mycoplasma* FSL-1 down-regulates IDO activity in airway ECs. Interestingly, TLR3 and TLR9 agonists namely polyI:C and CpG both down-regulated IDO activity, which might have implications in viral and bacterial infection. Some evidence has previously shown that ECs produce large amounts of nitric oxide (NO) through NO synthase in response to inflammatory and infectious stimuli (172, 173). NO production and TRP depletion regulate antimicrobial effector mechanisms *in vitro* (173, 180). Interestingly, NO directly inhibits IDO activity and expression through post-translational regulation, and increase intracellular proteasomal IDO degradation (172, 181). Therefore, given our transcriptional changes in IDO level in ECs, we assumed that the reduction of IDO activity and expression in this study is unlikely to be due to changes in NO production within ECs in response to TLRs and allergen extracts. Future studies should aim at elucidating the mechanism of IDO regulation via NO production by using inhibitors of proteasomal proteolytic activity. The down regulation of IDO activity in response to LPS in airway ECs is

opposite to the effect of LPS on IDO activity in human immune cells reported by previous studies (121, 177, 182, 183). This finding highlights the complexity of IDO regulation and fundamental differences in its activity between different cell types.

In the last few years, evidence has suggested that IDO serves an important immunosuppressive role during viral infections, such as HIV, influenza, and hepatitis B and C. In addition, a crucial role of IDO in bacterial infection and sepsis has been revealed (143, 144, 175). Diverse pathogens have developed strategies to use the IDO pathway in their favour to counteract immune responses. In particular, uropathogenic *E. coli* can attenuate innate responses to epithelial infection by inducing IDO in ECs of the urinary tract (141). Conversely, IDO can also serve an immune-protective role by affecting pathogen growth and dissemination. For instance, a reduction of IDO in ECs by IFN receptor deficiency affects the immune response against *Mycobacterium* infection (136). In addition, the IFN-γ/IDO pathway has been implicated in the replication of diverse viruses, parasites, and bacteria in human ECs, including *Staphylococcus aureus*, *Toxoplasma gondii,* measles virus, cytomegalovirus, and herpes simplex virus (172, 173, 184, 185). Moreover, IDO from ECs is also linked to immune responses against fungal infection, such as with *Aspergillus*

fumigatus (175, 176). Previous study suggested that although IFN-γ increased IDO activity in the Hela EC line, LPS did not enhance IDO activity synergistically with IFN-γ (174). It is known that IDO gene has two regulatory elements including interferon stimulated response elements (ISRE) and a gamma activation sequence (GAS) element that are required for the induction of IDO activity by IFN-γ (174, 186). Furthermore, Robinson *et al*. showed that TNF-α synergistically enhances the IDO activity induced by IFN-γ at the level of transcription in human ECs through binding of STAT1 to GAS and IRF-1 to ISRE (170). Moreover, the same study showed the possibility of a third GAS element (GAS-3) as an IFN-γ-responsive region which could account for some of the observed differences in IFN-γ responsiveness (170). In our study, there was an inconsistency between Calu-3 cells and primary HBECs treated with both IFN-γ and LPS which suggested that there may be different regulatory elements existing in IDO promoter that could be involved in this inconsistency. Future study should aim at elucidating the molecular mechanism responsible for gene expression including regulatory elements of IDO gene promoters in Calu-3 cell lines and primary cells that confer responsiveness to pro-inflammatory cytokines.

Additionally, our data show that diverse allergen extracts were able to down-regulate IDO activity in human airway ECs,

suggesting a key role of IDO in allergic responses and infection. Previous studies have provided evidence that IDO might have a protective role in various experimental models of airway allergic inflammation through different mechanisms (155, 177, 187, 188). Interestingly, increased susceptibility to infection has been associated with the development of asthma related diseases (46). Accordingly, it would be interesting to explore the role of IDO and specific IDO-derived metabolites from ECs in modulating other immune and non-immune cells in an allergy context under infection conditions which will provide new evidence into the importance of EC-derived IDO in mediating immune responses via suppression of unnecessary immune reactions.

One of the main aims of this study was to investigate the importance of IDO expression in ECs in mediating immune regulatory activities. Therefore, it was also important to examine the influence of IDO produced by ECs on T cell activation, which is the focus of chapter 3. Although using primary ECs represent a desired model to mimic *in vivo* physiological conditions, they have a limited life span, slow proliferation rate. Also, they are costly to maintain as well as they require substantial expertise in isolating and culturing them with a significant amount of time and to differentiate them at air-liquid interface (ALI) (189) which limit their use as

respiratory epithelium model in this study. Given these limitations, Calu-3 cell lines were used as an alternative to primary ECs. Despite the limitations associated with cell lines as models of respiratory epithelium, including lack of the physiological relevance, Calu-3 cell lines were widely used as a substitution of primary ECs because they are well-differentiated and characterized cell line compared with the other bronchial cell lines and they exhibit many of *in vivo* features of bronchial epithelium when they grow at ALI (120, 190, 191). Moreover, given that our preliminary data in terms of down-regulation of IDO activity and expression in response to TLR ligands and allergen extracts in both primary ECs and Calu-3 cell lines, we justify using this cell line to generate the majority of the data in subsequent chapters of this study.

Chapter 3: Elucidating the immune-modulatory role of IDO expression in epithelial cells in mediating T cell proliferation

3.1- Introduction

ECs lie at the interface between the host and the environment and represent the first line of defence against airborne pathogens and allergens (37, 66). Due to the expression of different pattern recognition receptors and their ability to produce a plethora of cytokines and chemokines, ECs can modulate and coordinate immune responses. Furthermore, it is well established that the cross-talk between ECs and DCs is critical in orchestrating immune responses to airborne antigens and constitutes a key element in maintaining mucosal homeostasis (3, 94, 95). DCs are the prominent APCs involved in the primary activation of naïve T cells, and several DC subsets can inhibit T cell responses (192, 193). In addition, ECs have been shown to modulate T cell responses (94, 95). In particular, airway ECs play a central role in controlling the function of lung DCs and influencing T cell activation and differentiation by increasing the recruitment, maturation and activation of DCs through the secretion of diverse chemokines

(98, 194, 195) and cytokines (196, 197). In addition to the ECs, alveolar macrophages are one of the main cells come into contact with the bacteria and produce IDO. Previous data have shown that IFN-γ induced the activation of IDO in macrophages and inhibited the growth of group B streptococci (198). IFN-γ induce-macrophages produce large amount of KYN metabolites, especially QA (199) and exhibited antimicrobial activity by killing or inhibit *Toxoplasma gondii* and *Chlamydia psiuci* via IDO-dependent manner (200). Moreover, it was found that increased activity of KYN pathway enzymes, following systemic immune stimulation and HIV infection, associated with macrophage infiltration, lead to acceleration of the local formation of QA (201). It has been shown that 3-HAA, an IDO metabolite, induces apoptosis via hydrogen peroxide formation in monocytes-derived macrophages under inflammatory or other pathophysiological conditions (202).

Despite their expression of MHC-II, human airway ECs not only promote allogeneic CD4+ T cell proliferation, but conversely induce T cell hypo-responsiveness (203). Previous findings have suggested that ECs inhibit T cell activation through the induction of Treg which suppress unnecessary adaptive immune responses (204). Furthermore, murine colonic and lung ECs can inhibit antigen presenting cell-

induced T cell proliferation. This effect appeared to depend upon cell-cell contact (204-206) and was attenuated by pre-treating ECs with IL-4 (206) or by viral infection (204).

Mounting evidence has shown the importance of airway ECs in driving Th2 allergic response through influencing the function of DCs. Data from *in vivo* mouse studies have demonstrated the crucial role of TLR4 signalling in ECs in Th2 sensitisation to HDM and LPS (207, 208). A recent study provided evidence that direct contact between ECs and DCs is essential for inhibiting *in vitro* T cell responses against allergens (209). Collectively, these data suggest a key role of airway ECs in maintaining a tolerogenic environment in the lungs.

Some studies have suggested the role of IDO-expressing immune cells in regulation of T cell responses and controlling autoreactive T cells *in vivo and in vitro* (159, 162, 210). Furthermore, IDO has been implicated in maternal-foetal tolerance and defence against foetal rejection through suppression of maternal T cell responses (162). Most previous data have demonstrated the role of IDO-expressing immune cells on T cell responses/activation. In the lung, DCs play a prominent role in controlling allergic inflammation through upregulation of various costimulatory molecules that involved in T cell activation or Tregs differentiation (94, 211, 212).

Moreover, tumour cells can produce sufficient KYN metabolites to supress the induction of T cell mediated anti-tumour responses (213). Thus, it is reasonable to hypothesise that IDO from ECs serves as one of the main mediators of T cell regulation in airways. Although some evidence suggests a role for TGF-β produced by lung ECs in decreasing T cell proliferation (204), the exact mechanism for such EC-mediated suppression of T cell responses has remained elusive.

The focus of this chapter is to evaluate the role of airway ECs in controlling immune responses via the modulation of T cell function, in particular T cell activation, and to evaluate the role of IDO in this process.

3.2- Materials and Methods

3.2.1- Lung epithelial cells

Calu-3 cells (ATCC® HTB55™, human lung epithelial adenocarcinoma cell line) were maintained in DMEM-F12 (Sigma-Aldrich) containing 10% v/v heat inactivated FBS, 100 U/ml penicillin - 100 µg/ml streptomycin (1% v/v pen/strep), 2 mM L-glutamine and 1% v/v non-essential amino acid solution (Sigma-Aldrich).

3.2.2- Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from fresh blood samples of healthy volunteers by density gradient centrifugation on Histopaque (Sigma-Aldrich). Cells were washed twice with PBS, and 8 × 10^5 cells/ 600 µl were co-cultured with 6 × 10^5 cells/ml Calu-3 cells in co-culture complete medium (i.e. with all supplements); 1:1 of DMEM-F12: RPMI-1640 (Sigma-Aldrich).

3.2.3- Gene silencing

Calu-3 cells were transfected with IDO1-specific small-interfering RNA (siRNA; GE Healthcare, UK) according to the manufacturer's instructions. Briefly, cells were transfected on day 1 with IDO1 siRNA at a concentration of 50 nM in the

culture supernatants, using a non-targeting siRNA as a negative control. Cells were then incubated for 6 hours in complete media without penicillin/streptomycin in a low-attachment tissue culture plate (Corning) and grown for 3 days for highest transfection efficiency with minimal effects on cell viability. The efficiency of IDO down-regulation was assessed by quantitative IDO-activity assays and conventional PCR for IDO1 mRNA expression, as described in chapter 2.

3.2.4- Co-culture conditions

Around 6 × 10^5 cells/ml control or IDO^{low}-ECs (i.e. ECs with low IDO expression after gene silencing with IDO1 siRNA) were cultured in transwell plates with a 0.4-μm pore size (Scientific Laboratory Supplies, Ltd.). After reaching confluency, PBMCs (8 × 10^5 cells/600 μl) were cultured in the bottom chamber in the presence of an immobilised anti-CD3 antibody (2 μg/ml; Sigma-Aldrich) which required for TCR activation and induce T cell proliferation (204). In some experiments, ECs were exposed to TLR ligands. In addition, IDO metabolites (KYN or 3- 3-HAA) or the vehicle control (HCl; all from Sigma-Aldrich) were added to the PBMCs in the lower chamber (Figure 3.1). Cells were then collected after 3–6 days for bromodeoxyuridine (BrdU) proliferation assays, and supernatants were stored at -80ºC for cytokine analysis.

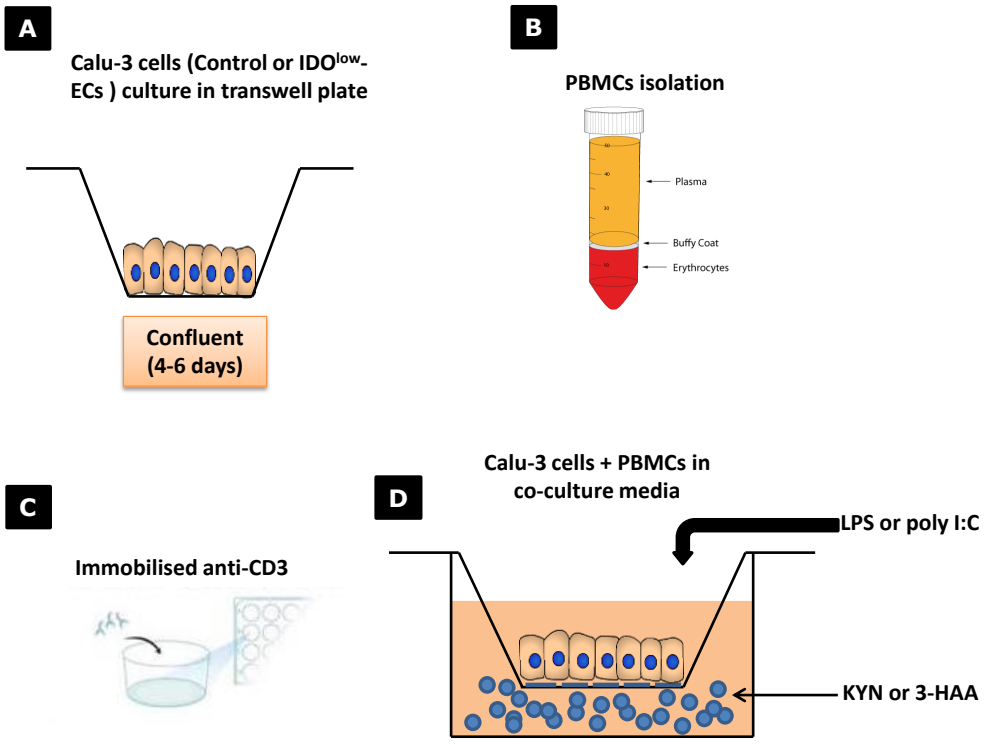


Figure 3.1: Schematic diagram of the procedure for co-culturing of ECs and PBMCs in transwell plate. (A) Calu-3 cells (6 × 10^5 cells/ml) were cultured in 0.4-µm pore size transwell plates for 4-6 days. (B) and (C) PBMCs were isolated from fresh blood and in the same time wells were activated with immobilised anti-CD3 antibody (2 µg/ml) for 90 minutes at 37°C. (D) Confluent layer of Calu-3 cells were co-cultured with anti-CD-3 stimulated PBMCs (8 × 10^5 cells/600 µl). In some experiments, Calu-3 cells were exposed to TLR ligands or IDO metabolites were added to the PBMCs in the lower chamber.

3.2.5- Proliferation assays

Cell proliferation was determined using the BrdU-Elisa Assay (Calbiochem, USA), according to the manufacturer's instructions. Two types of controls were used to ensure the validity of the experiments; cells without BrdU, and tissue culture media alone with BrdU (i.e. containing no cells) were used as the background and blank respectively.

3.2.6- Cytokine measurements

IFN-gamma cytokine concentrations in culture supernatants were assessed at 24 and 48 hours post-treatment using the Human IFN-gamma DuoSet ELISA (R&D Systems, UK), following the manufacturer's instructions. Absorbance was measured using a spectrophotometer, and the results were expressed in pg/ml based on a standard curve.

3.2.7- Determination of cell viability and apoptosis

The annexin V-FITC Apoptosis Detection Assay, calcium-dependent phospholipid-binding protein with a high affinity for phosphatidylserine (Beckman Coulter) was used to measure cell viability. Briefly, following stimulation, PBMCs were collected and washed with ice-cold PBS. Subsequently, the cell pellets were re-suspended in 200 µl of ice-cold, diluted binding

buffer. Cells were incubated with 25 µg/ml annexin V-FITC and propidium iodide (PI) on ice for 15 minutes in the dark. As a positive control, cells were incubated with PBS containing 3% formaldehyde for 30 minutes on ice, washed, and re-suspended in annexin V-FITC and PI. Then, 400 µl of binding buffer was added to each tube, mixed gently, and analysed by flow cytometry (Beckman Coulter) within 30 minutes.

3.2.8- High Performance Liquid Chromatography (HPLC)

HPLC was performed to measure KYN and 3-HAA production, using Calu-3 supernatants after TLR ligands and allergen extracts engagement. Protein was precipitated in culture supernatant samples by the addition of 5-volumes of ice-cold 0.1% (v/v) formic acid in methanol followed by pulse vortex mixing. Samples were then incubated (2-16h at -20°C) and supernatants were recovered by centrifugation (3min, 15kRCF), dried to residue using vacuum centrifugation and subsequently re-suspended in 80 µl of 5% (v/v) acetonitrile, 0.1% formic acid in water just prior to analysis. Stock solutions of standards were prepared in dimethyl sulfoxide (DMSO) and calibration standards were prepared by dilution in 5% (v/v) acetonitrile. Samples (20 µl) were analysed using binary gradient reversed-phase HPLC using a C18 column (Phenomenex Luna C18, 3 µm, 2.1x50 mm) with a gradient of

5 to 100% (v/v) acetonitrile in 0.1% formic acid in water developed over 4 minutes using a Waters 2700 sample manager and Jasco 2085 pumps with eluate directed to a triple quadrupole mass-spectrometer (Waters, Quattro Ultima) equipped with analytical Electrospray ion-source. Components were detected using Multi-Reaction-Monitoring of quantifier and validator transitions in positive ion mode. All instrument control, calibration and data processing was performed using Waters MassLynx 4.0 software.

3.2.9- Statistical analysis

Data are expressed as the mean ± standard error of mean (SEM). Statistical differences were determined using Student's *t* test to compare 2 groups, and one-way ANOVA was performed to compare 3 or more groups. Tukey's post-hoc test was applied, and $*p \leq 0.05$ was considered statistically significant. GraphPad Prism 6 software (GraphPad Software, La Jolla, Calif) was used for all statistical analysis.

3.3- Results

3.3.1- Human airway ECs (Calu-3 cells) inhibit T cell activation in a contact-independent manner

Previous studies have shown that murine ECs can inhibit T cell proliferation (9-11). To evaluate whether human airway ECs can also inhibit T cell proliferation, airway ECs were cultured in transwell plates to confluency, and then co-cultured with anti-CD3 stimulated PBMCs to activate TCRs, and proliferation was assessed after 3–6 days in culture. The data showed that the presence of airway ECs significantly inhibited T cell proliferation (Figure 3.2 A) and IFN-γ production (Figure 3.2 B) in a contact-independent manner. Furthermore, preliminary experiments in regards to cell-cell contact were investigated. Non-significant reduction of T cell proliferation was found (data not shown). Therefore, it is suggested that the inhibitory effect of airway ECs on T cell proliferation could be mediated via soluble factors.

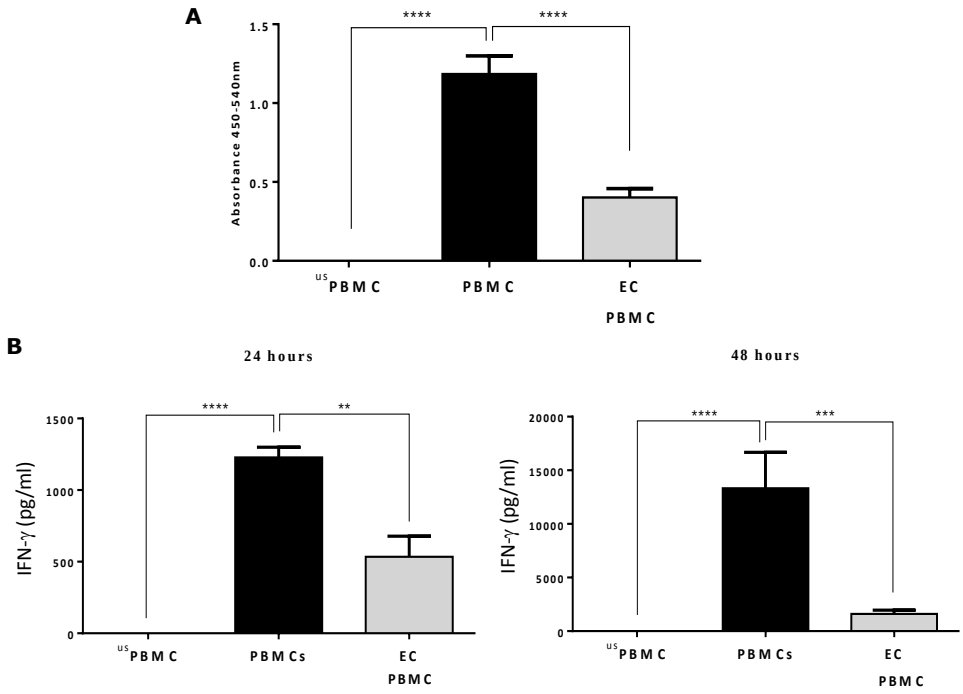


Figure 3.2: Inhibition of PBMC proliferation and IFN-γ production by human airway ECs. (A) Airway ECs (Calu-3 cells) were cultured to confluency and then co-cultured with human PBMCs stimulated with 2 μg/ml of anti-CD3, without cell-cell contact (performed in transwell plates with a 0.4-μm pore size). After 3 days, PBMC proliferation was assessed by performing BrdU assays. (B) The IFN-γ concentration in culture supernatants after 24 and 48 hours was measured by ELISAs. PBMCs alone were used as a control. US, unstimulated. The data shown are representative of 3 independent experiments. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$.

3.3.2- TLR3 and TLR4 engagement affect airway EC-mediated suppression of T cell activation

Data from chapter 2 had shown that TLR agonists simulating bacteria or viral stimulation could suppress IDO activity in airway ECs. Next, experiments were performed to address whether the same TLR agonists could influence the inhibitory

effect of airway ECs on T cell activation. Airway ECs were exposed to TLR4 and TLR3 ligands (LPS and poly I:C respectively) for 72 hours in co-culture with anti-CD3 stimulated PBMCs. After 3 days of co-culture, the cells were collected for BrdU proliferation assays, and supernatants were harvested for cytokine analysis. Interestingly, LPS and poly I:C treatment enhanced T cell proliferation, compared with that observed in control EC/PBMC co-cultures (Figure 3.3 A). This finding correlated with the IFN-γ production levels observed after LPS and poly I:C inoculation for 24 and 48 hours (Figure 3.3 B). Although IFN-γ levels in response to Poly I:C were slightly enhanced, they were not statistically significant (p = 0.0588) at 48 hours. These findings clearly suggested that airway ECs could lose their ability to regulate T cell proliferation in response to different TLR agonists as mimic bacterial or viral infection.

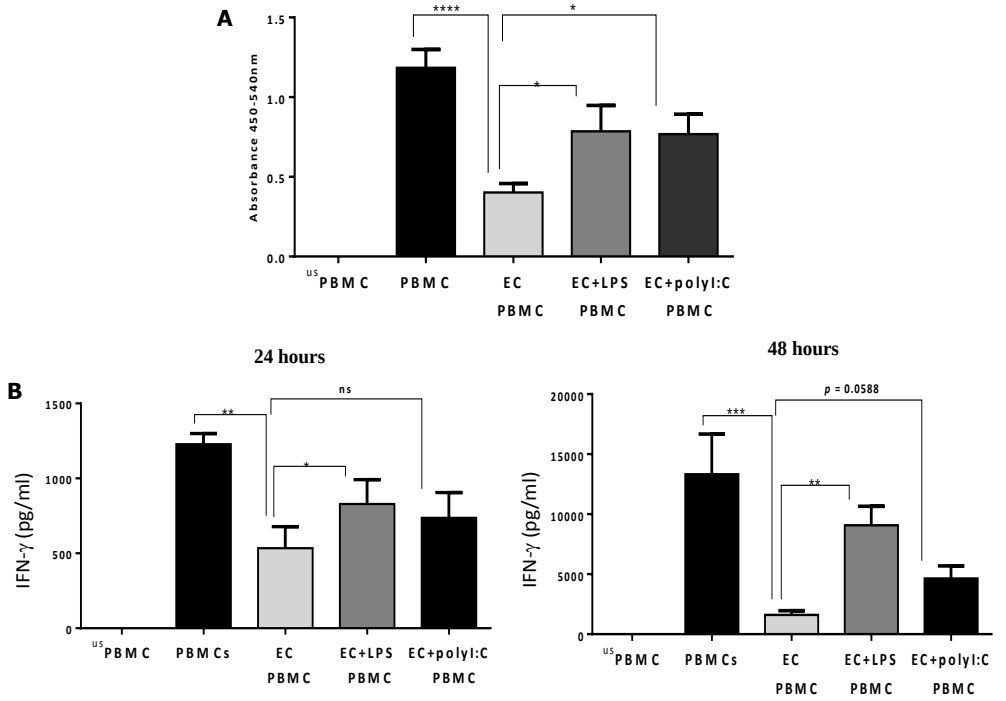


Figure 3.3: Influence of TLR-activated airway ECs on PBMC proliferation. (A) Human airway ECs (Calu-3 cells) were co-cultured with PBMCs stimulated with 2 µg/ml of anti-CD3 in the presence of 10 µg/ml of LPS or poly I:C. After 3 days, proliferation was assessed by performing BrdU assays. (B) IFN-γ production in the co-culture supernatants was quantified by performing ELISAs. US, unstimulated. The data shown are representative of 3 independent experiments. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

3.3.3- IDO contributes to airway EC-mediated suppression of T cell activation

Previous findings have suggested the critical function of ECs as a potent inhibitor of T cell proliferation, as well as the role of TGF-β produced by ECs in modulating T cell proliferation (204). However, the exact mechanism by which ECs inhibit T cell proliferation remains unclear. Given the crucial role of ECs

in modulating immune responses in the airways in the context of IDO activation, we hypothesised that the inhibition of T cell proliferation by airway ECs could be mediated by IDO. Therefore, the potential role of IDO in mediating the inhibitory effect of airway ECs over T cells was studied. IDO^{low}-ECs were generated by knocking-down expression of the IDO1 gene in airway ECs using siRNA technology. As shown in Figure 3.4 A, IDO activity and IDO1 mRNA expression significantly decreased after gene silencing (IDO^{low}), compared with the control cells that express higher IDO levels, demonstrating that IDO1 gene expression was reduced. Subsequently, IDO^{low}-ECs and PBMCs pre-treated with an anti-CD3 antibody were co-cultured, and PBMC proliferation was assessed 3 days later. Figure 3.4 B shows that the presence of airway ECs significantly inhibited T cell proliferation, but that inhibition was not evident after IDO silencing. Moreover, IFN-γ production after 24 and 48 hours was investigated. As expected, IFN-γ levels were significantly decreased in the presence of airway ECs, and IFN-γ production was restored in the presence of IDO^{low}-ECs, compared with that observed in control cells (Figure 3.4 C). Collectively, these results demonstrated the important role of IDO produced by ECs in inhibiting T cell activation.

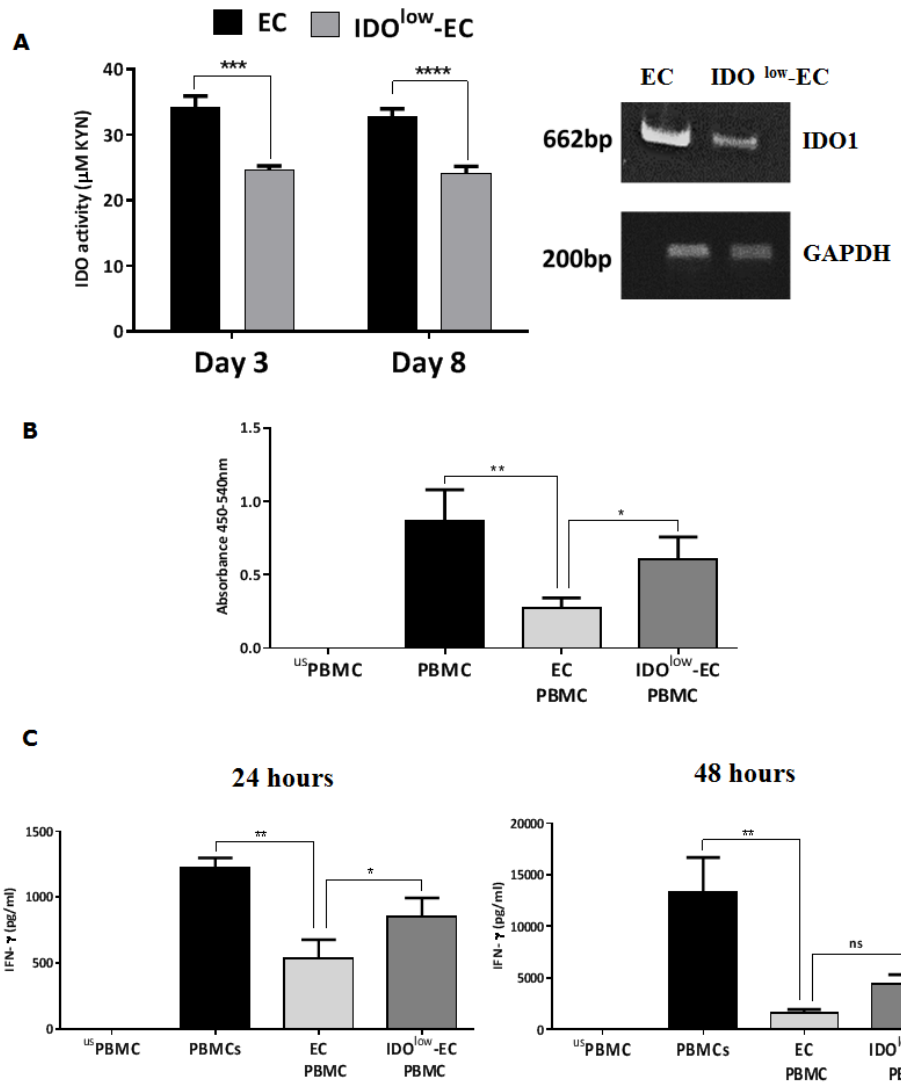
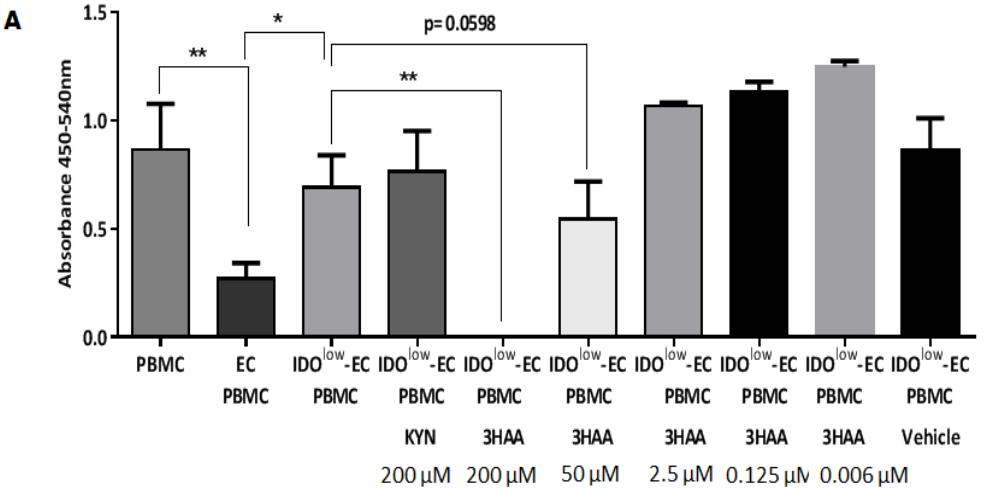


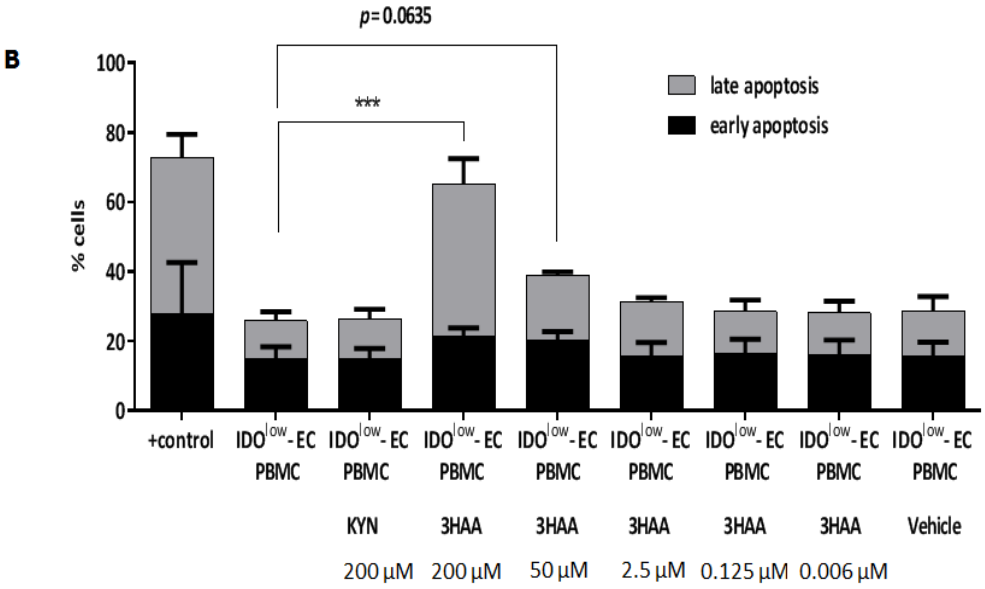
Figure 3.4: Role of IDO-expressing human airway ECs in inhibiting PBMC proliferation and IFN-γ production. (A) Airway ECs (Calu-3 cells) were transfected for 6 hours with 50 nM IDO1 siRNA, using a non-targeting siRNA as a negative control. The efficiency of IDO down-regulation after transfection was assessed by measuring IDO activity and mRNA expression (n = 3). (B) Control ECs (i.e. IDO^{high}) and IDO^{low}-ECs were co-cultured with PBMCs activated with an anit-CD3 antibody (2 µg/ml). After 3 days, proliferation was assessed by performing BrdU assays (n = 4). (C) IFN-γ levels in supernatants of the co-cultured cells were quantified by performing ELISAs after 24 and 48 hours (n = 3). US, unstimulated. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

3.3.4- 3-HAA, but not KYN, affects T cell proliferation by inducing apoptosis

KYN and 3-HAA are by-products of IDO enzymatic activity and play crucial roles in mediating many of the immunological effects of the KYN pathway (177, 210). Thus, to better understand if either of these IDO metabolites is involved in suppressing T cell activation, the effects of both KYN and 3-HAA were tested on T cell proliferation. IDO^{low}- ECs were incubated with 200 μM of KYN and (200 – 0.006 μM) varying concentrations of 3-HAA for 3 days, after which T cell proliferation was assessed. As shown in Figure 3.5 A, 200 μM and 50 μM of 3-HAA inhibited T cell proliferation; however, with reducing 3-HAA concentrations, its inhibitory effect on cell proliferation could be titrated out. However, KYN appeared to have no effect on proliferation, even at high concentration. It is well known that KYN, 3-HAA, and other downstream metabolites of IDO can cause cellular toxicity and apoptosis via the production of intracellular ROS or other mechanisms (147, 214). Therefore, to test whether the suppression of T cell proliferation by 3-HAA was due to induction of cell death, we further quantified the percentage of cells going through apoptosis. This could be either an early phase of apoptosis in which the plasma membrane remains intact but exposes phosphatidylserine (PS) on the cell surface to mediate its

recognition by phagocytes or late apoptosis (i.e. secondary necrosis) in which the plasma membrane becomes permeabilised (215). Our data showed no effect on cell viability in the presence of KYN or low concentrations (<50 μM) of 3-HAA compared with the control (IDO^{low}-ECs/PBMCs). However, there was significant induction of apoptosis in response to 200 μM of 3-HAA but only slight induction in the presence of 50 μM 3-HAA (Figure 3.5 B and C). These findings suggested that KYN does not affect T cell proliferation, whereas 3-HAA can inhibit T cell proliferation by inducing apoptosis. While, this data suggest that 3-HAA, but not KYN, might account for the observed suppression of T cell proliferation in these experiments, the role of other IDO metabolites cannot be ruled out. Moreover, the direct effect of KYN and 3-HAA on single culture of PBMCs (without Calu-3 cells) was examined. The data showed the same effect of co-culture condition by which 3-HAA but not KYN inhibited T cell proliferation via inducing apoptosis (Appendix 2).


A
Absorbance 450-540nm
1.5
1.0
0.5
0.0
*
**
**
p= 0.0598
PBMC
EC PBMC
IDO^low-EC PBMC
IDO^low-EC PBMC KYN 200 µM
IDO^low-EC PBMC 3HAA 200 µM
IDO^low-EC PBMC 3HAA 50 µM
IDO^low-EC PBMC 3HAA 2.5 µM
IDO^low-EC PBMC 3HAA 0.125 µM
IDO^low-EC PBMC 3HAA 0.006 µM
IDO^low-EC PBMC Vehicle


B
p= 0.0635
***
late apoptosis
early apoptosis
% cells
100
80
60
40
20
0
+control
IDO^low-EC PBMC
IDO^low-EC PBMC KYN 200 µM
IDO^low-EC PBMC 3HAA 200 µM
IDO^low-EC PBMC 3HAA 50 µM
IDO^low-EC PBMC 3HAA 2.5 µM
IDO^low-EC PBMC 3HAA 0.125 µM
IDO^low-EC PBMC 3HAA 0.006 µM
IDO^low-EC PBMC Vehicle

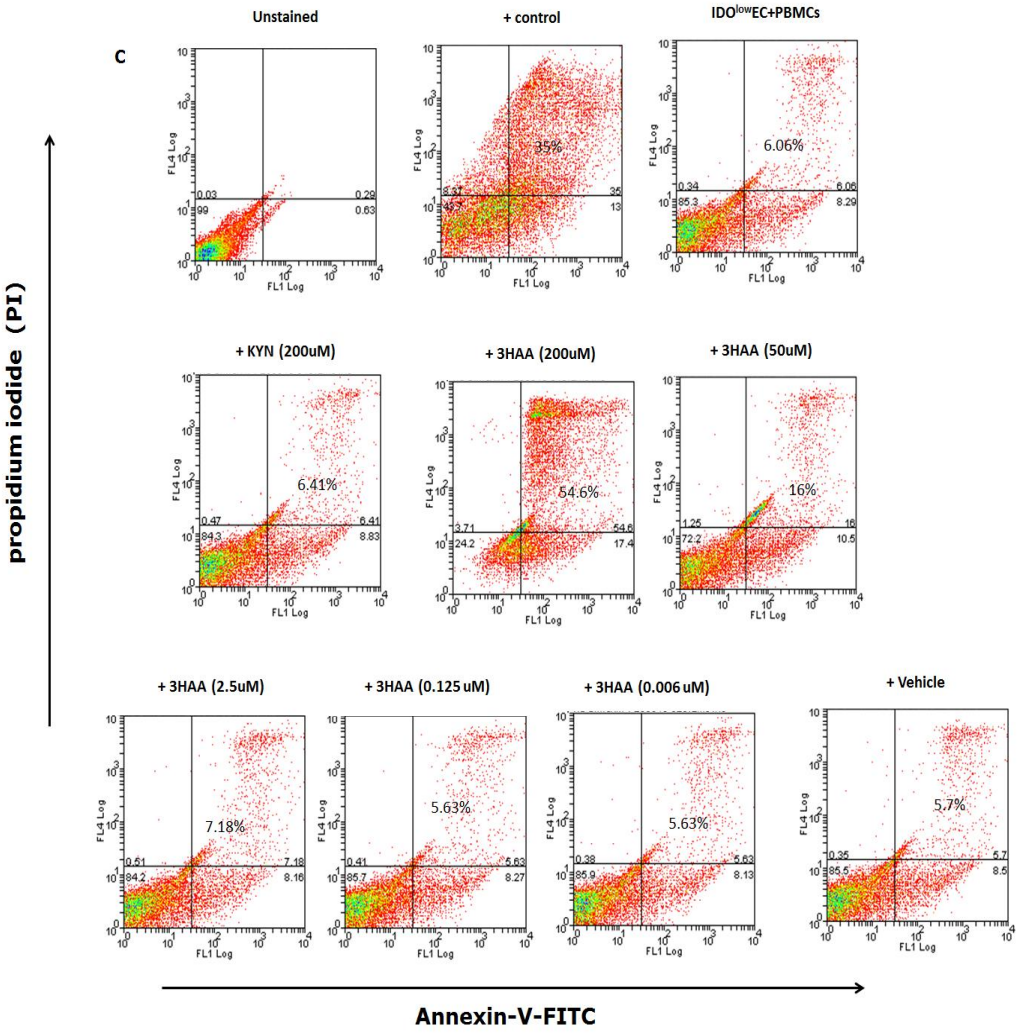


Figure 3.5: The metabolite 3-HAA, but not KYN, affects PBMC proliferation by inducing apoptosis. (A) ECs with low IDO expression (IDO^{low}-ECs) were co-cultured with PBMCs stimulated with 2 µg/ml of anti-CD3 antibody in the presence of 200 µM KYN, decreasing 3-HAA concentrations or with vehicle control (i.e. HCL) for 3 days, and then proliferation was quantified with a BrdU proliferation kit. (B) Cells viability after incubation with KYN and decreasing doses of 3-HAA was assessed by an Annexin V-FITC Apoptosis Detection Assay showing early (annexin-V-FITC+PI-) and late (annexin-V-FITC+PI+) apoptotic cells. Formaldehyde (3%) was used as a positive control. The data shown are representative of 3 independent experiments. (C) Dot plot representations of annexin-V-FITC and PI staining in the presence of KYN and 3-HAA (one experiment representative of three). $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

3.3.5- Down-regulation of 3-HAA production in Calu-3 cells, but not KYN production, as measured by HPLC in response to TLR ligands and allergen extracts

To further evaluate the effect of IDO metabolites, the concentrations of KYN and 3-HAA secreted into culture supernatants in response to various TLR ligands and allergen extracts was analysed by HPLC. The data showed a significant decrease of 3-HAA production (Figure 3.6 A) and no significant difference of KYN production (Figure 3.6 B) in response to the allergens (10 µg/ml) and TLR agonists (TLR4, TLR9, TLR2/6, and TLR3) tested compared with the control. This observation clearly supported the result shown above that 3-HAA, but not KYN, reduced T cell proliferation. Interestingly, the basal level of KYN production was much higher compared with the basal 3-HAA level. Although 3-HAA suppression occurred in response to TLR ligands and allergen extracts, which supported previous data generated in this study, the values were close to the limit of the detection. Hence, further investigation is required.

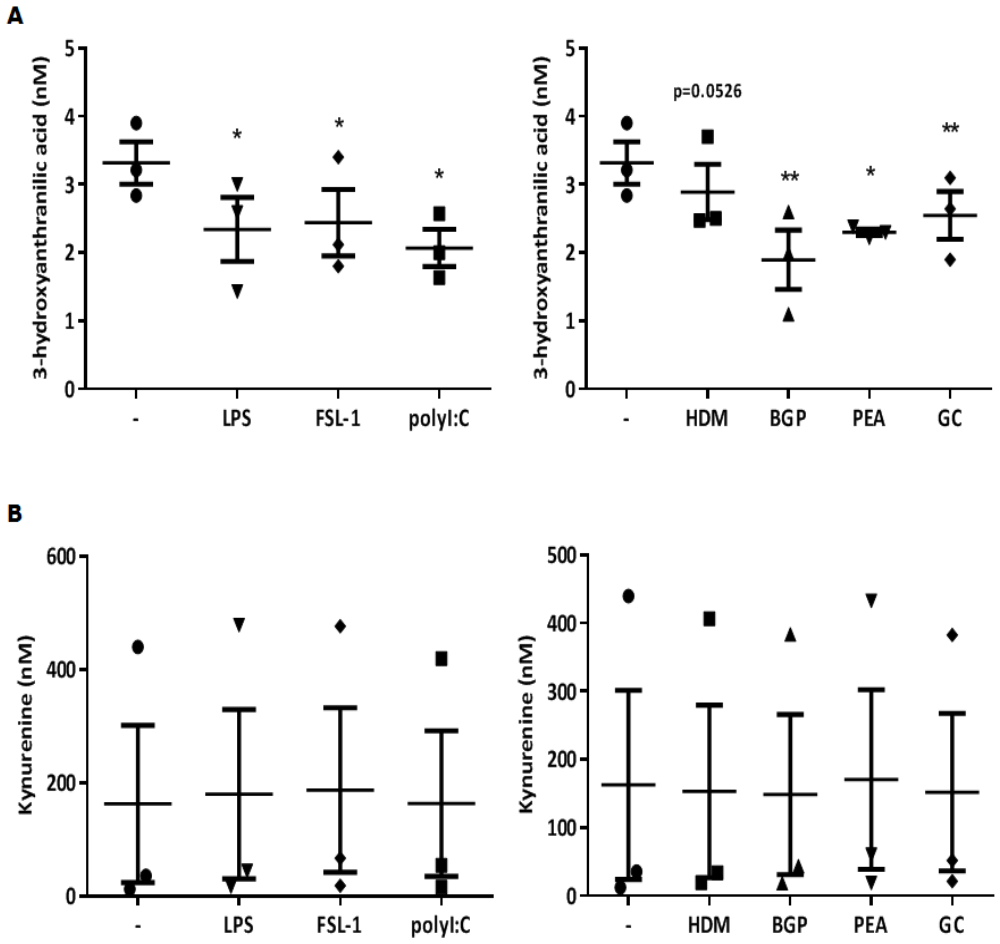


Figure 3.6: HPLC detection of IDO metabolites (KYN and 3-HAA) produced by human airway ECs. Calu-3 cells were cultured to confluency and pre-incubated for 24 hours with 10 µg/ml of allergen extracts (HDM, BGP, PEA, or GC) or TLR agonists; 2 µg/ml of TLR4:LPS; 10 µg/ml TLR2/6:FSL-1; or 0.1 µg/ml TLR3:poly I:C). The 3HAA (A) and KYN (B) concentrations were detected in culture supernatants by HPLC. The data shown are representative of 3 independent experiments.*$p \leq 0.05$, **$p \leq 0.01$.

3.4- Discussion

Lung ECs are one of the main cellular components of innate immunity and provide primary protection against airborne pathogens and allergens. ECs are in close contact with different immune cells, including DCs and intraepithelial lymphocytes, which collectively contribute to the stimulation of adaptive immune responses (94, 216). It has been recognised that ECs can directly regulate conventional and unconventional T cell responses (204, 205, 217, 218). However, the mechanism by which ECs mediate such immune regulation, particularly their effect on T cell proliferation, is still scarcely defined. The focus of this study was to analyse the role of human airway ECs in modulating T cell responses and to evaluate the impact of TLR ligands and allergens on such immune regulation. After investigating the pattern of IDO expression in airway ECs in response to TLR ligands and allergen extracts in previous chapter, we hypothesised that airway ECs could modulate human T cell activation via IDO expression. In this study, the data showed that airway ECs could suppress T cell activation (i.e. T cell proliferation and IFN-γ production) in a contact-independent manner. Within this context, it has been suggested that murine lung ECs can suppress T cell activation and induce regulatory T cells (204). This observation clearly supports our results showing that

human airway ECs can inhibit T cell activation, which could suppress undesirable adaptive immune responses; however, the role of regulatory T cells remains to be established. Interestingly, the levels of T cell proliferation and IFN-γ production were partially recovered when airway ECs were exposed to different TLR agonists, which were used as mimics for bacterial and viral infection conditions. Therefore, it is possible that under these conditions the immune balance in the airways is disrupted, facilitating the development of an inflammatory immune response. However, given the absence of some controls such as a single culture of PBMCs stimulated with LPS or poly I:C, there is also a possibility that the inhibitory effects on PBMCs proliferation and IFN-γ production were independent of ECs, which can be further explored in the future.

As previously discussed, ECs produced-TGF-β has mediated the suppression of T cell activation to some degree (204). They confirmed the effect of TGF-β by adding anti-TGF-β antibody to the ECs/T cells/DCs co-culture and resulted in partial restitution of T cell proliferation (204). However, having investigated the expression of IDO in airway ECs in previous chapter, which is down-regulated in response to TLR ligands, it is reasonable to assume that IDO produced by ECs could play an important role in modulating T cell activation. Accordingly,

the role of IDO in modulating T cell activation by generating IDO^{low}-ECs using gene silencing was evaluated in this chapter. The data showed that, compared with control cells that express high amounts of IDO, IDO^{low}-ECs were significantly less efficient in suppressing T cell proliferation. These data were also reflected in the levels of IFN-γ produced in the culture supernatants. Collectively, these data suggested that IDO produced by ECs could play a role in EC-mediated suppression of T cell responses.

IDO-induced tryptophan degradation results in the production of different metabolites, including KYN, 3-hydroxykynurenine, anthranilic acid, 3-HAA, and quinolinic acid, among others. KYN and 3-HAA are the main metabolites that account for IDO-dependent immune-suppressive effects (147, 158, 219, 220). Accordingly, the focus in this study was on KYN and 3-HAA, as they are 2 of the most extensively studied IDO metabolites (150, 151, 158, 210). Our data showed significant suppression of T cell proliferation by 3-HAA, but not by KYN, demonstrating their differential roles in mediating the effect of airway ECs on T cells. These data suggested that airway ECs play a crucial role in maintaining lung homeostasis. However, after infection – mimicked by treatment with TLR agonists – the immune balance in the airways was disrupted in an IDO-dependent manner, which could facilitate the development of

pro-inflammatory immune responses. Because infection, particularly viral infections, are major triggers of airway inflammation and asthma exacerbations (46), these data might have implications in asthma-related diseases. This possibility is further supported by data showing that IL-4 can block the inhibitory effect of ECs on T cell proliferation (206) and that Th2 cytokines (including IL-4 and IL-13) can also inhibit IDO activity (157).

Airway EC-mediated T cell inhibition may occur through the induction of T cell apoptosis, anergy, or tolerance, or through the modulation of DC functions. To address this question, the ability of IDO-derived metabolites to induce T cell apoptosis was evaluated. Our data showed that 3-HAA, but not KYN, induced T cell apoptosis, which could explain the impairment in T cell proliferation in response to 3-HAA. Previous data have suggested an association between 3-HAA and induction of apoptosis in different mouse models, although the associated mechanism remains unclear (147, 158, 214, 221, 222). Apoptosis has been associated with caspase activation (147, 214), ROS induction (147), cytochrome C release from mitochondria (214), and the depletion of intracellular GSH (222). However, other authors have shown that 3-HAA induces PDK1 activation, which inhibits NF-κB activation, leading to the

death of activated Th2 cells and the inhibition of experimental asthma (158). In contrast, 3-HAA can prolong corneal allograft survival by reducing T cell proliferation and inducing regulatory T cell development (221). Moreover, another study provided evidence that the induction of IDO activity in addition to high IDO metabolite concentrations (particularly 3-HAA) reduces lung allograft injury as a result of suppressed CD4+ and CD8+ T cell responses and impaired TCR activation (223). Finally, in an experimental model of autoimmunity, 3-HAA was shown to increase apoptosis and decrease proliferation of splenic activated T cells and to alter cytokine production from a Th1 profile to a protective Th2 profile (224). Collectively, these data suggest a key role for metabolites derived from the TRP catabolism pathway, particularly 3-HAA, in controlling T cell functions and subsequent immune responses. However, IDO-derived metabolites from airway ECs might have an effect, not only on T cells, but also on different immune cells such as DCs, macrophages, and B cells, although this remains to be established.

As previously discussed, diverse IDO-derived metabolites have been shown to account for IDO immune-suppressive effects (150, 151, 158). However, there is no evidence showing the presence of such metabolites in ECs. Accordingly, IDO metabolites, in particular KYN and 3-HAA, were measured by

HPLC in airway ECs stimulated with diverse TLR agonists and allergen extracts. Intriguingly, the data showed differential regulation in IDO-derived metabolites, reflected in a reduction in 3-HAA production and an increase in KYN production. The data in this study was reported as KYN levels since all metabolites in IDO pathway are often referred as kynurenines. However, IDO activity was quantified using a colorimetric assay that measure diverse indole-based metabolites including KYN and 3-HAA levels. Accordingly, these measurements account for a mixture of metabolites and not KYN levels only which could explain the inconsistency of HPLC determination of KYN and colorimetric assays. It is also important to highlight that the basal levels of KYN were higher than those of 3-HAA. In this context, it would be of interest to study the expression levels (after stimulation and under resting conditions) of diverse enzymes in the KYN pathway involved in KYN and 3-HAA metabolism, such as kynurenine hydroxylase and kynureninase, among others.

In conclusion, the results of this study underscore the important role of airway ECs in controlling immune responses through the direct modulation of T cell functions via production of IDO. However, using PBMCs in the co-culture with airway ECs was somewhat artificial since physiologically those ECs do

not encounter PBMCs. Therefore, BAL leukocytes should instead be used for future studies.

Given the pivotal role of IDO-derived metabolites from DCs in modulating immune responses in the airways (155, 177, 182) and evidence describing the key role of EC-derived cytokines in modulating DC behaviour (46), studying how the IDO pathway affects cross-talk between DCs and airway ECs in the context of allergy merits further study. Such investigations would help to better understand pathologies such as asthma and to develop better therapeutic strategies to combat Th2-related diseases.

Chapter 4: Development and characterisation of a 3D co-culture comprising human lung epithelial cells, fibroblasts and dendritic cells

4.1- Introduction

The respiratory system is constantly exposed to harmful micro-organisms and environmental factors, and ECs are the predominant cells that come into contact with inhaled particles. As such, these cells are considered the first barrier that interfaces between the external and internal environment to regulate mucosal homeostasis (37, 94). Notably, ECs comprise a chemical, physical, and immunological barrier that protects the host from microbial challenges. Specifically, the secretion of mucus and various anti-oxidants and anti-microbial molecules provides a chemical barrier (225), while the physical barrier consists of tight junction formation via the production of intracellular junction proteins, including claudin 1–5, occludins, and cytoplasmic ZO-1–3, as well as the transmembrane adhesion proteins E-cadherin and β-catenin (13, 59, 77). Lastly, ECs provide an immunological barrier via the production of immune-stimulatory and immune-modulatory mediators, such as cytokines, chemokines and

growth factors, as well as immune-regulatory enzymes such as IDO (226, 227).

Compromising the EC barrier results in structural changes within ECs and modification of EC function to communicate with neighbouring mesenchymal cells and other innate and adaptive immune cells via cell-cell or paracrine contact (207, 228, 229). In the airway lining, a layer of fibroblasts is located close to the basal surface of the epithelium and can contribute to pulmonary inflammation and airway remodelling through activation of ECs (57, 228). Indeed, patients suffering from airway diseases exhibit significant changes in the architecture of the airway wall, including increased deposition of ECM proteins, such as collagen, MMPs, and FN in the active site of the airway wall and increased expression and production of cytokines and growth factors, such as transforming TGF-β and EGF, which influence the synthesis of ECM proteins in fibroblasts/myofibroblasts (57, 230). Furthermore, eosinophils play an important role in airway remodelling through production of TGF-α and TGF-β, and by enhancing airway smooth muscle cell proliferation (231, 232). Compared to healthy airways, the airway layers of patient with pathological conditions such as asthma, CF, and COPD exhibit architectural alterations. That are characterised by sub-epithelial layer

thickening, goblet cell and smooth muscle cell hyperplasia, and angiogenesis (230).

At the crossroads of innate and adaptive immune responses, and alongside airway ECs, DCs play a central role in host immune defence by linking innate and adaptive immunity (3). Several studies have shown that cross-talk between ECs and DCs is crucial in driving Th2 allergic responses (6, 95, 233). ECs rapidly detect and recognise allergens via PARs, TLRs, and possibly CLRs (35, 96, 234). This recognition results NF-κB activation and subsequent expression of cytokines and chemokines, including TSLP, GM-CSF, IL-33, IL-25, IL-1, chemokine C-C ligand (CCL)2, and CCL20, which attract and activate innate and adaptive immune cells (3). While such T cell activation leads to optimal clearance of some pathogens (e.g. helminthic parasites) under normal physiological conditions, this process can lead to chronic inflammation and tissue damage in individuals with pathological conditions (e.g. allergic diseases) (235).

Most studies of the human airway are carried out using cells grown as 2D monolayers. Although ECs can be cultured at the ALI in a 2D platform, and this system could potentially be used to gain information regarding cellular interactions, cells cultivated in this manner do not accurately mimic the

phenotypes, cellular arrangements, and histological features of cells *in vivo*. Accordingly, 3D co-culture models provide a more *in vivo*-like system that exhibit a greater resemblance to physiological conditions (109, 236). One of the primary requirements of the 3D model is to grow cells on a scaffold comprised of ECM proteins, such as collagen, fibrinogen, elastin, and laminin, which supports cell differentiation and facilitates cross-talk between cells (109, 110). In addition, hydrogels can also provide a cellular environment resembling that of native ECM (237). Notably, different cell types prefer distinct 3D cell matrices. For example, cultivation of skin ECs on a porous micro-fibre scaffold results in the production of cells that display a greater morphological resemblance to the basement membrane of barrier cells, such as skin epithelium *in vivo* (238). Several studies including those from our group have reported that co-cultivation of ECs with fibroblasts results in accelerated differentiation of lung ECs in 3D models compared with that observed in mono-cultures (120). ECs co-cultured with fibroblasts have also been shown to be more efficient at healing and recovery after physical insult than 2D and mono-cultured ECs (59, 120, 239). It is therefore reasonable to suggest that 3D co-culture of lung ECs with relevant structures [e.g. fibroblasts, ASM cells], and effector cells (e.g. DCs and mast cells) could better mimic *in vivo*

conditions. Such models could provide a useful platform for studying the biology of different cell types in the context of airway diseases, such as allergic and pathogen-induced inflammation, as well as for disease modelling and testing of novel drugs.

In this chapter, the IDO-mediated immune-regulatory properties of differentiated ECs were elucidated in mono-cultures, co-cultures, and in the 3D model that offers a better representation of physiological conditions.

4.2- Materials and Methods

4.2.1- Cell lines and primary cell cultures

Calu-3 cells (ATCC® HTB55™) were maintained in DMEM-F12 as described in chapter 2, section 2.2.1. Immortalized human bronchial epithelial cells (iHBECs) were grown in keratinocyte serum-free media (Invitrogen) with 0.02% v/v epidermal growth factor, 0.5% v/v bovine pituitary extract (BPE), and 1% v/v pen/strep. MRC-5 cells (ATCC® CCL171™, human lung fibroblast cell line) were maintained in Minimum Essential Medium Eagle (MEM; Sigma-Aldrich) with 10 % v/v heat inactivated FBS, 1% v/v pen/strep, and 2mM L-glutamine (Sigma-Aldrich). iHBCs was a kind gift from Prof Jerry Shay (University of Texas South Western Medical Centre). The nature and the source of iHBCs described previously (240). pHBECs were maintained in BEGM and carried out as described in chapter 2, section 2.2.2. All pHBECs from healthy and asthmatic subjects were used in passage 3. For each experiment, around 5 to 7 samples were cultured and at least 3 of them were characterised successfully. The nature of the healthy and asthmatic volunteers was clarified as shown in Appendix 3.

4.2.2- Culturing of cells on transwell® inserts (TWI)

Calu-3 cells (2 × 10^5 cells/ml) were cultivated in complete media on TWIs (Figure 4.1) containing polyester membranes (12 mm diameter, 0.4 µm pore size) in 12-well plates. One ml of medium was also aliquoted into each well of the plate, in the basolateral side of the TWI. After 24 hours, the medium was replenished. After three to five days, when cells reached 80–90% confluence, the medium was removed from the apical side to allow the monolayer to grow at the ALI. At this time, the medium was also removed from the basolateral side of the TWI; 250 µl of this medium was then mixed with 250 µl fresh medium and returned to the well. For Calu-3/MRC-5 co-cultivation experiments, 2 x 10^5 cells/ml of Calu-3 and MRC-5 cells were inoculated on the apical and basolateral side of the TWI (i.e. in the tissue culture plate), respectively. Primary HBECs (4 × 10^4 cells/200 µl) were cultured in bronchial epithelial differentiation medium (BEDM) on TWI containing polyester membranes (6.5 mm diameter, 0.4 µm pore size) in 24-well plates. BEDM comprised 1:1 mixture of bronchial epithelial basal medium (BEBM) supplemented with 50nM retinoic acid and all BEGM™ SingleQuots™ Kit (Clonetics™ Airway Epithelial Cell Systems, Lonza) except triiodothyronine, and DMEM containing 45,000 mg/litre glucose, L-glutamine, $NaHCO_3$, pyridoxine HCl, and phenol red (Sigma-Aldrich).

BEDM (500 µl) was also added to the lower chamber of the transwell plate. After three to five days, when cells were 100% confluent, the medium was removed from the apical side to allow the monolayer to grow at the ALI, and the medium in the lower chamber was replaced with 500 µl of fresh BEDM. This was repeated for all subsequent media changes (every 2–3 days).

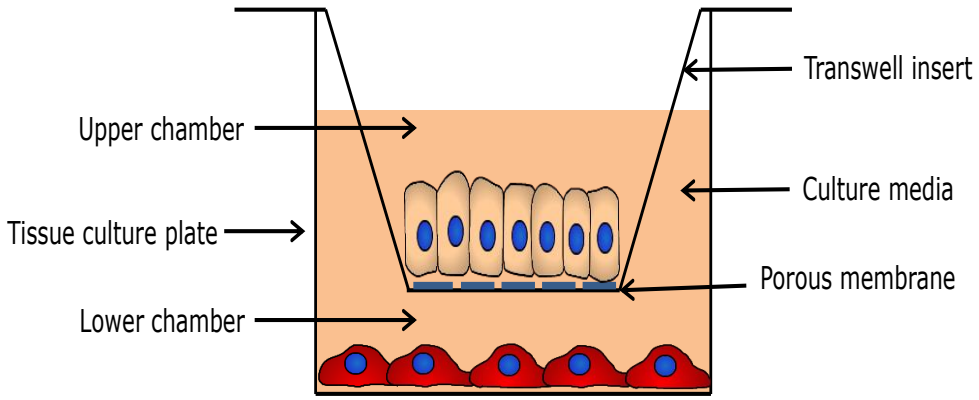


Figure 4.1: TWI system for the in vitro co-culture model. For the TWI co-culturing model, ECs and fibroblasts were cultivated in the upper and lower chambers (basolateral side) of wells in 12-well transwell plates, respectively. The chambers were separated by porous 2D membranes, and cultivation at the ALI was achieved by removing the media from the upper chamber. The apical surface of the ECs was thereby exposed to the air while the basolateral surface was exposed to the culture medium within the bottom chamber.

4.2.3- Cultivation of cells on PET electro-spun scaffolds (3D model)

PET scaffolds were prepared by electro-spinning. Briefly, a 10% (w/v) polymer solution was generated by dissolving PET

in a mixture of 99% (v/v) trifluoroacetic acid (TFA; Thermo Fisher Scientific), and 99% (v/v) dichloromethane (DCM; Thermo Fisher Scientific) overnight at RT. The solution was then loaded into a syringe (BD Biosciences), which was subsequently fitted to a syringe pump-driver (Harvard Apparatus Ltd.), and subjected to electrospinning at a constant flow rate of 0.5 ml/hour at 14 Kv for 4 hours. The resulting scaffold was collected on an aluminium collection plate, allowed to air-dry overnight, and then removed from the collection plate (Figure 4.2). For cell culture preparation, the scaffold was cut to a size of 2 cm^2, sterilised by exposure to 254 nm ultraviolet light for 15 minutes per side, and transferred to 12-well non-tissue culture plates. Each scaffold was secured by placing a steel ring on top, and then further sterilised by incubation with ethanol for 15 minutes followed by a sterilising solution comprised of an 1% v/v antibiotic/antimitotic and penicillin/streptomycin (pen/strep) solution diluted in PBS overnight (37°C, 5% CO_2). The following day, the sterilising solution was removed, and the scaffolds were washed with PBS and submerged in the appropriate cell culture media during the preparation of the cell suspension. Three hundred microliters of Calu-3 and MRC-5 cells were seeded onto separate PET scaffolds (inside of the steel rings) at a density of 5×10^5 cells/ (240 nm ±70) scaffold (1.67×10^6 cells/ml)

and incubated for 4–5 days at 37°C in a humidified atmosphere with 5% CO_2. Subsequently, the scaffolds were transferred to a polytetrafluoroethylene ScaffHolder (manufactured in-house) for growth at the ALI (Figure 4.3). For the co-culture model, a scaffold layer containing Calu-3 cells was cultured on top of the layer of MRC-5 cells, while for the tri-culture model, a scaffold layer containing immature DCs was inserted between the Calu-3 and MRC-5 cell layers on the same ScaffHolder (Figure 4.4).

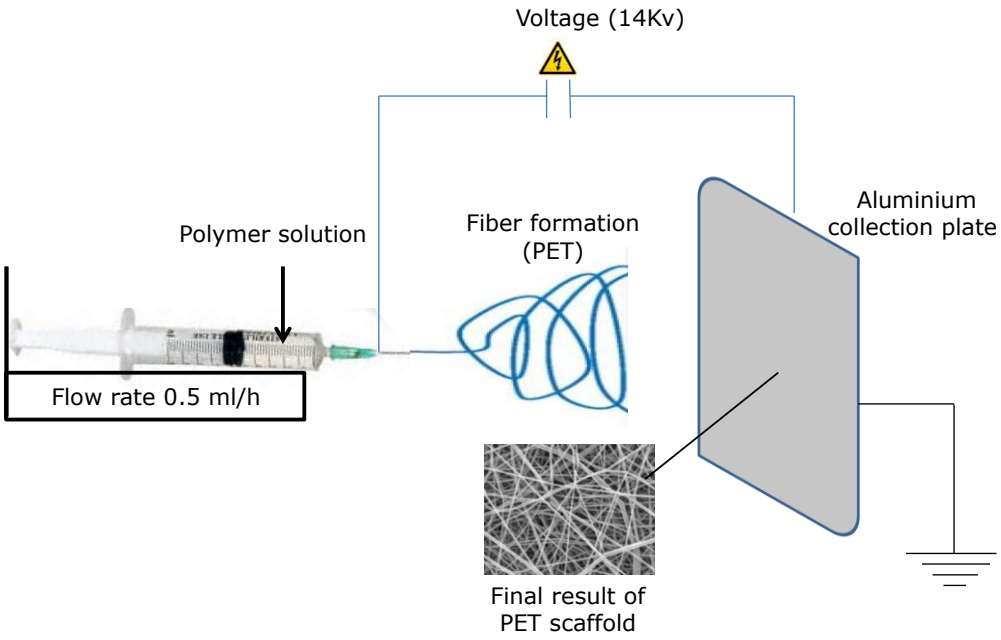


Figure 4.2: Schematic diagram of the electrospinning technique. A polymer comprised of PET dissolved in a solvent mixture of TFA and DCM was loaded into a syringe. The polymer was subsequently passed through the needle tip, which was exposed to a high electrical voltage. The charged jet of polymer fibres was ejected from the needle tip toward a grounded collection plate.

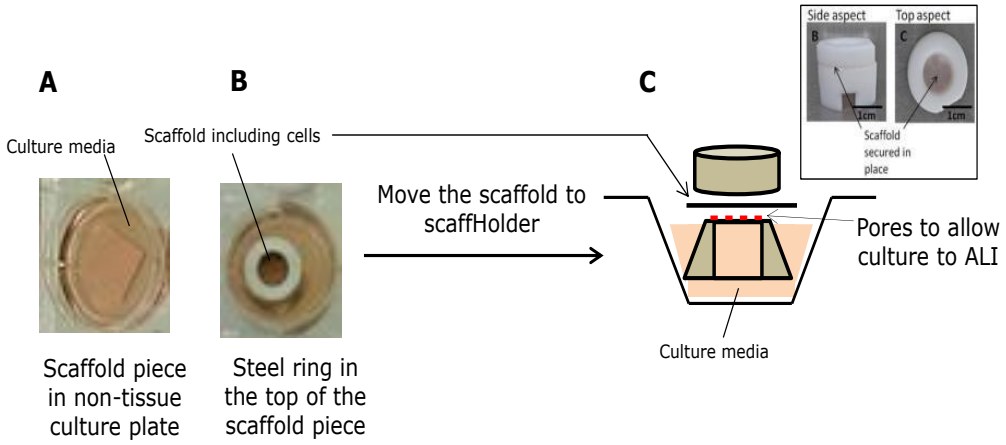


Figure 4.3: 3D engineering of lung tissue constructs. (A) Pieces of electro-spun PET scaffolds were submerged in complete media in 12-well non-tissue culture plates. (B) Steel rings were placed on top of the scaffolds to maintain submergence of the scaffold and to define the cell seeding area. Cells were then cultured on the scaffold for 5 days to ensure confluence. (C) Subsequently, scaffolds containing cultivated cells were moved to ScaffHolders to enable cell growth at the ALI. Each ScaffHolder contained pores to maintain exposure of the basolateral surface of the cell layers to the growth medium. For multi-culturing, multiple layers were inserted within the ScaffHolder and assembled to mimic *in vivo* condition.

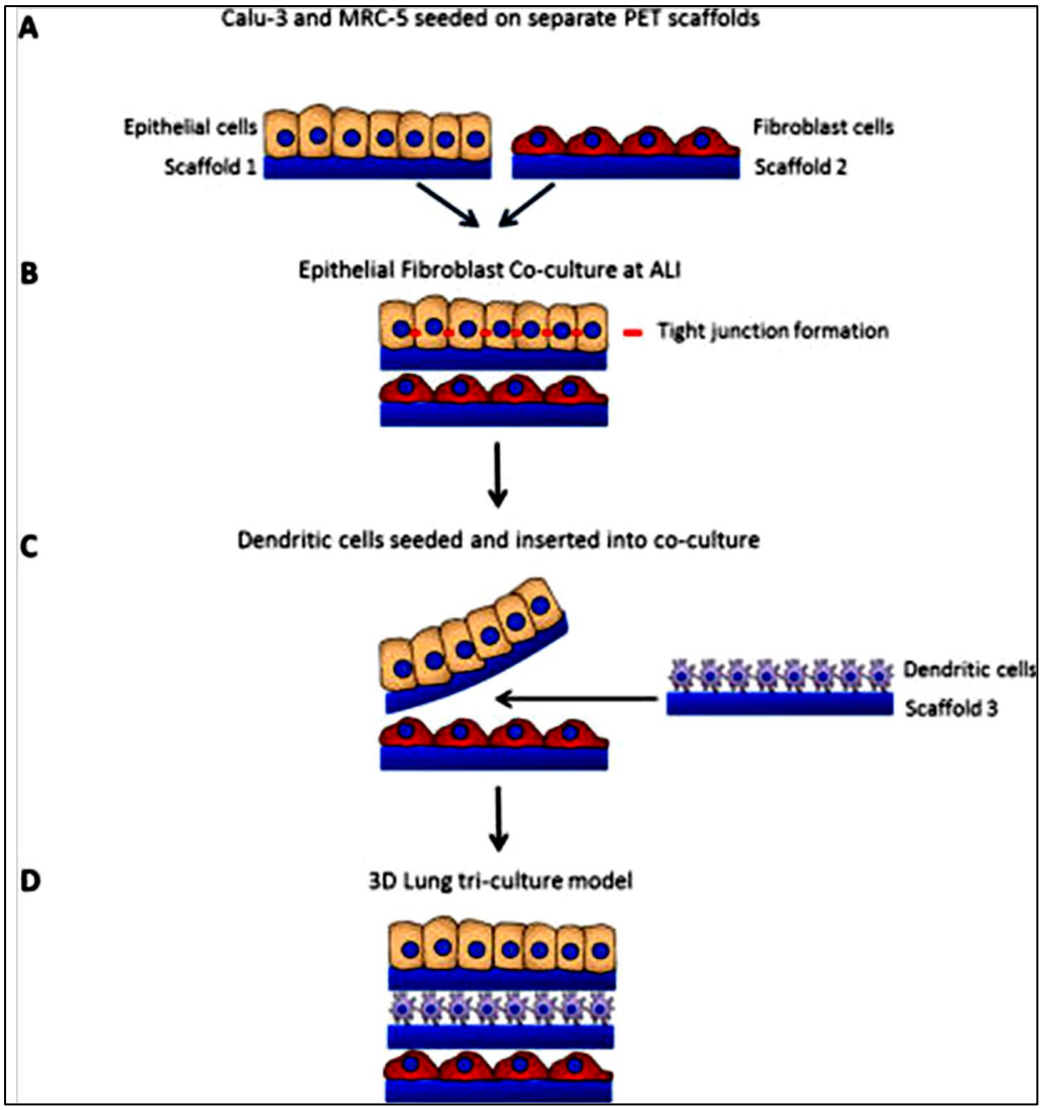


Figure 4.4: Schematic depiction of 3D co-culture model of airway lining. (A) Calu-3 and MRC-5 cells were inoculated onto separate scaffolds. (B–D) Scaffolds containing distinct cell types were layered to create a multi-culture airway epithelial tissue model. Image adapted from (120).

4.2.4- Trans-epithelial electrical resistance (TEER) measurements

ECs were grown to confluence and then cultivated at the ALI, as described above. TEER measurements were taken using an EVOM epithelial volt-ohm-meter (World Precision

Instruments). Specifically, readings were taken from the culture medium every 2–3 days after rehydrating the apical membrane with 500µl of fresh medium and another 500µl or 1.5ml of medium added into the basolateral chamber of TWI or scaffold respectively for 30 minutes. The STX-2 electrode was sterilised in 70% ethanol prior to each measurement.

4.2.5- Histological analyses

Cells were fixed by incubating overnight in 10% neutral buffered formalin diluted in PBS at 4°C and embedded in paraffin wax using a TP1020 automated tissue processor and a EG1150H embedding centre (Leica Microsystems). Cross-sections were then prepared (10 µm sections) using a RM2145 microtome (Leica Microsystems), deparaffinised in xylene for 5 minutes, rehydrated using graded concentrations of ethanol, and stained with haematoxylin and eosin (H&E; 5 minutes per stain), or with alcian blue (pH 2.5; Merck Millipore) and 0.1% nuclear fast red [(Raymond A Lamb Laboratory Supplies (Thermo fisher)] for 5 minute each. Sections were again dehydrated with ethanol and cleared with xylene, and then mounted using DPX mounting medium (Raymond A Lamb Laboratory Supplies). Mounted sections were viewed using a Zeiss Axioplan light microscope, and images were captured with a MicroPublisher 5.0 RTV camera (QImaging). Images

were processed using Openlab software (Agilent Technologies).

4.2.6- Generation and phenotype analysis of human DCs

PBMCs were obtained from buffy coat samples harvested from healthy volunteers (National Blood Service, Sheffield, UK) by density gradient centrifugation on Histopaque columns (Sigma-Aldrich). PBMCs were then stained with a 1:1 dilution of trypan blue (Sigma-Aldrich) in PBS and enumerated using a haemocytometer (Hawksley), and $CD14^+$ cells (monocytes) were isolated from the samples by positive-selection using a magnetic cell separation system (MACS; Miltenyi Biotech). Briefly, PBMCs were re-suspended in MACS buffer (0.5% FBS and 2 mM EDTA in PBS) and incubated with mouse anti-human CD14 monoclonal antibodies conjugated to magnetic beads (Miltenyi Biotech) for 15 minutes at 4°C. Subsequently, cells were centrifuged, re-suspended in MACS buffer, and passed through a MACS® cell separator column. Thereafter, the column was washed three times with MACS buffer. After the third wash, CD14-negative cells were eliminated and the column was removed from the magnetic stand and placed on a new sterile tube. MACS buffer was then passed through the column to collect $CD14^+$ cells. At this point, the purity of each monocyte preparation was evaluated by subjecting an aliquot of the cells (2×10^5 cells/ml) to staining with a CD14-FITC

antibody followed by flow cytometry analysis. The purity of all preparations was greater than 90%. For generating immature monocyte-derived dendritic cells (Mo-DCs), cells were seeded (1×10^6 cells/ml) in a 24-well plate (Corning Life Sciences) in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% v/v FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (1% v/v pen/strep), and 2 mM L-glutamine. Monocyte differentiation into DCs was carried out by cultivating for six days in the presence of 50 ng/ml GM-CSF and 250 U/ml IL-4 (both from R&D Systems). On day three of culturing, fresh RPMI medium containing cytokines was added to the cell cultures. On day six, immature DCs were harvested and used for appropriate assays.

Phenotypic analyses of $CD14^+$ monocytes (Day 0) and DCs (Day 6) were performed via flow cytometry. Briefly, 1×10^5 cells/ml were washed with cold PBS and cold PBA (0.5% BSA and 0.1% NaN_3 in PBS), and incubated for 20 minutes at 4°C in the dark with specific monoclonal antibodies conjugated to either fluorescein isothiocyanate (FITC), phycoerythrin (PE), or phycoerythrin cyanin 5.1 (PC5) (Table 4.1), according to the manufacturer's instructions. Cells were then washed twice with PBA, fixed in 0.5% v/v formaldehyde (Sigma-Aldrich) in PBS, and subjected to flow cytometry analysis (Beckman Coulter).

Table 4.1: Antibodies used for flow cytometry analysis of monocytes and dendritic cells

Antibody	Source	Isotype control	Source
CD14-FITC	Beckman Coulter	Mouse IgG2a	Beckman Coulter
CD11c-PE	Beckman Coulter	Mouse IgG1	Beckman Coulter
HLA-DR-PC5	Beckman Coulter	Mouse IgG1	Beckman Coulter
CD80-FITC	Beckman Coulter	Mouse IgG1	Beckman Coulter
CD209-PE	Beckman Coulter	Mouse IgG1	Beckman Coulter
CD83-FITC	Beckman Coulter	Mouse IgG2b	AbD Serotec
CD86-PE	Beckman Coulter	Mouse IgG2b	Biolegend
CD40-PE	Beckman Coulter	Mouse IgG1	Beckman Coulter
CD206-PC5	BD Bioscience	Mouse IgG1	Beckman Coulter

4.2.7- Immunocytochemistry

ECs and fibroblasts were washed once with PBS and fixed in 4% (v/v) paraformaldehyde (PFA, Electron Microscopy Sciences, Hatfield, PA, USA) diluted in PBS for 20 minutes at RT or overnight at 4°C. Samples were then washed three times with PBS (3 × 5 minutes) at RT, permeabilised for 20 minutes with 0.15% (v/v) Triton X-100 (Sigma-Aldrich) in PBS at RT, and blocked with 10% goat serum (Sigma-Aldrich) in PBS for 30 minutes at RT. Subsequently, cells were stained by incubation with a mouse monoclonal antibody specific to human ZO-1 (1:200 dilution, clone1A12; Invitrogen) or with a purified rabbit polyclonal anti-ZO-1 (mid) antibody (1:100; Invitrogen), a mouse anti-pan-cytokeratin monoclonal antibody (PK110; 1:100; Santa Cruz Biotechnology), and a rabbit polyclonal anti-vimentin antibody (1:100; Abcam) for

one hour at RT or overnight at 4°C, depending on the manufacturer's protocol. Samples were then washed with PBS (3 × 5 minutes) at RT and incubated with a rhodamine red X-labelled goat anti-mouse antibody (1:250; Invitrogen), an Alexa Fluor 488-conjugated goat anti-rabbit antibody (1:100; Invitrogen), and an Alexa Fluor 488-conjugated goat anti-mouse IgM (μ chain; 1:100; Invitrogen) for 30–45 minutes in dark at RT. Cells were again washed with PBS (3 × 5 minutes) at RT, stained with DAPI (1:20,000; Invitrogen) for 5 minutes for labelling of nuclei, washed with PBS (3 × 5 minutes) at RT, and embedded in FluorSave anti-fade medium (Calbiochem, Billerica, MA, USA) for approximately 5 minutes. Finally, cells were mounted on glass slides using drop of fluorescence mounting medium (Sigma-Aldrich) and covered with coverslips.

For analysis of DCs migration, cells were harvested on day six of cultivation, stained with Hoechst nuclear stain (5 μg/ml) for 30 min at 37°C, inoculated on PET scaffolds (3×10^5 DCs per scaffold), and incubated overnight at 37°C with 5% CO_2. Scaffold-containing cells were then inserted between two blank scaffolds, incubated overnight (37°C, 5% CO_2), and stimulated with lipopolysaccharide (LPS; 0.1 μg/ml) for 24 hours. Unstimulated DCs in blank scaffold assemblies and on single scaffolds were included as controls. Immunostained samples

were visualised by fluorescence (Leica DMRB) or confocal microscopy (Leica SP2) using 20×, 40× (oil), and 63× (glycerol) objective lenses at laser excitation wavelengths of 570, 492, and 345 nm.

4.2.8- IDO enzymatic activity assay

IDO activity was evaluated in the culture supernatants harvested from submerged, differentiated ECs (i.e. ALI) and from cells subjected to 3D cultivation as described in chapter 2, section 2.2.3. For disruption of tight junction proteins, cells were treated with 60 U/ml of papain (Sigma-Aldrich). Papain solution was prepared with L-cysteine (5 mM, Sigma-Aldrich) to reconstitute the cysteine active site. L-cysteine alone was used as a control for papain.

4.2.9- Statistical analysis

Data are expressed as means ± SEM. Student's *t* test was utilised to compare statistical differences between two groups, while one-way ANOVA was utilised to compare three or more groups. Tukey's post-hoc test was applied to all results, and $*p \leq 0.05$ was considered statistically significant. GraphPad Prism 6 software (GraphPad Software, La Jolla, Calif) was used for all statistical analysis.

4.3- Results

4.3.1- Scaffold fabrication for the *in vitro* 3D cultivation model

The first step in developing the immunocompetent 3D model of airway ECs was fabrication of a porous scaffold that could support the growth and differentiation of ECs as well as fibroblasts and DCs. Preliminary studies focused on scaffold fabrication, and PET was selected as a non-biodegradable polymer for scaffold production by electrospinning. The electrospinning procedure is associated with numerous variable conditions, such as electrical charge, flow rate, distance between syringe needle and collection plate, and solvent volume, which influence the structural properties of the resulting scaffold sheet. Figure 4.5 shows a scanning electron microscopy (SEM) image of the nanometre-scale PET scaffold and its morphological similarities to decellularised human lung tissue (from healthy sections of lung tissue obtained from patients undergoing surgical procedures at Nottingham University Hospitals NHS Trust after informed consent and ethical approval). Thus, PET electro-spun scaffolds (with an average thickness of 60 ± 10 μm) exhibit optimal physical properties including structural strength (as a non-biodegradable polymer) with stability, which support long-

term cell cultures and enable inoculation of individual cell types onto separate scaffolds. In this study, we examined the incorporation of lung-associated ECs and fibroblasts into the 3D model. Figure 4.6 depicts immunocytochemical staining of pan-cytokeratin and vimentin in ECs and fibroblasts, respectively. These images indicate that the cells attached to and spread evenly over the scaffold.

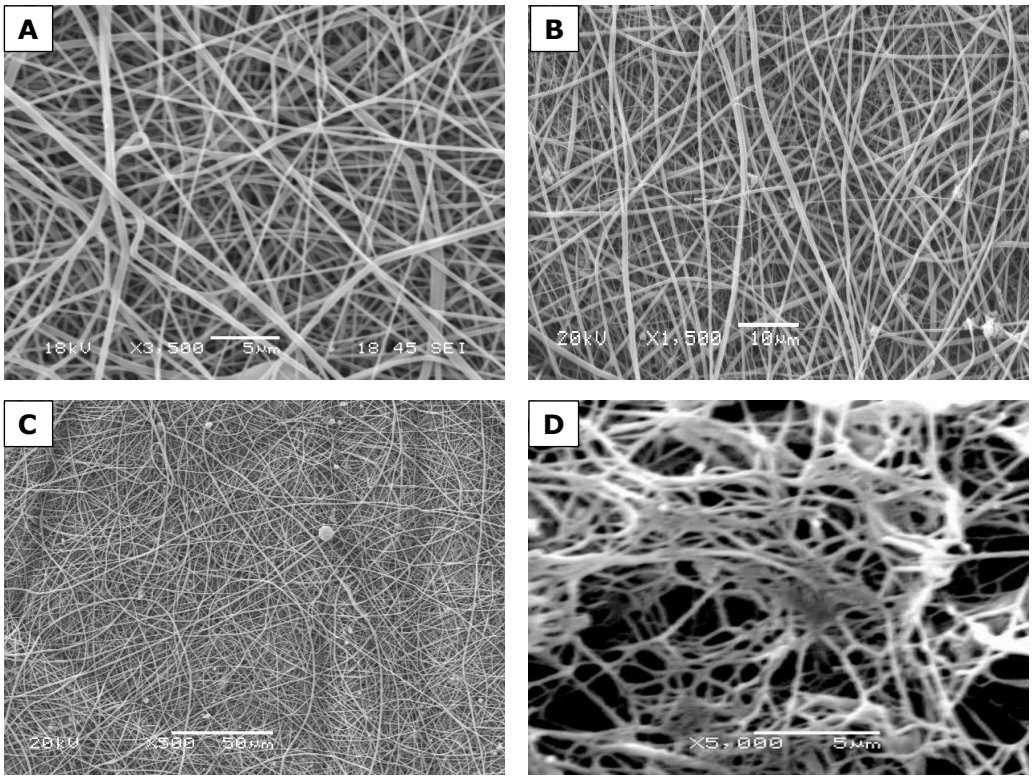


Figure 4.5: Morphological similarities between electrospun PET scaffolds and decellularised lung tissue. (A–C) SEM images of PET scaffolds with scale bars of 5 μm, 10 μm, and 50 μm, respectively. Scaffolds were constructed by electrospinning 10% (w/v) PET suspended in a 50:50 mixture of TFA and DCM. Images are from three different experiments. (D) SEM image of decellularised human lung tissue revealing a porous network of fibers. Image was provided courtesy of Dr. Helen Harrington, University of Nottingham.

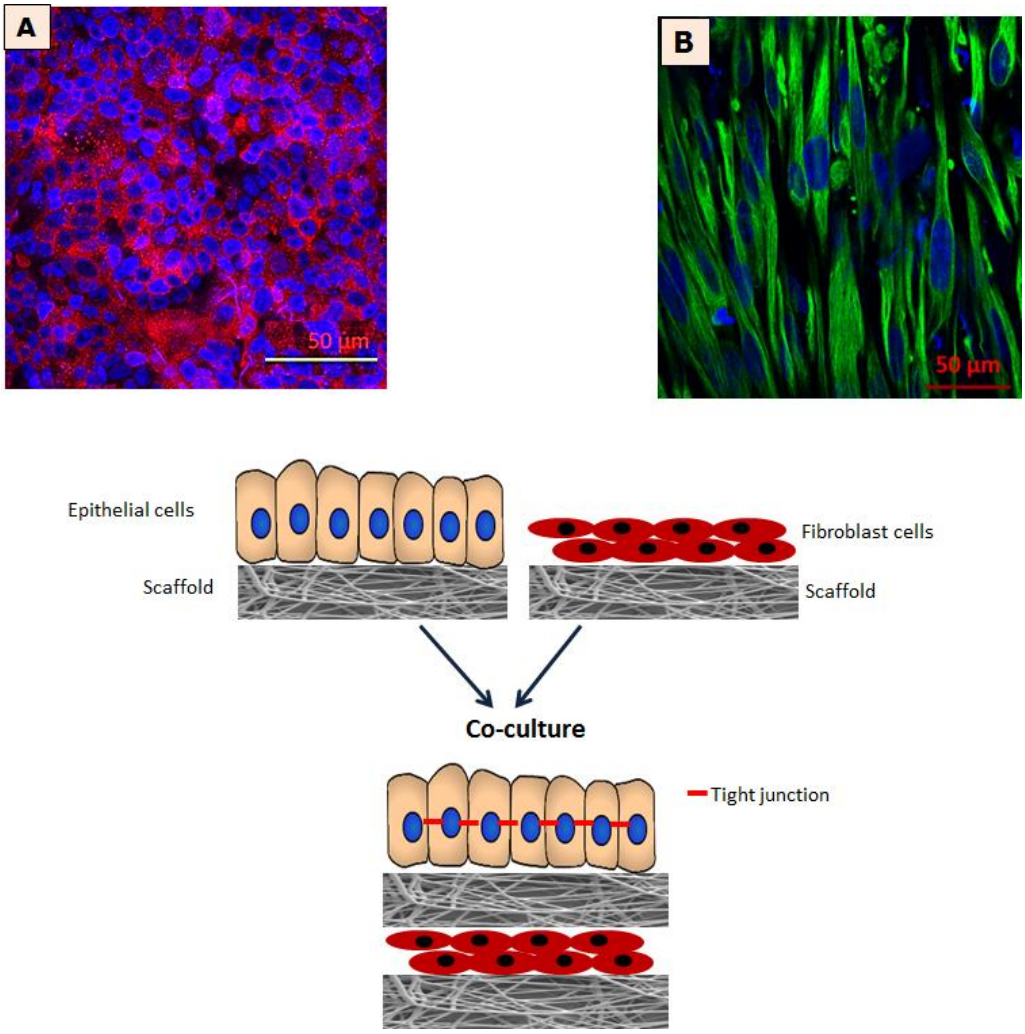


Figure 4.6: Immunocompetent 3D co-culture model. Confocal microscopy images of (A) Calu-3 ECs and (B) MRC-5 fibroblasts cultured on electro-spun PET scaffolds. Cells were cultivated on the scaffolds for up to one week and then stained with pan-cytokeratin- (red) and vimentin-specific antibodies (green), respectively. Cells were then treated with DAPI stain (blue) for labelling of nuclei and visualised using fluorescence microscopy with 40× (oil) objective lens. Data are representative of 6 different experiments.

4.3.2- Evaluation of barrier formation by ECs through TEER measurement and immunostaining of tight junction-associated proteins

Given their widespread use, TWI were used as a control for the PET electro-spun scaffolds. To induce differentiation of ECs at the ALI on TWI, the culture medium was removed from the apical chamber. Under these conditions, airway ECs (Calu-3) developed a robust barrier as evidenced by substantial increases in TEER values. The electrical resistance values increased starting on the first day of cultivation at the ALI and reached a plateau of ~830–900 Ω/cm^2 between days six and eight. Meanwhile, co-cultures of Calu-3 (apical side) and MRC-5 cells (basolateral side; i.e. within the well) produced markedly higher TEER values than single cultures (Figure 4.7 A).

To evaluate the barrier formation capacity of cells cultivated on PET scaffolds, Calu-3 ECs and MRC-5 fibroblasts were cultivated to confluency on respective scaffold layers. The two PET layers were then assembled in one ScaffHolder, with the ECs comprising the uppermost layer to enable differentiation at the ALI. Meanwhile, the fibroblast layer was positioned directly beneath the EC layer, and was maintained submerged in the culture medium to provide apical-basal polarity. The highest TEER value obtained from this co-culture model was

approximately 280 Ω/cm^2 after 14 days at the ALI. This value was significantly higher than that obtained from single cultures (~220 Ω/cm^2; Figure 4.7 B). These data indicate that ECs exhibited enhanced barrier formation and differentiation during co-cultivation than in single culture. Notably, however, immunostaining analyses detected high levels of ZO-1 protein expression in Calu-3 cells during both single and co-culturing at the time points at which peak TEER values were observed. Furthermore, ZO-1 exhibited the typical "chicken wire" pattern that is indicative of tight junction and barrier formation and integrity (Figure 4.7 C).

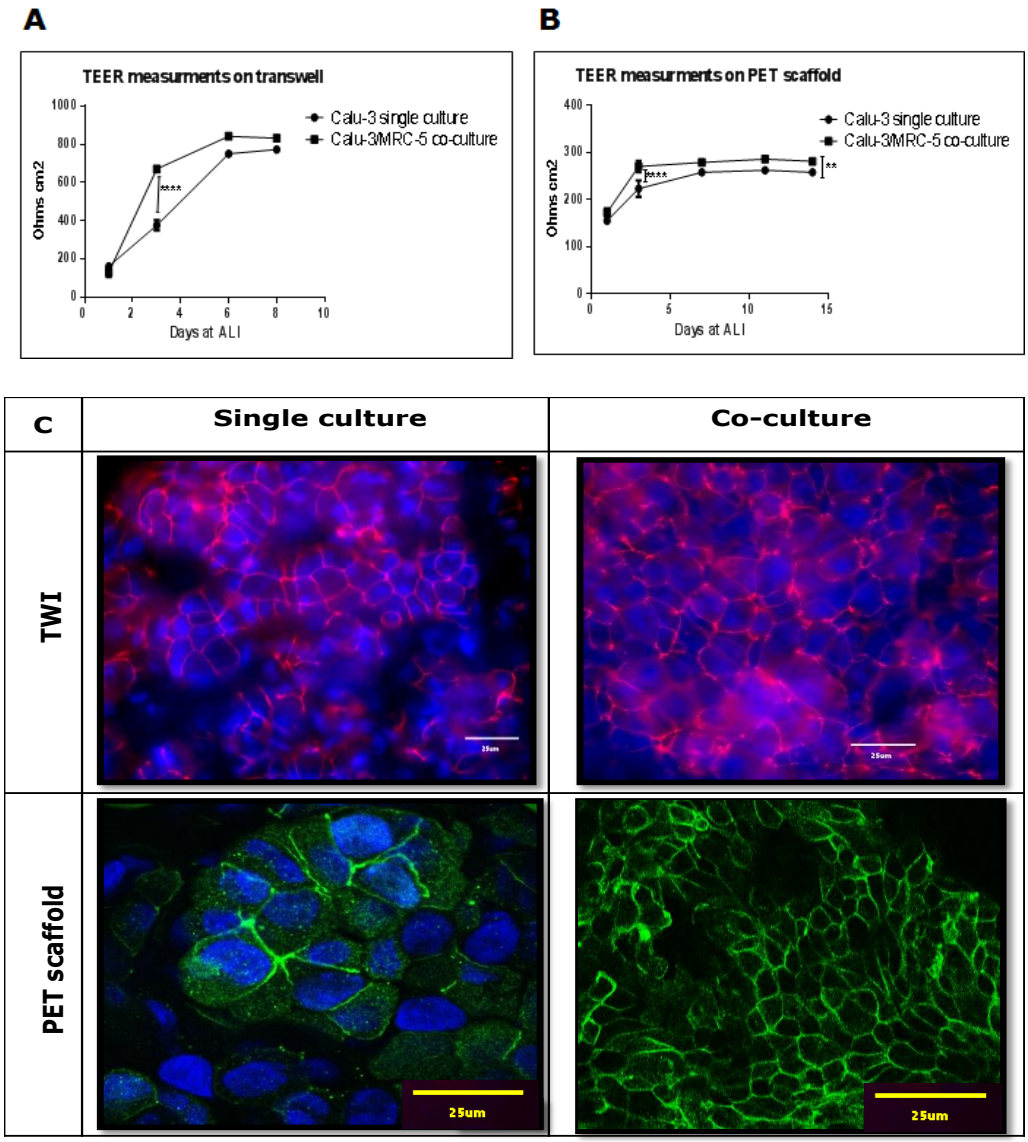


Figure 4.7: TEER measurements and immunostaining of tight junction proteins in Calu-3 cells and Calu-3 cells co-cultured with MRC-5 cells on TWI or PET scaffolds. (A and B) Graphic depictions of TEER values for mono-cultures of Calu-3 cells and Calu-3 cells co-cultivated with MRC-5 cells on (A) TWI or (B) PET scaffolds. (C) Fluorescence microscopy images of Calu-3 cells that were cultivated alone or in co-culture with MRC-5 cells and stained with antibodies specific to ZO-1. Red/green fluorescence depicts the intracellular localisation of ZO-1 in mono- and co-cultures grown on TWI and PET scaffolds, respectively. Cells were treated with DAPI stain (blue) for labelling of nuclei, and were visualised using 20× and 63× (glycerol) objective lenses. Data are representative of 6 different experiments. $^{**}p \leq 0.01$; $^{****}p \leq 0.0001$.

4.3.3- Differentiation and barrier formation of pHBECs

Having established that our airway EC line exhibits barrier formation and integrity in the single culture and co-culture model, we attempted to enhance the physiological relevance of our model by using primary ECs. For initial experiments, iHBECs were used to assess tight junction formation and the ability of cells to display *in vivo* epithelium characteristics, including secretory activity. Indeed, iHBECs exhibited mucin protein production, as confirmed via immunostaining with an anti-mucin 5AC [45M1] antibody (Figure 4.8 A). Furthermore, H&E staining analyses indicated that these cells formed multi-layered sheets and exhibited a cuboidal shape, which was more similar to their typical *in vivo* morphology (Figure 4.8 B). Thereafter, pHBECs from healthy donors were differentiated at the ALI for up to three weeks. These cells formed intact ZO-1 tight junctions and produced TEER values that reached a plateau (600–700 Ω/cm^2) between days 14 and 18 (Figure 4.9 A & C). Subsequent histological examination of the pHBECs detected multi-layered cell sheets with cuboidal morphology, ciliated cells, and goblet cells (Figure 4.10 A). In addition, these cells appeared to produce mucus, as evidenced by alcian blue staining analyses (Figure 4.10 C). Meanwhile, we also evaluated the characteristics of pHBECs harvested from asthmatic donors. Although certain asthmatic donors were

difficult to characterise, and their ECs did not readily form intact tight junctions, the ECs obtained from several donors generated high TEER values, formed normal ZO-1 tight junctions, and exhibited mucin production when cultivated on TWI (Figure 4.9 and 4.10 B & D).

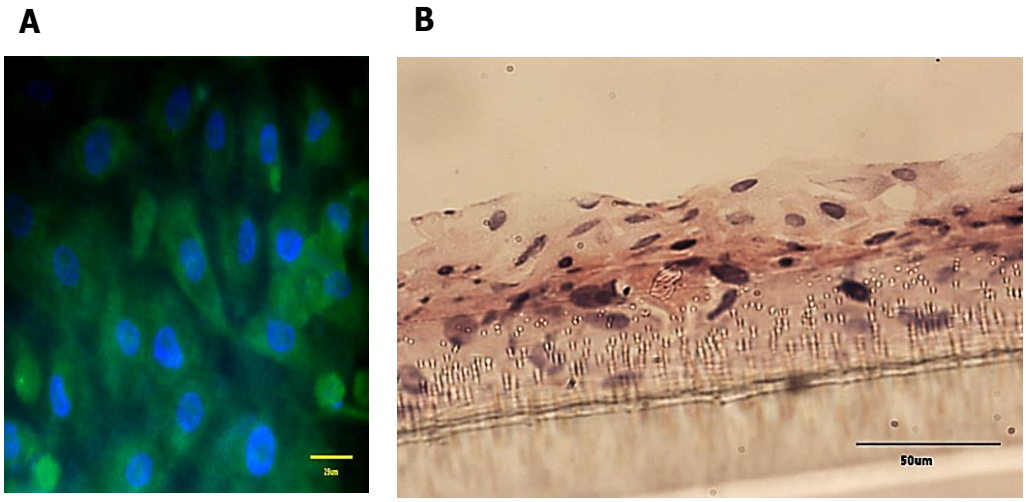


Figure 4.8: Mucin immunostaining and histology of differentiated iHBEC. iHBEC were cultured submerged in TWI for 4 days and then raised to ALI for up to 28 days. (A) Green fluorescence shows the production of mucin protein and the blue fluorescence shows DAPI-stained nuclei in iHBEC. (B) H&E staining shows a multi-layered and cuboidal shape of iHBEC with blue nuclei and varying shades of pink cytoplasm. Fluorescence and light microscopy were performed by using 63/40 times glycerol/oil objective lens respectively. Data shown are representative of 3 different experiments.

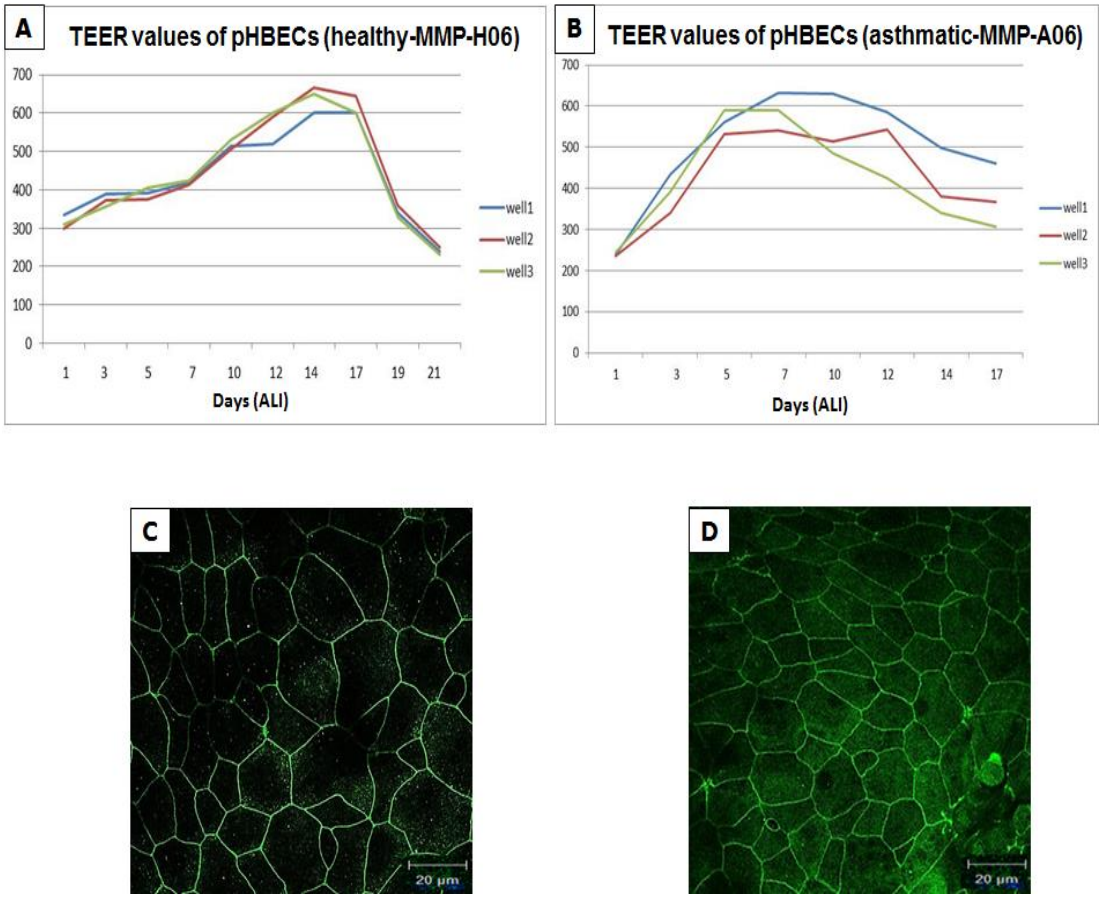


Figure 4.9: TEER measurements and immumnostaining of tight junction proteins in pHBECs (healthy and asthmatic). (A and B) Graphic depictions of TEER values for mono-cultures of pHBECs harvested from (A) healthy and (B) asthmatic patients cultivated on TWI. (C) The tight junction protein ZO-1 was visualised in HBECs harvested from (C) healthy and (D) asthmatic donors by immunostaining analysis. Green fluorescence represents the intracellular localisation of ZO-1 in HBECs during culturing on TWI. Confocal microscopy was performed using a 40× (oil) objective lens. Data are representative of 6 different experiments.

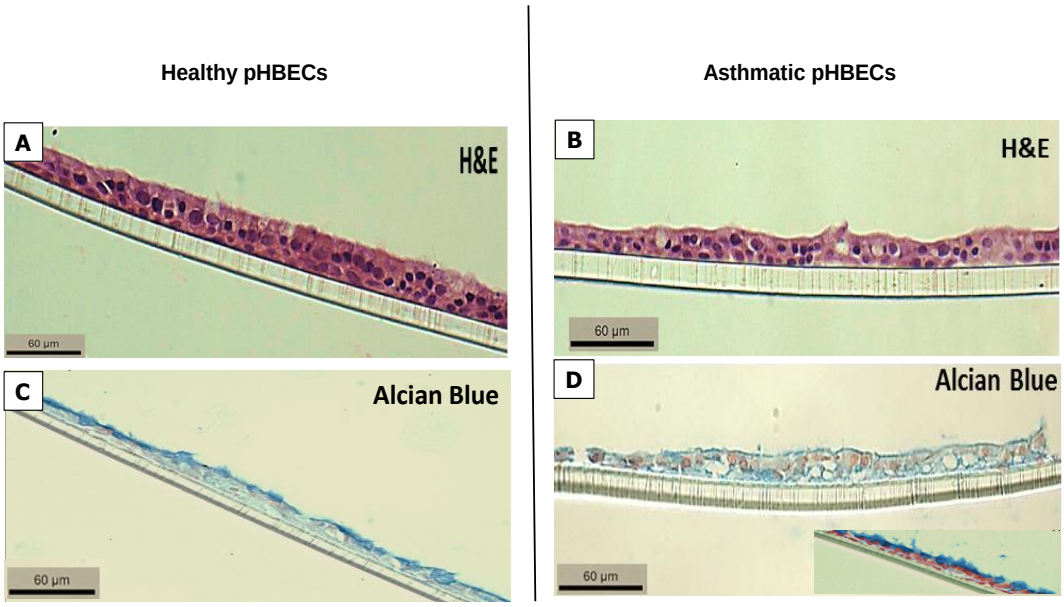


Figure 4.10: H&E, and alcian blue staining of paraffin-embedded pHBECs (healthy and asthmatic) cultured at ALI for three weeks. (A and B) Images of (A) healthy and (B) asthmatic pHBECs treated with H&E stain. Cytoplasmic contents appear purple while nuclei appear blue-black. (C and D) Images of (C) healthy and (D) asthmatic pHBECs treated with alcian blue (pH 2.5) for staining of mucins (blue stained material) and 0.1% nuclear fast red. Light microscopy was performed using a 20× objective lens. Data are representative of 3 different experiments.

4.3.4- Evaluation of the effects of papain on the breakdown of tight junction proteins

Papain is an allergen with cysteine protease activity. Previous findings suggested that papain disrupt ZO-1 tight junctions in airway ECs, as evidenced by a sharp decrease in TEER readings (120). In the current study, to investigate the effect of disruption of tight junctions on the immune-regulatory properties of differentiated ECs, Calu-3 cells were

differentiated at the ALI for approximately two weeks and then exposed to papain for 24 hrs in the presence or absence of LPS. IDO production levels were then evaluated as a marker for the immune-regulatory activity of ECs. As shown in Figure 4.11, treatment with LPS (added to either apical or basolateral side of EC layer) resulted in significant down-regulation of IDO activity. Notably, this reduction was even greater in the cells treated with both papain and LPS. These data indicate that full barrier formation may lead to reduced accessibility of LPS to TLR4 and potentially other PRRs. Accordingly, barrier degradation would result in increased accessibility of LPS to TLR4, and thereby to further reductions in IDO activity.

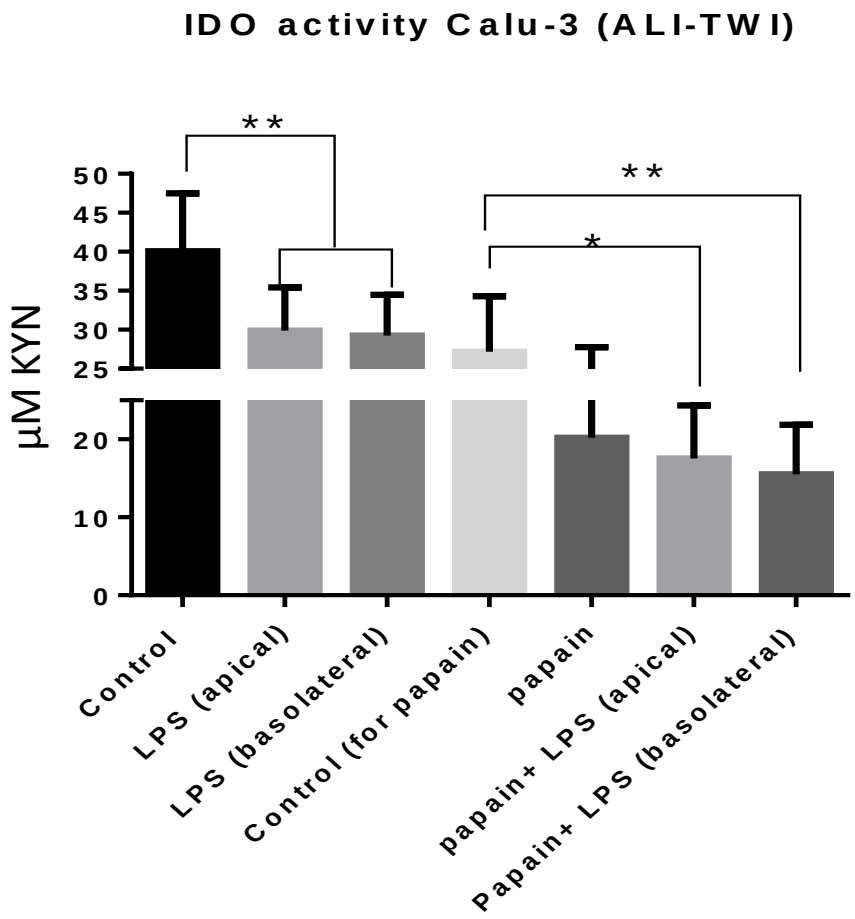


Figure 4.11: Down-regulation of IDO activity in response to LPS and after tight junction degradation via papain in airway ECs. Calu-3 cells were cultured on TWI at the ALI for up to 14 days and then incubated with media containing 2 µg/ml LPS on either the apical and basolateral side for 24 hours. To disrupt tight junctions, cells were incubated with 60 U/ml papain in the presence or absence of LPS. Papain was diluted with 5 mM L-cysteine to reconstitute the cysteine active site, and L-cysteine was used as a control for papain. IDO activity in the culture supernatants was then quantified. Data shown are representative of 3 independent experiments $**p \leq 0.01$; $***p \leq 0.001$; $****p \leq 0.0001$.

4.3.5- Differential regulation of IDO in airway ECs and fibroblasts during mono- and co-culturing (2D and 3D models)

In the present study, we examined IDO activity in three different cell types (ECs, fibroblasts, and DCs) during mono- and co-cultures to ascertain if the IDO activity of ECs is different in mono-culture than in Co-culture condition. For these analyses, Calu-3 cells and MRC-5 cells were cultured submerged in 12-well plates alone or together within the same well to enable cell-cell contact. These cells were also co-cultured on two sides of TWI to enable paracrine contact. As expected, stimulation with IFN-γ resulted in significant induction of IDO activity under all experimental conditions tested (Figure 4.12) and this IDO up-regulation was still significant in the mono-culture of either Calu-3 or MRC-5 in response to co-stimulation of IFN-γ with LPS or HDM compared with the control (Figure 4.12 A and B). In contrast, the patterns of IDO activity in response to LPS or HDM differed between the mono- and co-cultures. Specifically, there was a significant decrease in the IDO activity of the submerged Calu-3 cells, but not in that of the submerged MRC-5 cells or the Calu-3/MRC-5 co-cultures, in response to LPS and HDM compared with the control.

Most debates regarding the *in vivo* immune-regulatory role of IDO are based on observations made in mice; however, these data cannot always be extrapolated to humans. Likewise, data obtained from 2D cell cultures or monocultures are not always relevant to *in vivo* conditions. Therefore, to achieve more *in vivo*-relevant system, in terms of cell cross-talk and function, we evaluated IDO activity in differentiated ECs at ALI co-cultured with fibroblasts. For these experiments, Calu-3 cells were differentiated at the ALI on TWI (2D model) or on PET electro-spun scaffolds (3D model) in the presence or absence of MRC-5 fibroblasts (co-cultivation), and IDO activity in the culture supernatants was measured. Although treatment with IFN-γ resulted in significant induction of IDO activity in the mono-cultures as well as the 2D and 3D co-culture models, we detected differential regulation of IDO activity in response to LPS (Figure 4.13). Specifically, treatment with LPS resulted in significant down-regulation of IDO activity in submerged Calu-3 monocultures and in Calu-3 cells differentiated on TWI compared with the unstimulated cells. Moreover, compared to stimulation with IFN-γ alone, co-stimulation with IFN-γ and LPS resulted in markedly reduced IDO activity in differentiated ECs in both the 2D and 3D culture models. In contrast, exposure to IFN-γ and LPS resulted in up-regulation of IDO activity in ECs cultivated in the 3D co-culture model.

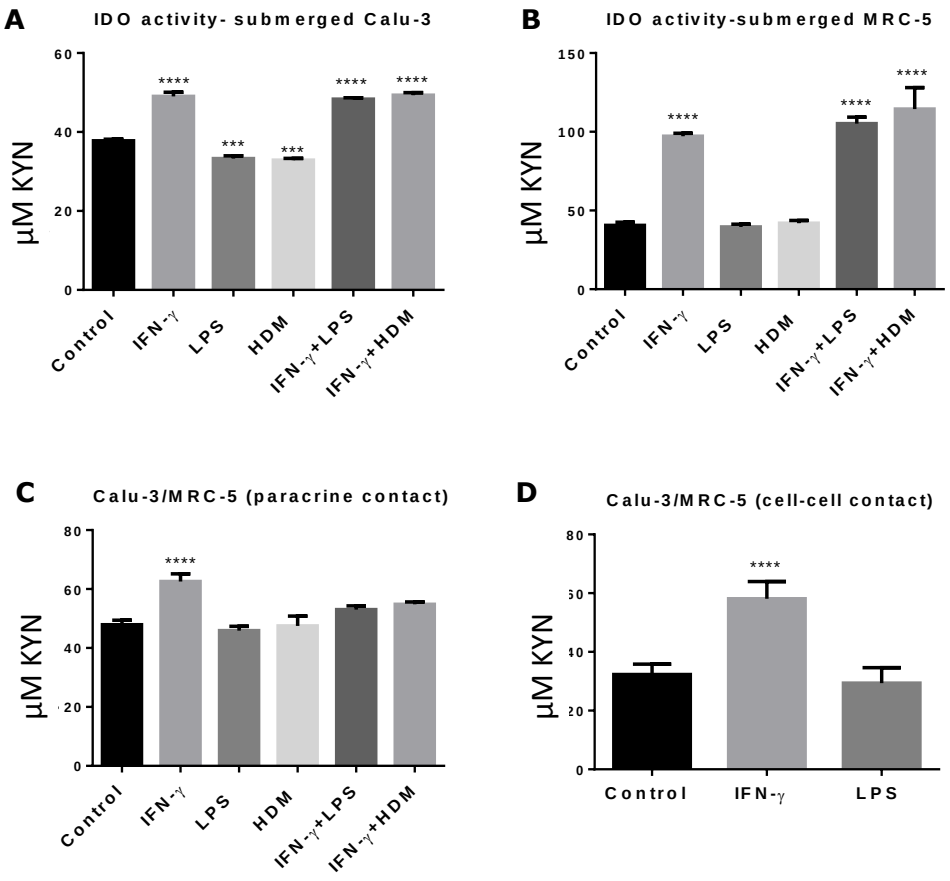


Figure 4.12: Differential regulation of IDO activity in Calu-3 epithelial cells and MRC-5 fibroblasts. (A) Calu-3 cells, (B) MRC-5 cells, and co-cultures of Calu-3 and MRC-5 cells in (C) paracrine contact (carried out on TWI) or (D) direct cell-cell contact were cultured submerged together in the same well to confluency. Cells were then incubated for 24 hours with 10 ng/ml IFN-γ, 2 µg/ml LPS, 10 µg/ml HDM, or a combination of IFN-γ and LPS or HDM. Data are representative of 3 independent experiments. ***$p \leq 0.001$; ****$p \leq 0.0001$.

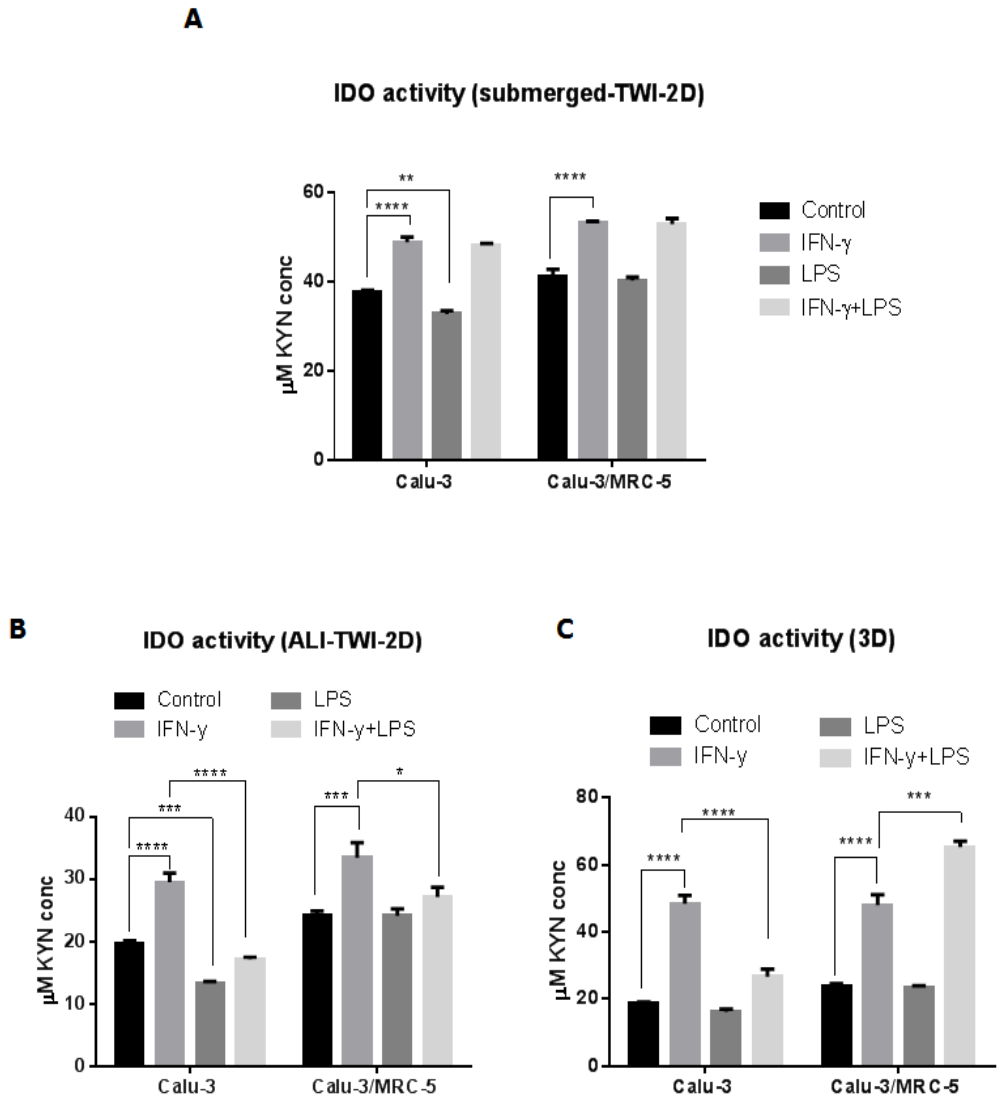


Figure 4.13: Comparison of IDO activity between mono- and co-cultures in the 2D and 3D models. (A–C) Calu-3 mono-cultures and Calu-3 cells co-cultured with MRC-5 cells were (A) cultured submerged to confluence on TWI, (B) cultivated at the ALI for 8–10 days, or (C) cultured on PET scaffolds (i.e. 3D model) for 4–5 days and grown at the ALI for up to 2 weeks. Cultures were then incubated with 2 µg/ml LPS, 10 ng/ml IFN-γ, or a combination of LPS and INF-γ for 24 hours. Supernatants were then collected for IDO activity measurement. Data are representative of 3 independent experiments $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$.

Lastly, we investigated the levels of IDO activity in differentiated primary ECs. Although submerged pHBECs and submerged Calu-3 cells exhibited similar patterns of down-regulation of IDO activity in response to LPS or HDM (Figure 4.14), there were non-significant differences in IDO activity in response to LPS or HDM in pHBECs (Figure 4.14 A), which was consistent with the regulation of IDO activity observed in the 3D single culture model of Calu-3 cells (Figure 4.13 C). Interestingly, the basal concentration of KYN in differentiated primary ECs was much higher than that in submerged ECs. Collectively, these data highlight the differential regulation of IDO activity in Calu-3 cells cultivated in the 3D model and during co-cultivation, which might represent a more physiologically relevant setting than other currently used models, compared with that of cells cultivated in the 2D model. These findings therefore suggest that both cell cross-talk and EC differentiation affect the immune-regulatory properties of ECs.

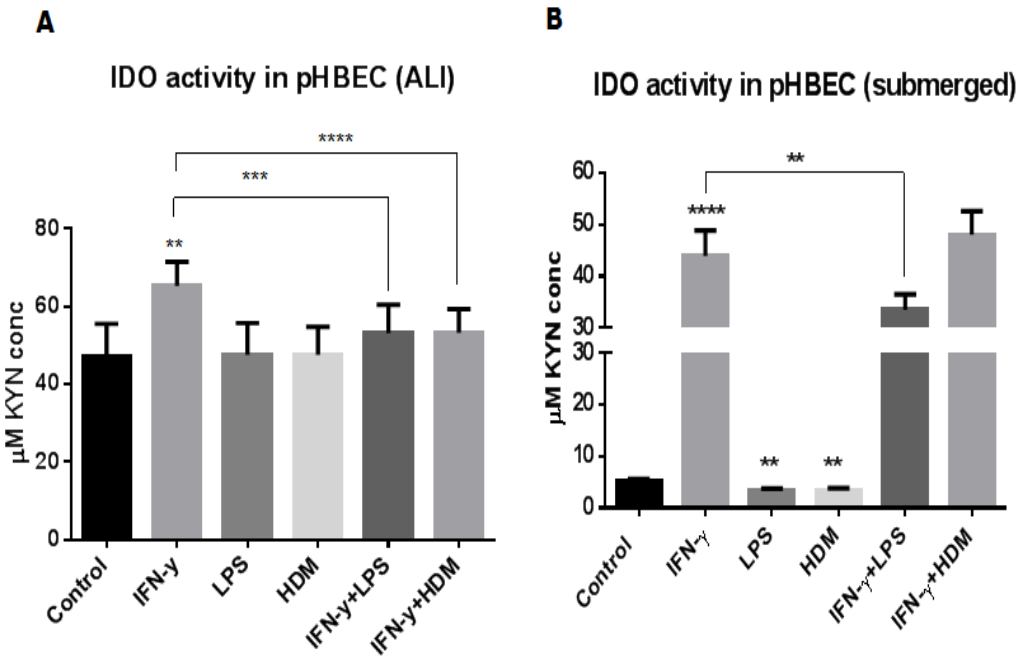


Figure 4.14: Regulation of IDOactivity in human lung primary ECs. (A and B) pHBEC were cultured (A) at the air-liquid interface (ALI) or (B) submerged on TWI for up to three weeks and then incubated for 24 hours in the presence of 10 ng/ml IFN-γ, 2 µg/ml LPS, 10 µg/ml HDM, or a combination of IFN-γ and LPS or HDM. Note the differential regulation of IDO activity among populations of submerged pHBEC (B). In addition, submerged pHBEC exhibited lower basal levels of IDO than those cultivated at the ALI. Data are representative of 3 independent experiments. $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$.

4.3.6- Phenotypic analysis of CD14+ monocytes and Mo-DCs

Prior to co-culturing with epithelial cells, human DCs were generated and characterised. Monocytes were isolated from PBMCs using CD14 magnetic beads and then cultured for six days in media supplemented with IL-4 and GM-CSF to induce

differentiation into immature Mo-DCs. To verify staining specificity against surface markers, cells were stained with monoclonal antibodies specific to CD14, CD11c, HLA-DR, CD80, CD209 (DC-SIGN), CD206 (MR), CD83, CD86, and CD40 in combination with their appropriate isotype-matched controls. As shown in Figure 4.15, monocytes exhibited high levels of CD14 expression on the first day of isolation (Day 0). Conversely, as expected, expression of this marker was not detected on DCs (Day 6). Meanwhile, immature DCs expressed high levels of C-type lectin receptors [mannose receptor (CD206) and DC-SIGN (CD209)], CD11c (specific marker for myeloid DCs), and CD40 (co-stimulatory molecule required for DC maturation), and low levels of the classic activation/differentiation markers CD80, CD83, CD86, and HLA-DR. These expression patterns were consistent with the immature phenotype of these cells. Five different experiments of phenotypic analysis were shown in Appendix 4.

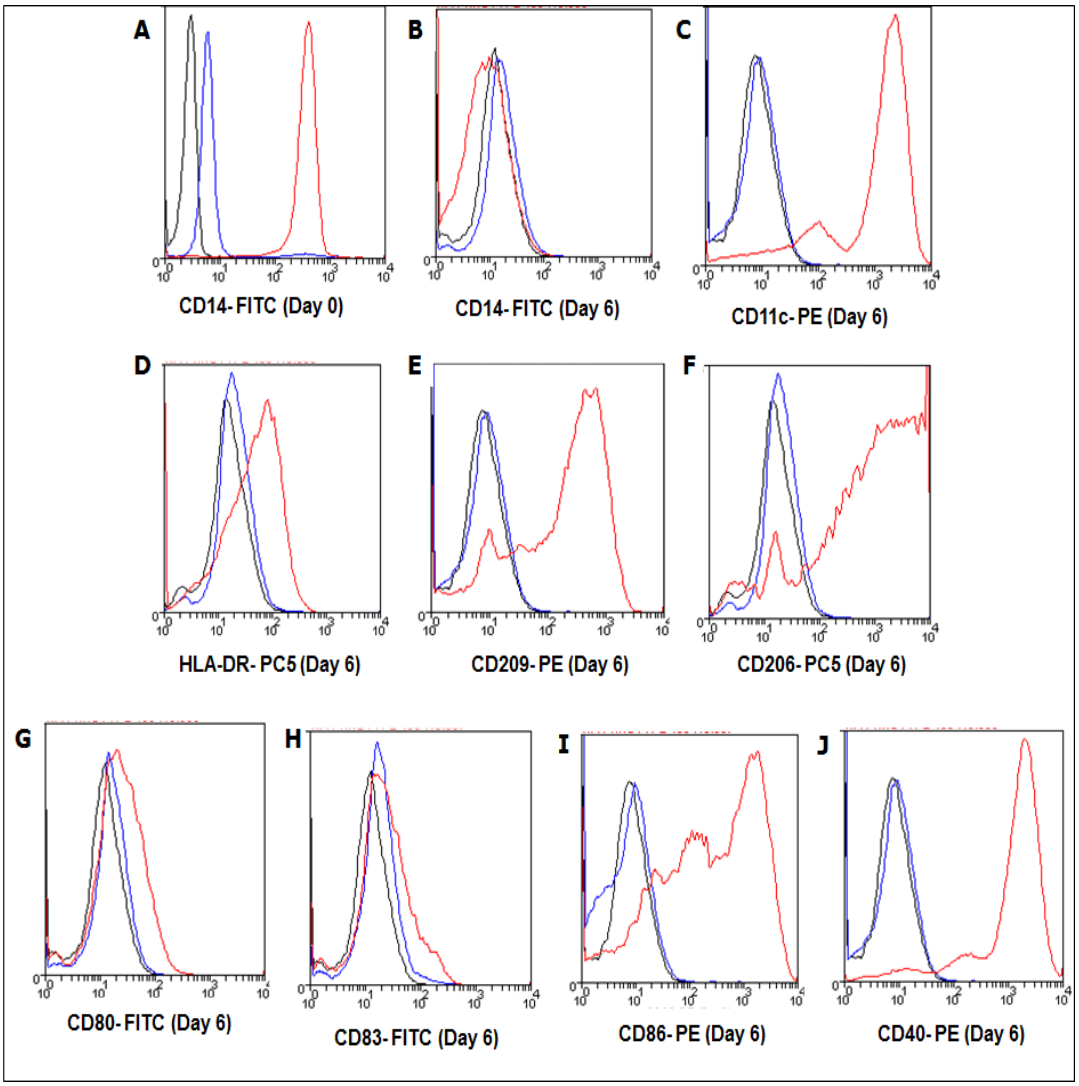


Figure 4.15: Phenotypic analysis of human monocytes and Mo-DCs. Cells were collected and stained with monoclonal antibodies specific to the indicated cell surface molecules. (A–J) Histograms depicting the number of cells exhibiting surface expression of each marker, respectively. Black lines represent unstained cells (autofluorescence), blue lines represent the isotype control, and red lines represent cells that stained positive for the indicated marker. Data are representative of 5 different experiments.

4.3.7- Evaluation of IDO activity and IDO1 protein expression in Calu-3 EC/DC co-cultures

The effects of interactions between Calu-3 cells and DCs on IDO expression were investigated by exposure of mono-cultures and co-cultures to LPS. On day 1, Calu-3 cells (2×10^5 cells/ml) were seeded into 24 wells plates and cultured submerged to confluence. Meanwhile, monocytes were isolated from buffy coats and cultured for six days in the presence of cytokines (IL-4 and GM-CSF) to generate immature Mo-DCs (Figure 4.16 A & B). For setting up co-cultures, DMEM-F12 medium was removed and DCs (2.5×10^5 cells/ml) were re-suspended in co-culture medium (50:50 mixture of DMEM-F12 and RPMI-1640) and cultured on top of Calu-3 monolayers. Cells were then exposed to 2 µg/ml of LPS for 24 hours, and IDO activity and IDO1 protein expression levels were measured in culture media and cell lysate samples, respectively (Figure 4.16 C & D).

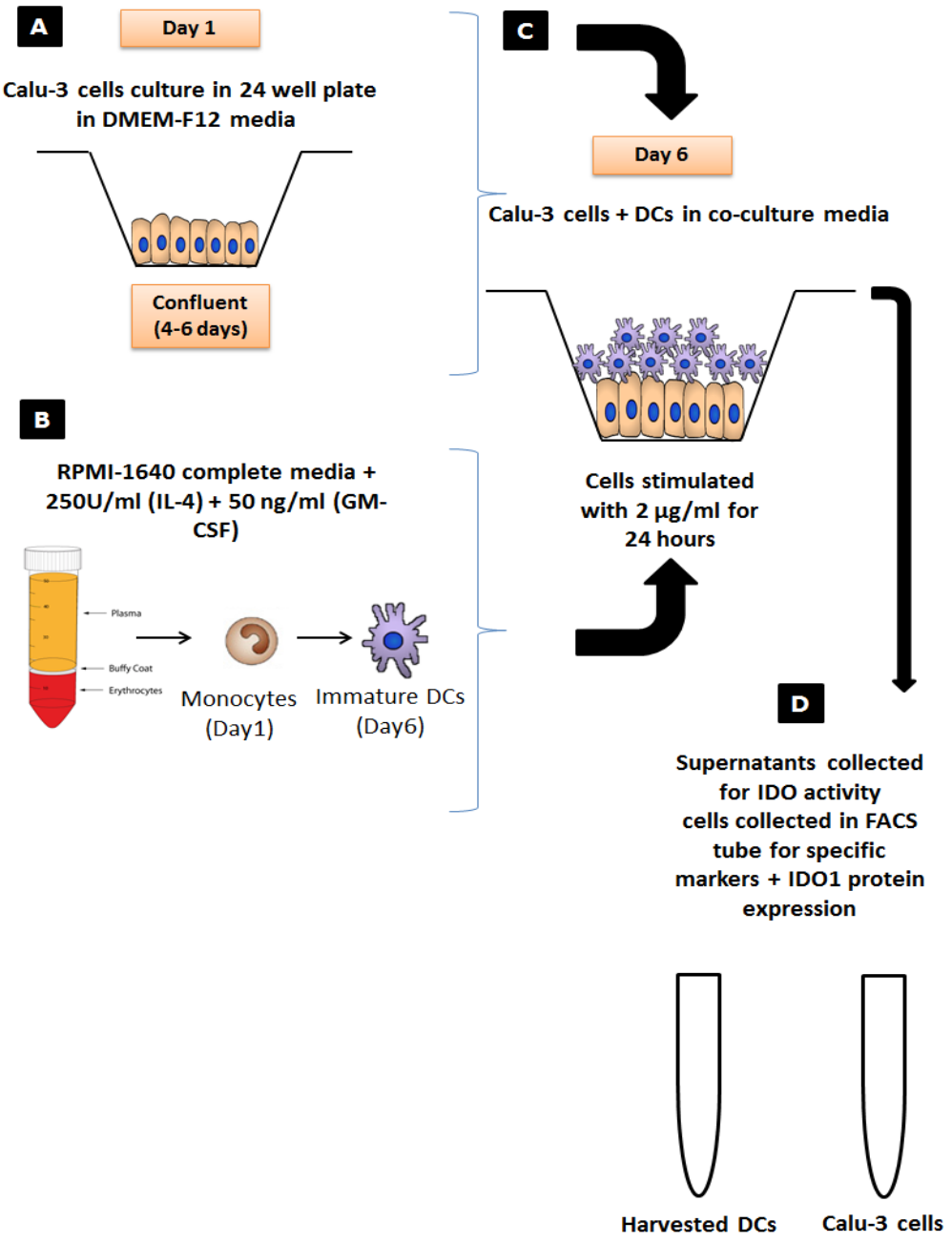
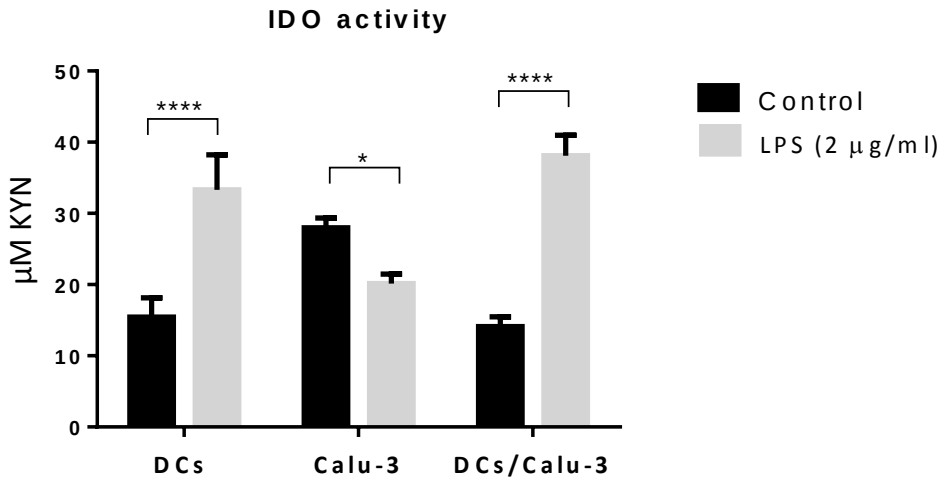


Figure 4.16: Schematic diagram of the procedure for co-culturing of ECs and DCs in 24-well plate. (A) on day 1, Calu-3 cells were cultured to confluence and (B) Mo-DCs were differentiated from Monocytes after they were isolated from PBMCs. (C) on day 6, DCs were harvested and transferred into the same well plate that include Calu-3 cells for co-culture condition (direct contact) and exposed to 2 µg/ml LPS for 24 hours. (D) After 24 hours, 200µl of the supernatant was collected for IDO activity, DCs were gently harvested, and then Calu-3 cells were trypsinised and collected for IDO1 protein staining.

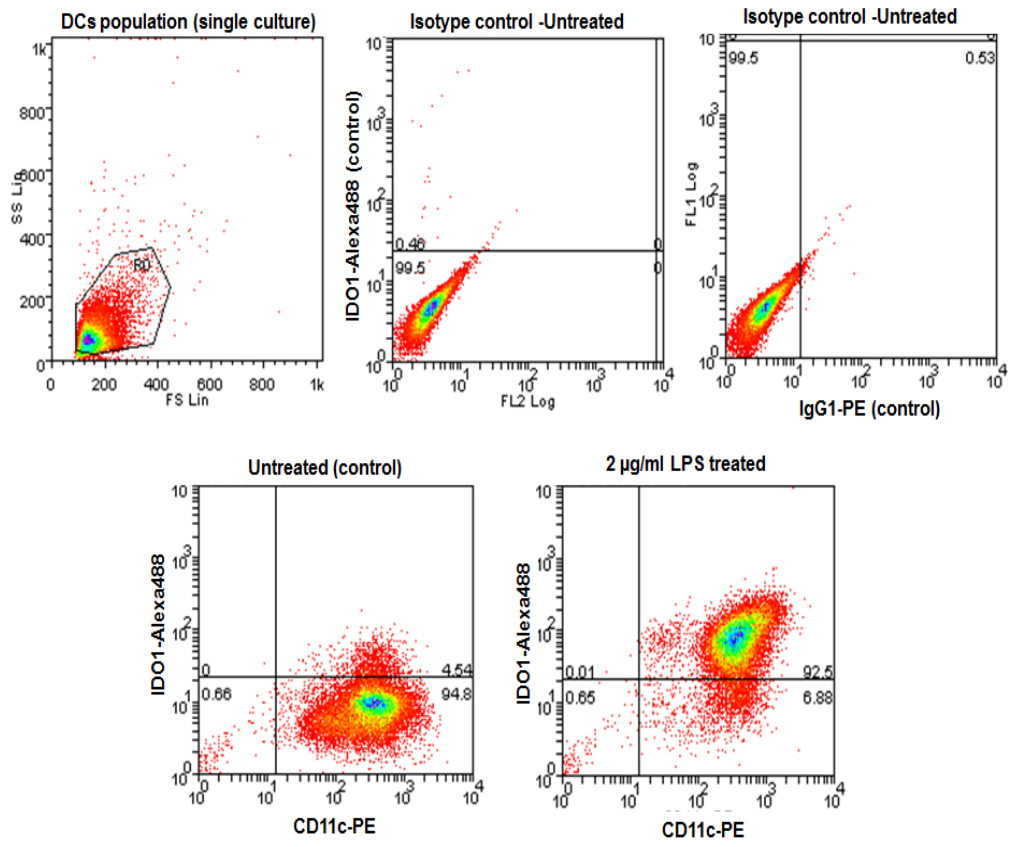
Changes in IDO activity and IDO1 protein expression in response to LPS in both mono- and co-cultures of Calu-3 cells and DCs are shown in Figure 4.17. There was a significant increase in IDO activity in response to 2 μg/ml LPS in DC mono-cultures. Conversely, mono-cultures of Calu-3 cells exhibited a significant decrease in IDO activity upon exposure to 2 μg/ml LPS. Notably, similar to the DC mono-cultures, co-cultures of Calu-3 cells and DCs exhibited a significant increase in IDO activity in response to LPS stimulation (Figure 4.17 A). Accordingly, LPS exposure resulted in marked increases on IDO1 protein expression in both the DC mono-cultures and in DCs co-cultivated with ECs (Figure 4.17 B & C). As expected, there was a reduction in IDO1 expression in Calu-3 mono-cultures upon LPS exposure; however, treatment with LPS resulted in significant induction of IDO1 expression in Calu-3 cells in co-culture (Figure 4.17 D & E). These effects became more obvious upon analysis of the percentages of IDO1-positive cells and the median fluorescence intensity (MFI) of each cell population (Figure 4.17 F & G). These data show that DCs could induce IDO expression in ECs during co-cultivation which could be due to the pro-inflammatory cytokines produced by DCs upon LPS exposure. This effect is of particular interest, as the co-culture model provides a more

physiological relevant representation of the cell communication that occurs *in vivo*.

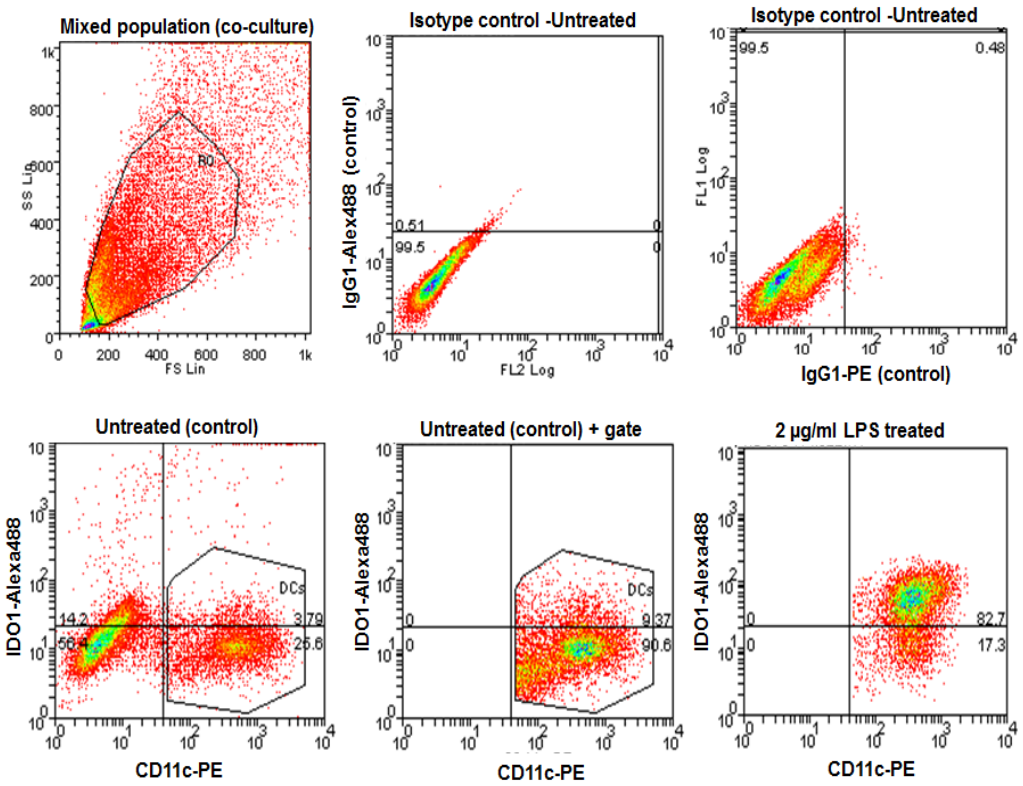
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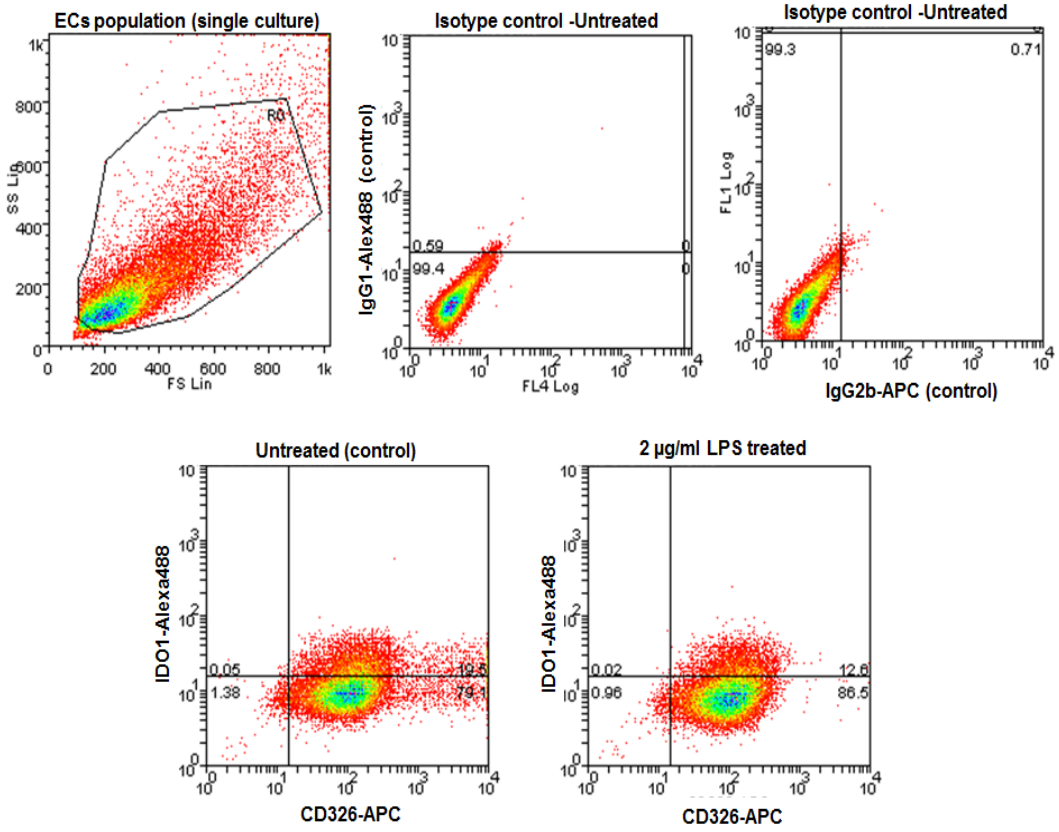
B


DCs population (single culture)
SS Lin
FS Lin
R1
Isotype control -Untreated
IDO1-Alexa488 (control)
FL2 Log
0.46
99.5
Isotype control -Untreated
FL1 Log
IgG1-PE (control)
99.5
0.53
Untreated (control)
IDO1-Alexa488
CD11c-PE
0.66
4.54
94.8
2 µg/ml LPS treated
IDO1-Alexa488
CD11c-PE
0.01
0.65
92.5
6.88

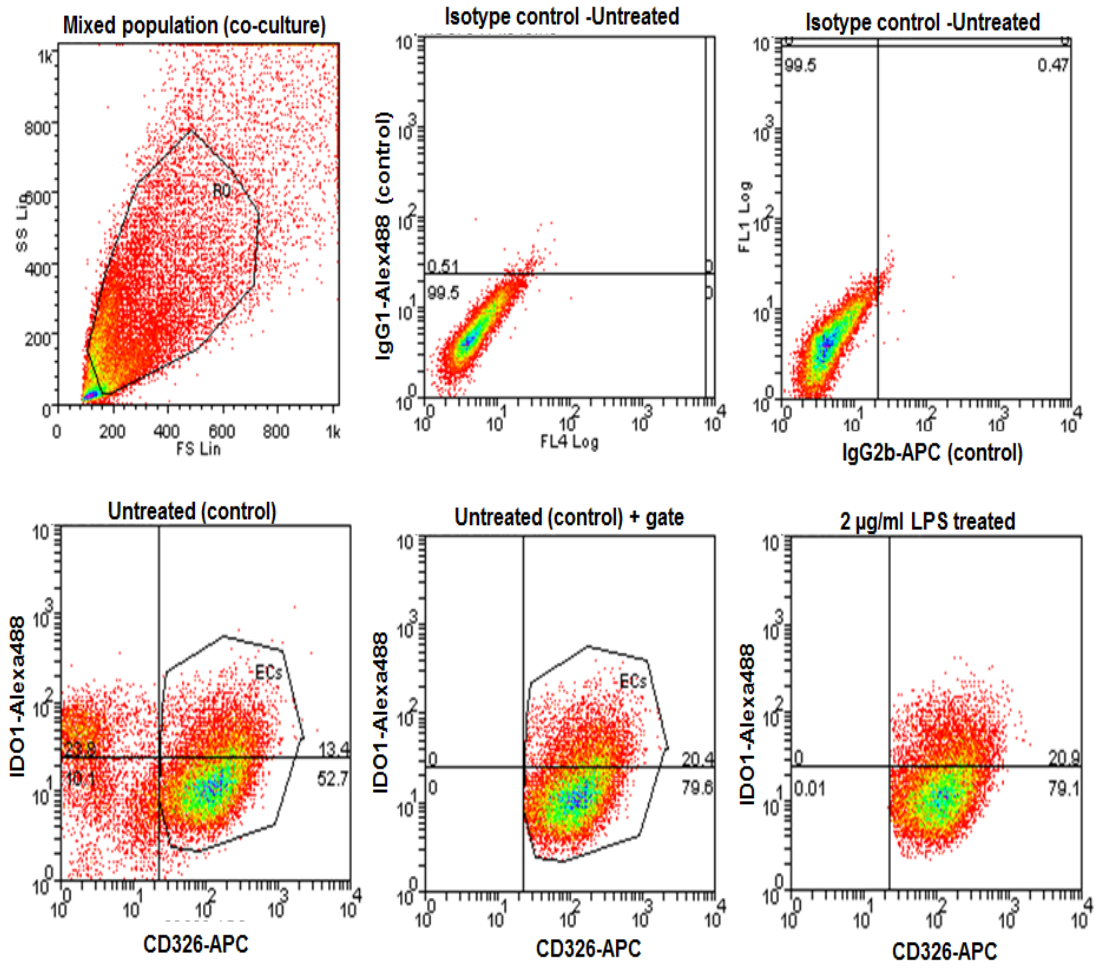
C


Mixed population (co-culture)
SS Lin
FS Lin
R0
Isotype control -Untreated
IgG1-Alexa488 (control)
FL2 Log
0.51
99.5
Isotype control -Untreated
FL1 Log
IgG1-PE (control)
99.5
0.48
Untreated (control)
IDO1-Alexa488
CD11c-PE
DCs
14.2
3.79
56.4
25.6
Untreated (control) + gate
DCs
9.37
90.6
2 µg/ml LPS treated
82.7
17.3

D


ECs population (single culture)
SS Lin
FS Lin
R0
Isotype control -Untreated
IgG1-Alexa488 (control)
FL4 Log
0.59
99.4
Isotype control -Untreated
FL1 Log
IgG2b-APC (control)
99.3
0.71
Untreated (control)
IDO1-Alexa488
CD326-APC
0.05
19.5
1.38
79.1
2 µg/ml LPS treated
0.02
12.6
0.96
86.5

E


Mixed population (co-culture)
R0
SS Lin
FS Lin
Isotype control -Untreated
IgG1-Alex488 (control)
FL4 Log
0.51
99.5
Isotype control -Untreated
FL1 Log
IgG2b-APC (control)
99.5
0.47
Untreated (control)
IDO1-Alexa488
CD326-APC
ECs
13.4
52.7
Untreated (control) + gate
20.4
79.6
2 µg/ml LPS treated
0.01
20.9
79.1

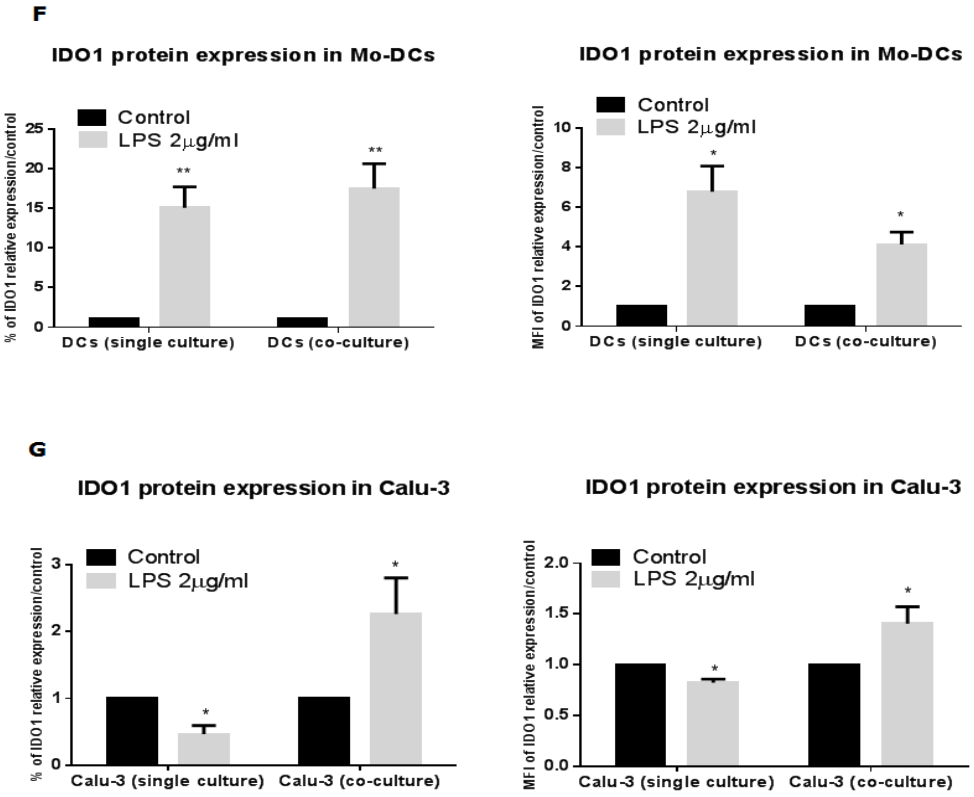


Figure 4.17: Differential regulation of IDO activity and IDO1 protein expression in EC/DC co-cultures. Calu-3 cells were co-cultured with fully differentiated DCs. Mono- and co-cultured cells were incubated with 2 µg/ml of LPS for 24 hours. Media and cells were then collected for analysis of IDO activity and IDO1 protein expression, respectively. (A) Graphic depictions of IDO activity in DC and Calu-3 mono-cultures and co-cultures. (B and C) Dot plot representations of IDO1 expression in DC mono-cultures and DCs co-cultured with Calu-3 cells. DCs were stained with IgG1-PE, IgG1-Alexa fluor488, CD11c-PE, and anti-hIDO-Alexa fluor488 antibodies and subjected to flow cytometry analysis. (D and E) Dot plot representations of IDO1 expression in Calu-3 mono-cultures and Calu-3 cells co-cultivated with DCs. Cells were stained with IgG2b-APC, IgG1-Alexa fluor488, CD326-APC (anti-body recognises human epithelial cells antigens), and anti-hIDO-Alexa fluor488 antibodies and subjected to flow cytometry analysis. (F and G) Graphic depictions of the percentages of DCs and Calu-3 cells (Cells %) expressing IDO1, and MFI of IDO1 expression in DCs and Calu-3 cells relative to that of the control, under mono- and co-culture conditions, respectively. *$p \leq 0.05$; **$p \leq 0.01$; ****$p \leq 0.0001$.

4.3.8- DC migration on PET scaffolds

Maturation and migration of DCs are essential for efficient cross-talk between DCs and T cells, and for the development of effective immune responses (120, 241). To assess the feasibility of obtaining cell-cell contact in the tri-culture 3D model, we first evaluated the efficacy of DC attachment and migration within the PET scaffold. Immature monocyte-derived DCs were cultivated for six days, stained with Hoechst nuclear stain (5 µg/ml) for 30 minutes at 37°C, and inoculated on PET scaffolds (3×10^5 cells/scaffold) overnight at 37 °C with 5% CO_2. Scaffolds containing cells were then inserted between two acellular scaffolds (i.e. scaffolds lacking cells), incubated overnight (37°C, 5% CO_2), and challenged with LPS (0.1 µg/ml) for 24 hours. Unstimulated DCs on single scaffolds and on scaffolds combined with acellular (blank) scaffolds were used as controls. As shown in Figure 4.18, while unstimulated DCs primarily remained stationary, LPS stimulation resulted in migration of DCs between the different layers of electro-spun scaffolds. These findings demonstrate definitively that our 3D model allows for migration of DCs, an essential cell type that migrates to epithelial cells *in vivo* to modulate immune responses (8, 207). Thus, 3D tri-cultivation of ECs, DCs, and fibroblasts allows for greater cell-cell contact than previously developed methods and provides an excellent model for

mimicking the *in vivo* cellular orientation and functional features of the human airway epithelium. While due to time limitations I was not able to fully characterise the 3D tri-cultures, it would be very interesting to investigate IDO regulation in such model to assess the influence of DC, fibroblasts and EC cross-talk on this key immune regulatory mechanism in airway epithelium.

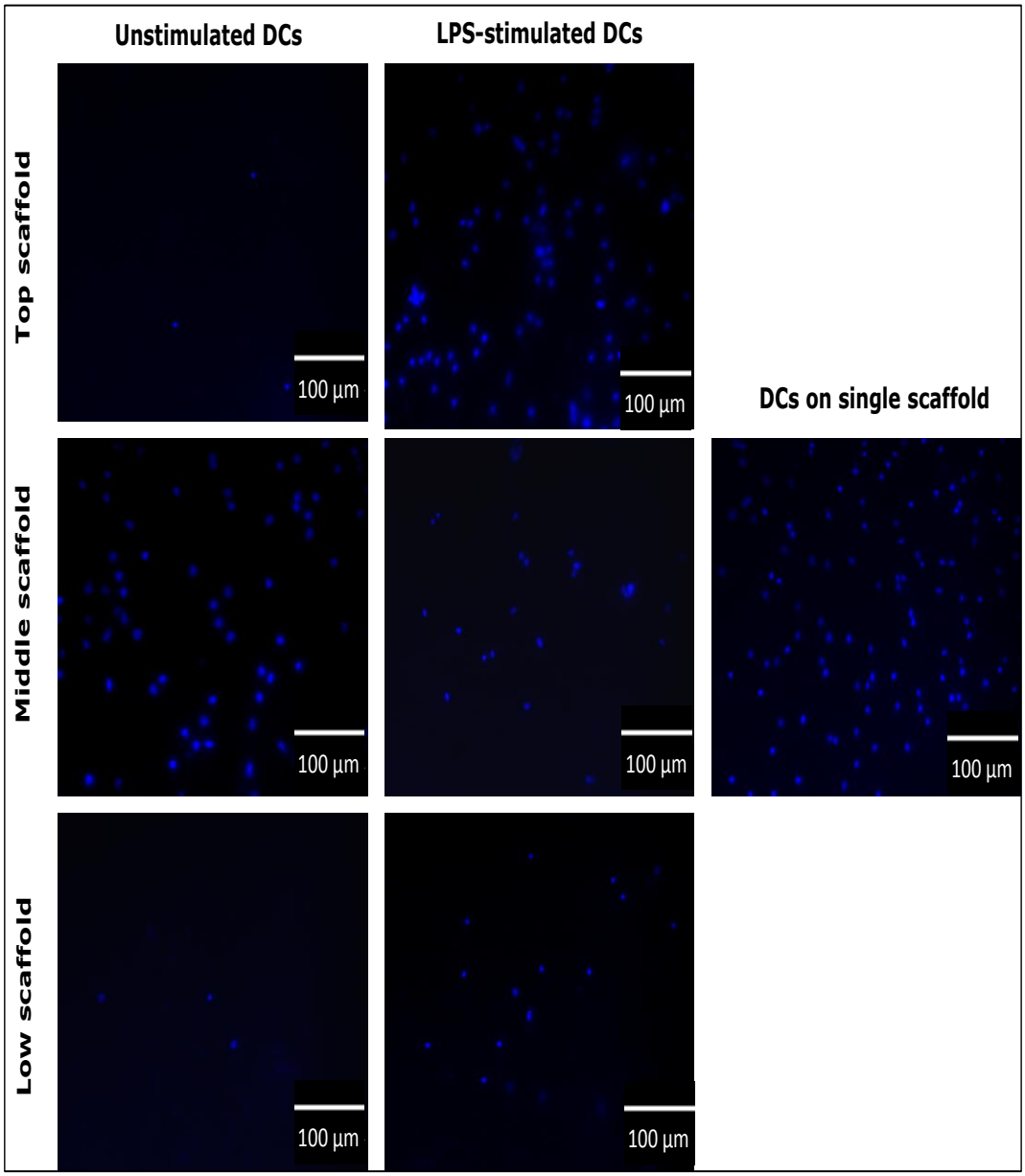


Figure 4.18: Fluorescence microscopy of DC migration on PET scaffolds. Immature DCs (day 6) were pre-stained with Hoechst nuclear stain for 30 minutes and challenged with LPS (0.1 µg/ml) for 24 hours. Unstimulated DCs on single scaffolds and on scaffolds combined with acellular (blank) scaffolds were included as controls. Fluorescence microscopy was performed at 20× magnification. Data shown are representative of 3 independent experiments.

4.4 Discussion

There is growing interest in achieving a greater understanding of the cross-talk between ECs and immune cells during the initiation and regulation of inflammatory reactions to various stimuli. ECs constitute a barrier surface that separates host tissues from the external environment. ECs constitute physical and biochemical barriers against microorganisms, and thus play an important role in regulating immune responses (233, 242). Accordingly, communication between ECs and the underlying stromal cells, as well as with DCs, is an important step in the regulation of immune reactions, including allergic responses (i.e. polarisation towards Th2 responses) (3, 6, 35). Studying such cell-cell communication in 3D co-culture models and comparing the findings with those obtained from 2D models could facilitate the characterisation of the mechanism underlying this cross-talk under *in vivo*-like conditions, and could therefore be used as a platform for disease modelling. It was previously shown that ECs cultivated *in vitro* are able to form barriers and polarised cohesive cell layers via the production of tight junctions, such as ZO-1 junctions, and the production of adherens junctions, respectively (243, 244). Furthermore, airway ECs exhibit the capacity to generate TEER readings that can be utilized to distinguish whether they have

differentiated when cultivated at the ALI, and whether they have generated functional tight junctions (120, 245, 246).

Using natural polymers that are constructed from constituent materials of the natural ECM is more suitable than synthetic polymer in terms of providing *in vivo* like conditions. Although there has been some success in the construction of these pure protein electro-spun scaffolds such as collagen (247, 248) and fibrinogen (247), they are not without limitations. Poor structural strength, low stability and batch-to-batch variation limits their use and reproducibility (120, 249, 250). Therefore, synthetic polymers such as poly glycolic acid (PGA), poly lactic acid (PLA), poly (lactic-*co*-glycolic) acid (PLGA) and PET offer the choice of well-defined batches with a greater range of mechanical and chemical properties than those of natural materials (120). In this study, airway ECs were differentiated on electro-spun PET scaffolds for 3D modelling in addition to TWI. One of the main advantages of PET scaffold is non-biodegradable polymer compared with other synthetic polymers which they are rapidly degraded via hydrolysis (251, 252). PGA is known to degrade in aqueous solutions within 4 days in PBS (251). Moreover, the breakdown products of PLA and PLGA degradation are organic acids (lactic and glycolic acid), the accumulation of these acids in cell culture media could cause the medium to become acidic which in turn could

adversely affect the cells. However, PET scaffold known to be more resistant and maintain its stability over the course of the experiment which for this reason was selected for this study. The porous PET scaffold fibres exhibit a structural resemblance to the native lung ECM, and are suitable for culturing individual cell layers per scaffold. Furthermore, utilisation of the scaffolds enables the separation and assessment of individual cell layers following experimentation.

Our findings demonstrate that Calu-3 cells and pHBECs are capable of expressing tight junctions (e.g., ZO-1 protein expression) and producing high TEER values when grown at the ALI. Despite lower TEER values of Calu-3 cultivated on scaffold compared to those cultivated on TWI, immunostaining for ZO-1 showed clear tight junction formation in Calu-3 cells cultured on PET scaffolds. This is consistent with results from previous studies showing that TEER values of cells grown on PLGA scaffolds were significantly lower than that of cells grown on TWI (253). Lower TEER values of PET cultures (3D) compared with transwell cultures (2D) could be due to the material upon which ECs are cultured. (120, 254). In addition, it has been shown that permeability data on TWI often have poor correlation to *in vivo* studies (255) which could be partially due to missing villous geometry in *in vitro* models (253). Moreover, the lower TEER values observed for cells

cultivated on the electro-spun scaffolds were similar to those obtained from ex-vivo samples of upper airway epithelium (120, 256, 257).

Several studies have shown that stromal cells play an important role in restoring barrier integrity after injury, potentially via acceleration of epithelial cell differentiation and promoting the expression of junctional proteins to repair and seal the injured epithelium (59, 258). These findings are consistent with our observation that ECs co-cultured with lung fibroblasts exhibited greater increases in TEER values than EC mono-cultures, indicating that ECs achieved full differentiation status faster in co-culture than in mono-culture. These data highlight the importance of paracrine interactions and cross-talk between cells in maintaining the barrier function of ECs.

While cells grown on transwell membranes exhibited paracrine cross-talk, the small pore size of these membranes enables only limited/ negligible cell-cell interaction and cell migration. The latter is of particular importance for migratory cells such as DCs. Indeed, migration to the site of inflammation and subsequently to the draining lymph node is one of the critical functions of DCs *in vivo* (8, 207, 259). Herein, our preliminary data highlighted the viability of DCs and their responsiveness when cultured on electro-spun scaffolds, as evidenced by their migration following LPS stimulation. Specifically, DCs seeded

on PET scaffolds and sandwiched between two blank scaffolds, which represented epithelial and fibroblast layers, respectively, were observed to migrate from the middle scaffold to the upper scaffold, particularly in response to LPS stimulation. These data provide evidence for the feasibility of co-culturing DCs with epithelial cells and fibroblasts on electro-spun scaffolds in the future. Collectively, our findings indicate that the 3D tri-culture model comprises a valuable tool for pharmaceutical research, particularly for evaluating drug uptake by DCs and drug delivery at the respiratory epithelium. Although the advantage of using natural polymers to mimic the natural ECM is invaluable, the time-consuming nature of this method limits the applicability of 3D tri-culture lung models (i.e. all three cell types together) to certain types of experiments. For future experiments, it would be interesting to incorporate an immune component into the 3D model and determine how each cell type influences the others' behaviours.

As previously discussed, IDO is a well-characterised prominent immune regulatory enzyme that modulates the activation status and phenotype of immune cells, and mediates antimicrobial activity via several molecular mechanisms, primarily through deprivation of L-TRP and its conversion into KYN metabolites (121, 135, 159, 213). In earlier sections of

this study (chapter 2 and 3), we investigated the regulation of IDO activity and expression in submerged ECs and observed significant down-regulation of IDO in response to a wide range of TLR ligands and allergen extracts in comparison to the control. In contrast, previous studies observed significant up-regulation of IDO in immune cells, particularly DCs, in response to stimuli both *in vivo* and *in vitro* (182, 260). In this chapter, we assessed IDO regulation in differentiated ECs in mono-culture and during co-cultivation with DCs and fibroblasts, and compared the levels IDO activity in submerged cells vs. cells grown at the ALI, in response to paracrine contact vs. cell-cell contact, and in 2D vs. 3D culture models. By studying such cell-cell communication in 3D co-cultures and comparing the findings with those obtained using the 2D model, it may be possible to characterise the immune-biology of these cells under *in vivo*-like conditions.

Accordingly, after evaluating tight junction formation, we quantified IDO activity in ECs. There were significant reductions in IDO activity in response to LPS in differentiated ECs cultivated on TWI. Using a papain-like cysteine protease to disrupt tight junctions in differentiated ECs resulted in even greater reductions in IDO activity after LPS stimulation, indicating that full barrier formation could lead to reduced accessibility to TLR4, and possibly to other PRRs, which should

be taken into account during data interpretation and experimental design. These data support recent observations that papain affects the skin barrier by promoting the expression of degrading tight junction proteins and inducing vasodilation, independent of its enzymatic activity, to promote TLR4 activation (261). Moreover, a separate study detected a relationship between the disruption of tight junctions (ZO-1) in respiratory epithelia and significant reductions in TEER values (120). It is widely recognised that there are limitations to *in vitro* models that generated from commercially 2D inserts including the surface upon which cells grow is planar and the cell−cell interaction is restricted due to the small pore size of the inserts (120). Although that both ALI and submerged culture of ECs has proved excellent models facilitating research into cells/paracrine communication (120, 262) and other respiratory diseases (263, 264), a limitation of this study is that using EC lines instead of primary cells. The fact is that some experiments of 3D model of primary ECs were conducted; however, poor attachment and proliferation of primary ECs on electro-spun scaffold (Appendix 5) limit their use and remains an avenue for future research.

Interestingly, the current study demonstrated that ECs cultured in the 3D model and co-cultured with fibroblasts exhibited differential regulation of IDO activity in which no

change of IDO in 3D model compared with significant decrease in 2D model in response to LPS stimulation. These differences are likely due to changes in orientation and expression pattern of TLR4 in ALI and in 3D compared to 2D cultures. Furthermore, while there were marked reductions in IDO activity and expression in mono-cultures of ECs in response to LPS treatment, ECs co-cultured with DCs exhibited considerable increases in IDO activity and expression under these same conditions compared to the control. It is widely recognised that stimulation of DCs by LPS induces the production of many pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-8 and IL-12 amongst other pro-inflammatory mediators (265-267). It is therefore possible that increased IDO activity in Calu3-DC co-cultures after LPS stimulation is driven by such pro inflammatory mediators. Although IFN-γ is one of the most potent inducers of IDO, IL-2 is also able to induce IDO activity and TRP degradation in peripheral blood mononuclear cells (268, 269). Moreover, cytokines including IL-1, IL-6 and TNF-α induces the expression of IDO in DCs in addition to IFNs (270, 271).

In conclusion, in this study we investigated the impact of EC differentiation status and co-culture with stromal and immune cells in 2D and 3D on an important immune-regulatory function namely IDO expression and activity. ECs cultivated in

isolation or co-cultures in 2D or 3D exhibited differential IDO regulatory properties and 3D cultures would be closer representation for physiological conditions than those cultivated in 2D culture. These data clearly highlighted the impact of EC differentiation status, their spatial orientation and cross-talk with other cells in the way the response to different stimuli. Given that 2D monolayers on artificial membranes can lose their original properties, including the physicochemical properties of the cell membrane, and that 3D co-cultures yield more physiologically relevant cellular interactions, which are important for cell behaviour, stability, and gene expression, application of our 3D model should enhance our understanding of epithelial pathophysiology and may lead to the development of improved therapeutic strategies (272, 273). Future efforts should focus on replacing EC cell lines in the 3D model with human primary ECs, which would provide an even better representation of the *in vivo* lung epithelium.

Chapter 5: The influence of disease status on IDO activity in the lung epithelium

5.1- Introduction

Chronic respiratory diseases, such as asthma and COPD, are generally thought to be caused by an abnormal immune response to environmental agents. The innate immune system plays a key role in the development of airway diseases. Airway ECs and innate immune cells are important components of the pathogenesis of chronic airway disease and are emerging targets for new therapies (62). Airway DCs fulfil the sentinel role in the adaptive immune response, and it has been proposed that changes in DC function (e.g. cytokine profile and surface phenotype) in response to allergen exposure or viral infection lead to asthma or other airway inflammation (274). Airway ECs act as the early and main responders to airborne allergens/pathogens and can modulate the subsequent immune responses. This notion first became apparent in studies showing that airway ECs may control immune cell infiltration into the airways during experimental asthma (275, 276). Since then, the role of ECs has been shown to be even more extensive in the airway: airway ECs monitor the external environment using PRRs including TLRs,

RIG-I-like receptors (RLRs), NLRs, PARs, and CLRs; and then orchestrate the innate immune response to inhaled allergens and infectious agents (35-37, 62, 277). A major consequence of PRR activation that may influence the development of airway disease is the induction of IFN-mediated signalling and the production of IFNs (278, 279). The activation of TLR signalling, in particular, results in the production of IFNs, in an immune response being mounted, and in the induction of antiviral resistance (280).

It is widely recognised that the respiratory epithelium is the largest surface of the human body that is in contact with the external environment. In support of an upstream role of airway ECs in driving inflammatory disease, a wide range of studies has shown that airway ECs respond to allergen challenge or viral infection by producing bioactive lipids, cytokines, and chemokines that control the trafficking, activation, and survival of immune cells (37, 62, 281). Moreover, the development of airway diseases demands the activation of innate immune cells such as DCs, natural killer cells and macrophages. These cells help to drive airway mucus production and airway hyper-reactivity via the activation of IL-33 signalling and the induction of IL-13 production (282, 283). IL-33 has been shown to induce the expression and production of Th2 cytokines *in vivo* and *in vitro* (284, 285) as well as it is

associated with mucus overproduction and goblet cell hypertrophy in the lungs and the gastrointestinal tract (285). Structural cells of various tissues, including fibroblasts, endothelial, ASM cells and ECs are the main source of IL-33 (285-287). It has been shown that IL-33 is expressed at higher levels in human endobronchial biopsies from asthmatic subjects compared with healthy controls which suggest the potential role of IL-33 in the pathogenesis of asthma, particularly that of the more severe phenotypes (285). Moreover, IL-33 plays a major role in protecting against helminth infection and reduces atherosclerosis by promoting Th2-type immune responses. However, IL-33 can also induce the pathogenesis of asthma by expanding Th2 cells and mediate atopic dermatitis and anaphylaxis by mast cell activation. Thus IL-33 could be a new target for therapeutic intervention across a range of diseases including allergic asthma (288). Liu *et al*. demonstrated that anti-IL-33 antibody can prevent the development of asthma in a mouse model and attenuated the damage to the airway epithelium (284). Collectively, airway ECs are crucial for the initiation of innate immunity, which in turn activates ongoing immune responses and drives the exacerbation and progression of the respiratory disease.

IDO has been widely recognised as a prominent immune regulatory enzyme capable of modulating immune cell activation status and phenotype through various molecular mechanisms including enzyme-dependent reduction of L-TRP and its conversion into bioactive KYNs (121, 146, 159, 221), or non-enzymatic cell signalling processes including tyrosine phosphorylation of IDO (123, 289). Through these biochemical signalling processes, IDO contributes to different physiological and pathological settings. Examples of such contributions include regulatory role of IDO in various physiological (e.g. pregnancy) and pathological conditions such as chronic inflammation, infectious disease, allergic, autoimmune disorders, transplantation, and cancer (123, 132). The expression of IDO has been reported for various tissue and cell types including macrophages, DCs (183, 290), neutrophils (291), eosinophils (292), endothelial cells (293), epithelial cells (141, 156, 173), fibroblasts (294), and smooth muscle cells (295); where IDO expression is either constitutive or stimulated by relevant inflammatory and immune stimuli. Constitutive IDO protein expression under normal physiological conditions occurs mainly in mucosal tissues and subsets of immune cells, and is significantly induced in response to inflammation and infection (123).

It is widely accepted that IDO represents a key immune regulatory enzyme by which innate and adaptive immune responses are modulated via several different biochemical mechanisms including L-TRP deprivation, activation of the GCN2-dependent signalling pathway (165, 296), activation of AhR signalling (150), the production of downstream metabolites that modulate immune cell actions (159), and IDO1 tyrosine phosphorylation, which induces immune tolerance through the conversion of CD4+ T-cells into immunosuppressive Tregs (297). These effector mechanisms, however, vary depending on the physiological and pathological conditions.

Several studies have revealed the importance of IDO in chronic inflammatory and autoimmune diseases. The induction of IDO in epithelial cells, for example, has been shown to limit the severity of colitis in experimental mice through Tregs-mediated immune suppression (298, 299), while the elevation of IDO activity and expression in DCs of diabetic mice via IFN-γ production has been shown to protect against diabetes (130, 300). An increasing body of data also demonstrates the modulatory role of IDO in inflammatory and allergic disorders such as suppression of experimental asthma by IDO. A murine *in vivo* study showed that the induction of IDO in epithelial cells in response to TLR9-ligand results in the inhibition of

Th2-mediated inflammatory responses, airway hyper-reactivity, and eosinophilia (156). Another study demonstrated a correlation between reduced IDO activity and expression and concomitant IL-17A induction and IL-10 reduction, resulting in chronic airway neutrophilic inflammation in COPD patients (301, 302). In terms of genetic disorders, it has been shown that IDO may play a role in CF via an imbalance of Th17 and Tregs as a result of dysfunctional IDO activity and expression in murine and human CF (303).

Collectively, the multifunctional role of IDO in health and diseases is becoming increasingly clear (123); however, it is important to understand all factors surrounding IDO immune control in both physiological and pathological contexts. The aim of the work described in this chapter was to investigate the regulation of IDO activity and expression in healthy and diseased epithelial cells and to evaluate the influence of diseased fibroblasts (COPD fibroblasts) on IDO activity in epithelial cells. Understanding IDO expression and regulation in different tissue/cell types in health and disease it would contribute to the elucidating its potential role in inflammatory responses in different pathologies and could enable the development of novel IDO-targeted therapeutic strategies.

5.2- Materials and Methods

5.2.1- Cell culture

5.2.1.1- pHBECs

pHBECs separated from bronchial brushings of healthy and asthmatic volunteers attending the Nottingham University Hospital Respiratory Clinic who provided informed written consent, were maintained in BEGM as described in Chapter 2, Section 2.2.2. Asthmatic individuals included those who had been diagnosed with asthma for 1–5 years and one individual who had been diagnosed with asthma for more than 5 years. The individual concerned was being treated with some medications such as prednisolone, salbutamol, Seretide, and was diagnosed with sinusitis as comorbidity (Appendix 3). All asthmatic subjects used in this study were non-allergic (304, 305). pHBECs from both healthy and asthmatic donors were differentiated in BEDM on TWI as described in Chapter 4, Section 4.2.2. All pHBECs from healthy and asthmatic subjects were used in passage 3. For each experiment, around 5 to 7 samples were cultured and at least 3 of them were characterised successfully.

5.2.1.2- Cystic fibrosis epithelial cells (CF-ECs)

Immortalised human bronchial ECs (IB3-1) were derived from a CF patient with a compound heterozygous mutation (ΔF508/W1282X) in the CFTR gene. The corrected counterpart of IB3-1 cells (i.e. IB3-1 cells stably transfected with cDNA encoding the CFTR protein by the adeno-associated virus type 2 inverted terminal repeat) are S9 cells. Both CF-ECs and their corrected counterparts were maintained in MEM media (Sigma-Aldrich) containing 10% v/v FBS, 100 U/mL penicillin-100 µg/mL streptomycin and 2.5 µg/mL amphotericin B. For sub-culturing, cells were incubated with 10 mL trypsin-EDTA solution (Sigma-Aldrich) for 5 minutes and then neutralised with MEM complete media. All experiments were performed under aseptic conditions in a laminar airflow cabinet. Herein, CF and non-CF cells were cultured to confluency and were then incubated with TLR ligands (2 µg/mL and 10 µg/mL LPS; 0.1 µg/mL poly I:C), 10 µg/mL allergen extracts, 10 ng/mL IFN-γ, or a combination of IFN-γ with TLRs or allergen extracts for 24 hours.

5.2.1.3- Primary fibroblasts

Human lung primary fibroblasts derived from lung biopsies of healthy and COPD patients were provided by the Nottingham University Hospital Respiratory Clinic. Primary fibroblasts outgrowth (Appendix 6). Primary fibroblasts were maintained

in DMEM media (Sigma-Aldrich) with 10% v/v FBS, 100 U/mL penicillin-100 µg/mL streptomycin, 4 mM L-glutamine, and 2.5 µg/mL amphotericin B. Cells were cultured in T-225 culture flasks (Corning Life Sciences) and were harvested once they reached 90% confluence. For sub-culturing, cells were incubated with 10 mL trypsin-EDTA solution (Sigma-Aldrich) for 2–4 minutes and then neutralised with DMEM complete media. All experiments were performed under aseptic conditions in a laminar airflow cabinet. For Calu-3 cell and primary fibroblast co-cultivation experiments, 2 x 10^5 cells/mL Calu-3 cells and primary fibroblasts (healthy or COPD) were inoculated on the apical and basolateral sides of the TWI (i.e. in the tissue culture plate), respectively. After the cells reached confluence, they were incubated with 2 µg/mL LPS, 10 µg/mL CpG, or 10 µg/mL poly I:C for 24 hours, after which the supernatants were collected for quantification of IDO activity.

5.2.2- Cytokine measurements

Supernatants were collected and stored at -20°C until analysis. Cytokine (IL-6, IL-8, and GM-CSF) concentrations were analysed using the ProcartaPlex Multiplex Immunoassay system (eBioscience) according to the manufacturer's instructions. Some data (IL-8 from ALI-differentiated

asthmatic pHBECs) were obtained using the DuoSet ELISA System (R&D Systems) according to the manufacturer's instructions.

5.2.3- IDO enzymatic activity assay

The IDO activity levels were evaluated in culture supernatants harvested from submerged, differentiated ECs (i.e. ALI) or primary fibroblasts (either healthy or diseased) and activity assays were carried out as described in Chapter 2, Section 2.2.3.

5.2.4- mRNA isolation, cDNA synthesis and PCR

Messenger RNA isolation and cDNA synthesis were performed using the μMACS One-Step cDNA Kit (Miltenyi Biotech), and PCR was carried out in a TC-312 PCR Thermocycler (Bibby Scientific, Ltd.) using Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific) as described in Chapter 2, Section 2.2.5, according to the manufacturer's instructions.

5.2.5- Statistical analysis

Data are expressed as means ± SEM. Student's *t* test was used to compare statistical differences between two groups, while one-way ANOVA was utilised to compare three or more

groups. Tukey's post-hoc test was applied to all results, and differences with $*p \leq 0.05$ were considered statistically significant. GraphPad Prism 6 software (GraphPad Software, La Jolla, Calif) was used for all statistical analysis.

5.3- Results

5.3.1- Differential regulation of IDO activity in healthy and asthmatic pHBECs

It was previously shown that IDO activity from pulmonary cells (not resident dendritic cells) could suppress Th2-mediated experimental asthma in mice (156) This confirmed by the induction of IDO in response to TLR-9 ligand which enhanced cell death of Th2 cells in the lung, as well as reduced the levels of Th2 cytokines (IL-4, IL-5, and IL-13) in the BAL fluid and this reduction was partially reversed by using IDO inhibitor (156). To better understand IDO expression and regulation in human asthma in this study, IDO regulation in primary ECs derived from healthy and asthmatic donors was investigated. pHBECs from healthy and asthmatic patients were cultured to confluency either submerged or ALI-differentiated for three weeks and incubated with IFN-γ, LPS, HDM or with co-stimulated with LPS + IFN-γ or HDM + IFN-γ for 24 hours. Although IFN-γ induced significant levels of IDO activity in both submerged and differentiated pHBECs from both healthy and asthmatic volunteers compared with the control (untreated cells), KYN concentrations were clearly higher in asthmatic pHBECs than in healthy pHBECs under each set of conditions (Figure 5.1 A and B). Statistical analyses of submerged *vs.*

differentiated pHBECs in healthy or asthmatic cells furthermore revealed that there was generally a significant increase in IDO activity in differentiated cells compared with undifferentiated cells (Figure 5.1 C and D). The higher levels of IDO in ECs from asthmatic individuals under steady state conditions and in response to different stimuli are intriguing. Such higher levels of IDO could act as a protective/regulatory mechanism in response to general heightened levels of inflammation in the airway microenvironment of asthmatics.

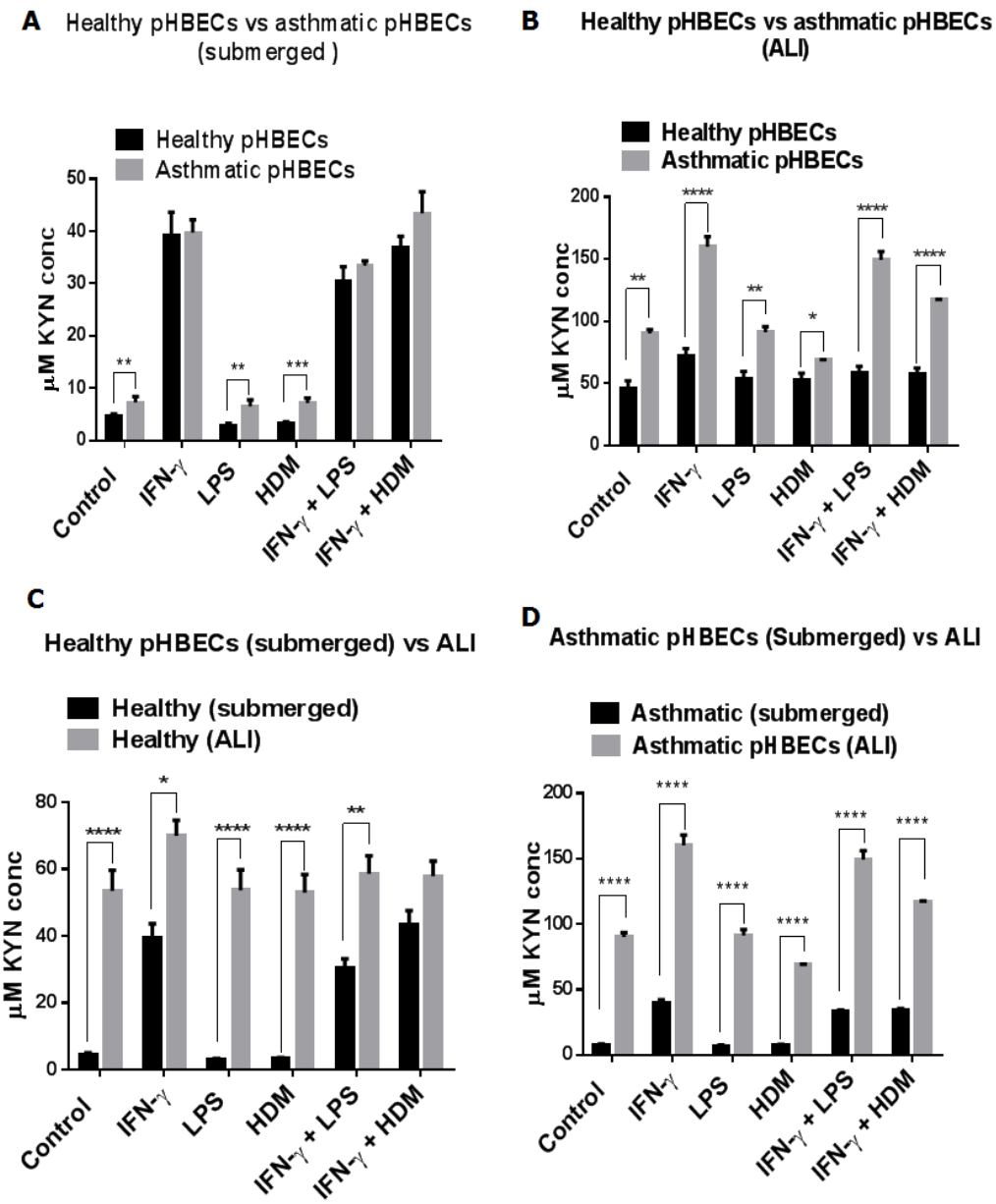


Figure 5.1: IDO activity in submerged and differentiated pHBECs from asthmatic and healthy donors. (A) pHBECs from healthy and asthmatic donors were cultured submerged to confluence or (B) differentiated by culturing at ALI on TWI for up to 3 weeks. The cells were then incubated for 24 hours with 10 ng/mL IFN-γ, 2 µg/mL LPS, 10 µg/mL HDM or with a combination of IFN-γ and LPS or HDM. (C and D) IDO activity was also compared between submerged and ALI-differentiated states in healthy and asthmatic pHBECs respectively. Data represent three independent experiments; *p ≤0.05, **p ≤0.01, ***p ≤0.001, and ****p ≤0.0001.

5.3.2- Changes in pro-inflammatory cytokine production in primary ECs from healthy and asthmatic donors

A variety of stimuli are recognised as inducers of pro-inflammatory cytokines such as IL-6, IL-8, and GM-CSF in airway ECs. These cytokines in the airway potentially contribute to the disease process and regulate inflammation via their effects on cell recruitment, activation, and survival (306, 307). GM-CSF, a pro-inflammatory cytokine produced by ECs via TLR triggering, activates DCs and thus plays an important role in the early activation of adaptive immune responses (37, 308). Some of these cytokines are also linked to IDO regulation. For example, in airway EC lines, IFN-γ induces IDO-mediated L-TRP degradation which leads to increased IL-6 and IL-8 responses and might be implicated in enhanced inflammatory responses to microbial challenges (309). The present data reveal that under submerged growth conditions, pHBECs from asthmatic donors had significantly lower basal levels of IL-6, IL-8 and GM-CSF in steady state condition and in response to different stimuli including LPS, HDM, IFN-γ and co-stimulation of IFN-γ with LPS or HDM compared with healthy pHBECs (Figure 5.2 A, B, and C). In polarised ECs grown at ALI, however, IL-6 and IL-8 were present at higher levels in asthmatic pHBECs than in healthy

pHBECs (Figure 5.2 D, and E); with no GM-CSF production by either cell type (data not shown). Furthermore, a comparison of cytokine production in healthy or asthmatic pHBECs between submerged and ALI-differentiated conditions revealed significantly lower levels of pro-inflammatory cytokine production in differentiated cells compared with submerged cells (Figure 5.3). It is believed that cells grown submerged have different morphological and physiological properties compared with well-differentiated cells. Differentiated cell layers form a polarised barrier which could have an impact on the differences between apical and basolateral secretions (310). We speculated that it could be a link between IDO and pro-inflammatory cytokine in which high level of pro-inflammatory cytokines in addition to high IDO activity in polarised ECs of asthmatic donor could play an important role in the pathogenesis of asthma in regard to act as a protective mechanism and lead to the recruitment and subsequent activation of diverse immune cells.

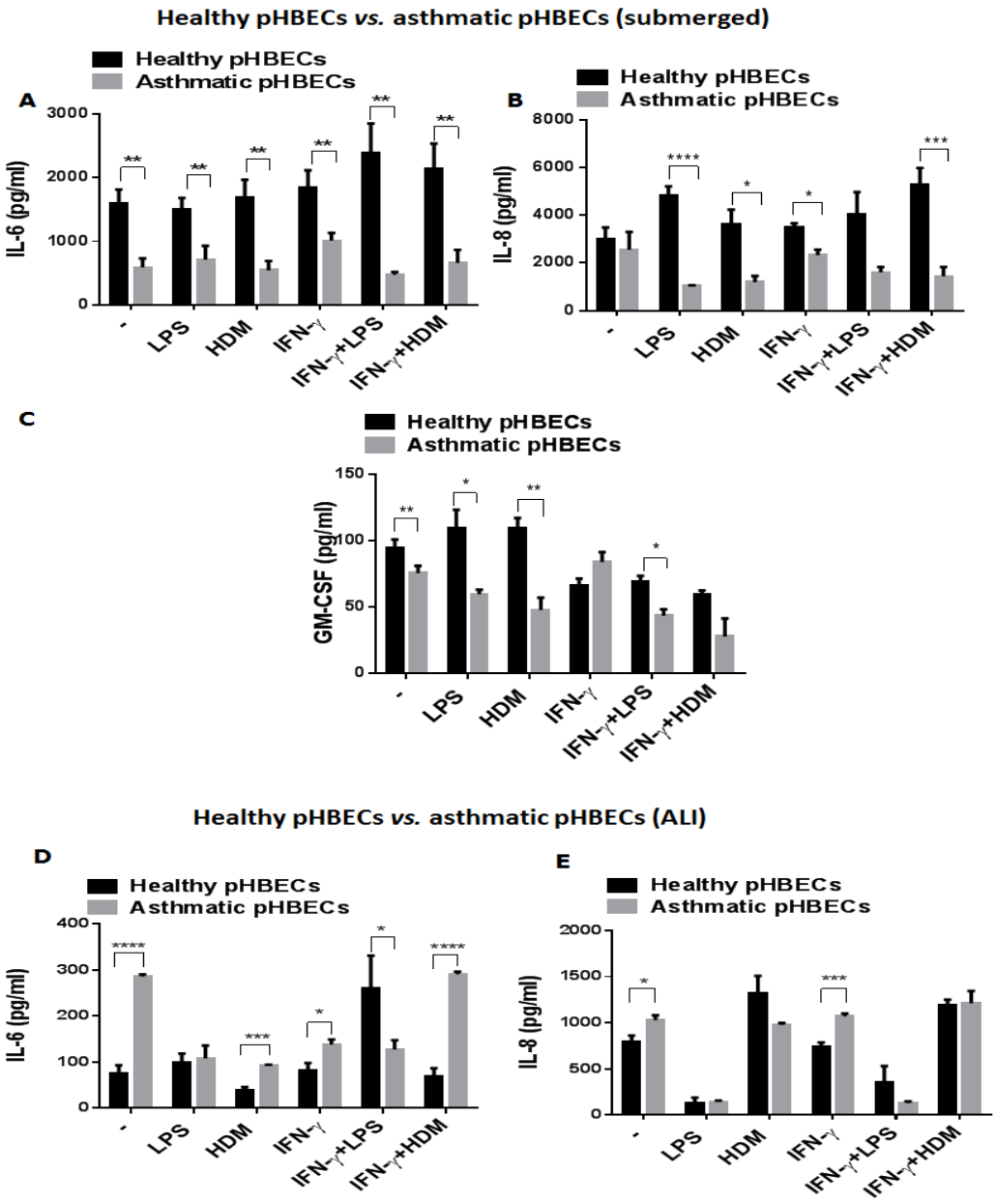
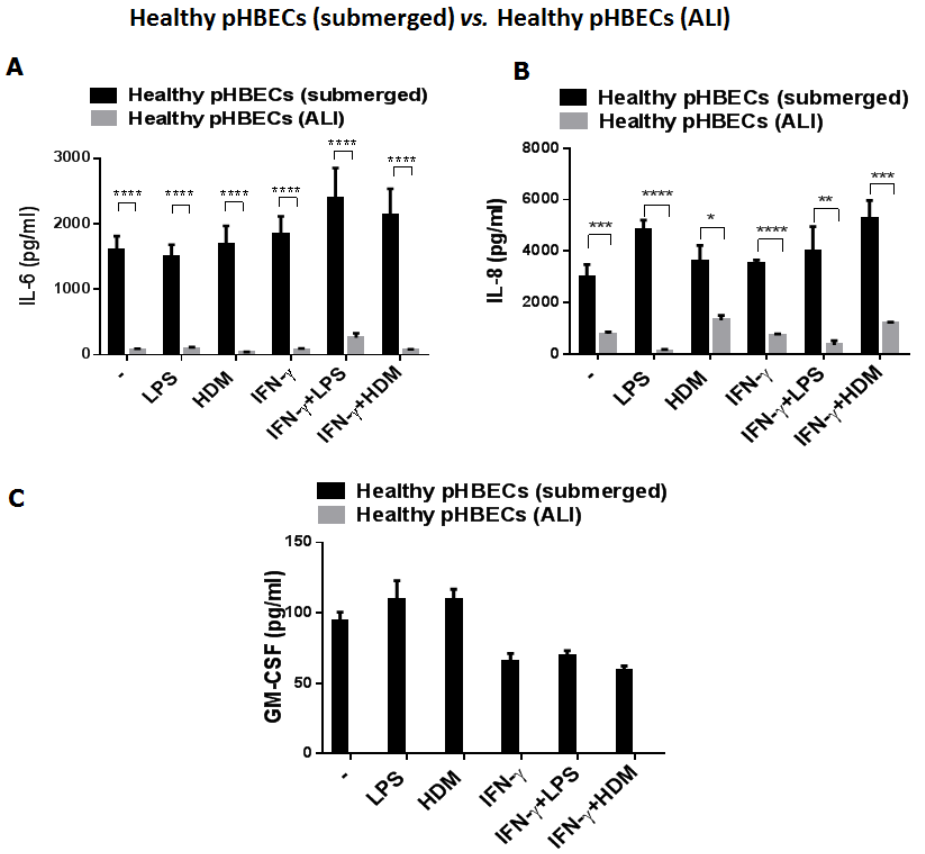


Figure 5.2: Cytokine production in healthy and asthmatic pHBECs. pHBECs were cultured submerged until confluence or were differentiated at ALI for three weeks. The cells were then incubated for 24 hours with 2 µg/mL LPS, 10 µg/mL HDM, 10 ng/mL IFN-γ, or with a combination of IFN-γ and LPS or HDM. IL-6 (A; D), IL-8 (B; E), and GM-CSF (C) production by submerged (A, B and C) or ALI-differentiated (D and E) healthy and asthmatic pHBECs. Cytokine concentrations in culture supernatants were quantified by multiplex microbead immunoassays. Data represent three independent experiments. *p ≤0.05, **p ≤0.01, ***p ≤0.001, and ****p ≤0.0001.


Healthy pHBECs (submerged) vs. Healthy pHBECs (ALI)
A
Healthy pHBECs (submerged)
Healthy pHBECs (ALI)
IL-6 (pg/ml)
3000
2000
1000
0
****
-
LPS
HDM
IFN-γ
IFN-γ+LPS
IFN-γ+HDM
B
Healthy pHBECs (submerged)
Healthy pHBECs (ALI)
IL-8 (pg/ml)
8000
6000
4000
2000
0
***
****
*
**
C
Healthy pHBECs (submerged)
Healthy pHBECs (ALI)
GM-CSF (pg/ml)
150
100
50
0

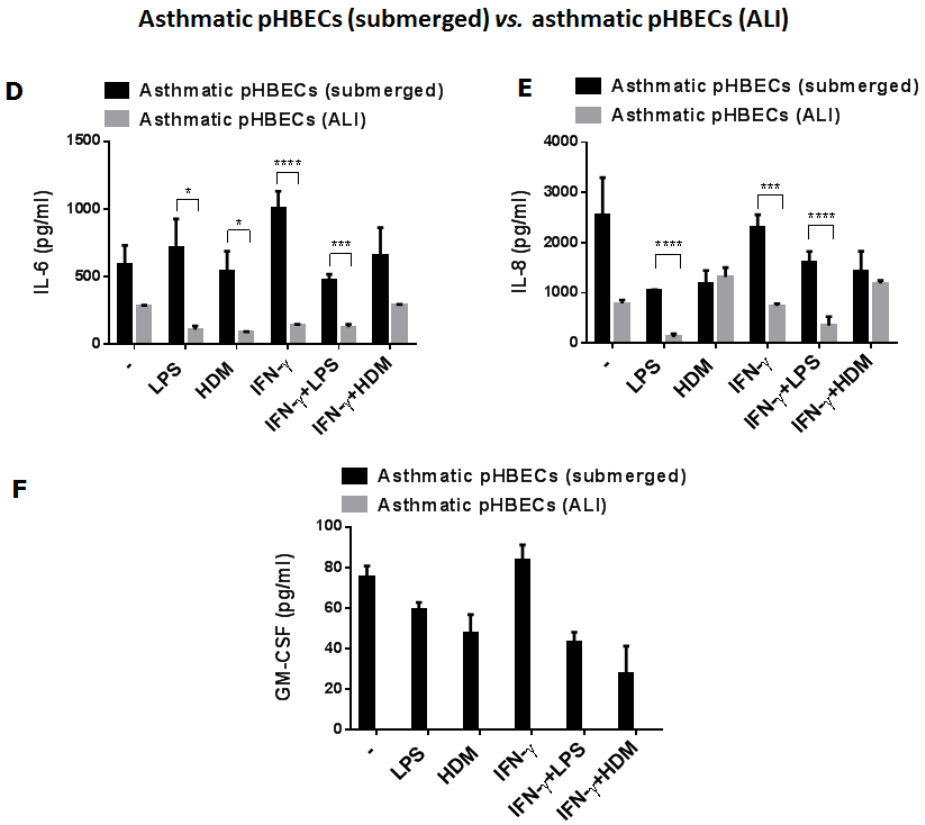

Asthmatic pHBECs (submerged) vs. asthmatic pHBECs (ALI)
D
Asthmatic pHBECs (submerged)
Asthmatic pHBECs (ALI)
IL-6 (pg/ml)
1500
1000
500
0
*
****
***
-
LPS
HDM
IFN-γ
IFN-γ+LPS
IFN-γ+HDM
E
Asthmatic pHBECs (submerged)
Asthmatic pHBECs (ALI)
IL-8 (pg/ml)
4000
3000
2000
1000
0
****
***
F
Asthmatic pHBECs (submerged)
Asthmatic pHBECs (ALI)
GM-CSF (pg/ml)
100
80
60
40
20
0

Figure 5.3: Cytokine production in submerged and differentiated pHBECs. pHBECs were cultured submerged until confluent or differentiated at ALI for three weeks and were incubated for 24 hours with 2 µg/mL LPS, 10 µg/mL HDM, 10 ng/mL IFN-γ, or with a combination of IFN-γ with LPS or HDM. IL-6 (A; D), IL-8 (B; E), and GM-CSF (C; F) by submerged *vs*. differentiated pHBECs from healthy (A, B and C) and asthmatic (D, E and F) volunteers. Cytokine concentrations in culture supernatants were quantified by multiplex microbead immunoassays. Data represent three independent experiments; *p ≤0.05, **p ≤0.01, ***p ≤0.001, and ****p ≤0.0001.

5.3.3- IDO activity in CF

A correlation between impaired IDO activity and expression in a murine CF model and the induction of lung dysfunction and inflammation has been demonstrated (303). In this study, the aim was to investigate IDO regulation in CF and non-CF HBECs in response to stimulation by IFN-γ as well as TLR ligands and allergen extracts. As shown in Figure 5.4, while IDO activity did not increase in response to stimulation with TLR ligands and allergens in both control and CF for unknown reasons, a significant increase in IDO activity was observed in response to IFN-γ in both cell types (Figure 5.4 A and B). A dose response and time course studies need to be done in the future to clarify the unresponsiveness to TLR ligands and allergens in both CF and non-CF ECs. The increased IDO

activity in response to IFN-γ was furthermore shown to correspond to an increase in IDO mRNA expression (Figure 5.4 C and D). Although the data was not entirely quantitative, we think it is still worthwhile using real time PCR for producing a quantitative result of mRNA expression.

Moreover, when comparing the KYN concentrations between CF and non-CF cells, significantly lower baseline KYN levels were observed in CF cells under steady state conditions or upon stimulation with IFN-γ (Figure 5.5). These findings are in line with a previous report of lower IDO levels in CF patients compared with controls (303). This could partly contribute to the higher predisposition to airway infection in CF patient such as *Aspergillus* infection due to a defective IDO in CF (303).

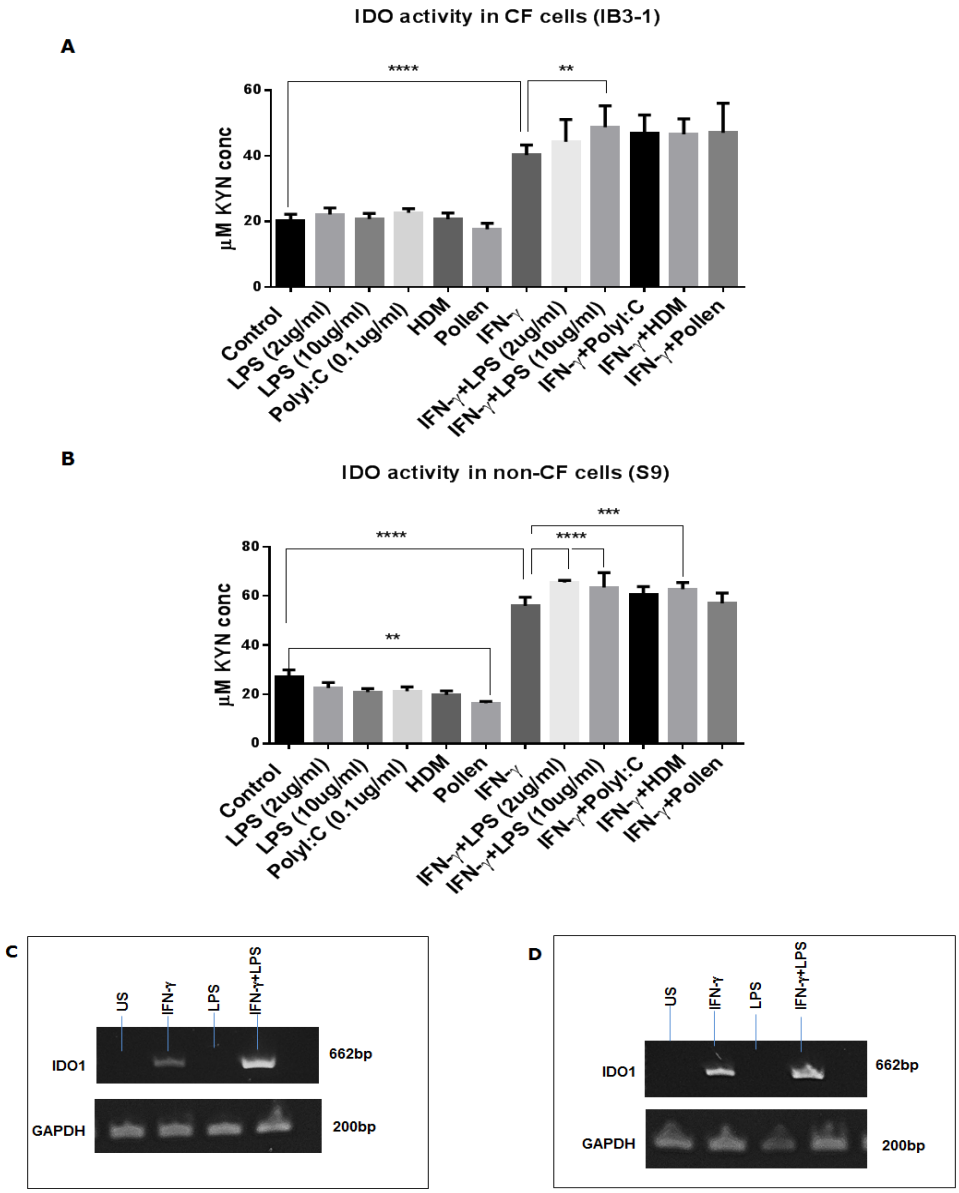


Figure 5.4: IDO activity and expression in CF and non-CF HBECs. (A) CF patient-derived immortalised HBECs (IB3-1 cells) and (B) their corrected counterparts (S9 cells) were cultured submerged to confluency and were then incubated with TLR ligands, 10 µg/mL allergen extracts, 10 ng/mL IFN-γ, or combinations of IFN-γ with TLRs or allergen extracts for 24 hours. (C and D) IB3-1 and S9 cells were cultured submerged to confluency and were then incubated with 10 µg/mL LPS, 10 ng/mL IFN-γ, or a combination of IFN-γ and LPS for 24 hours. Supernatants were collected for IDO activity measurements and the cells were processed for mRNA expression. GAPDH was used as a housekeeping gene. US, unstimulated. Data represent three independent experiments; ***p* ≤0.01, ****p* ≤0.001, and *****p* ≤0.0001.

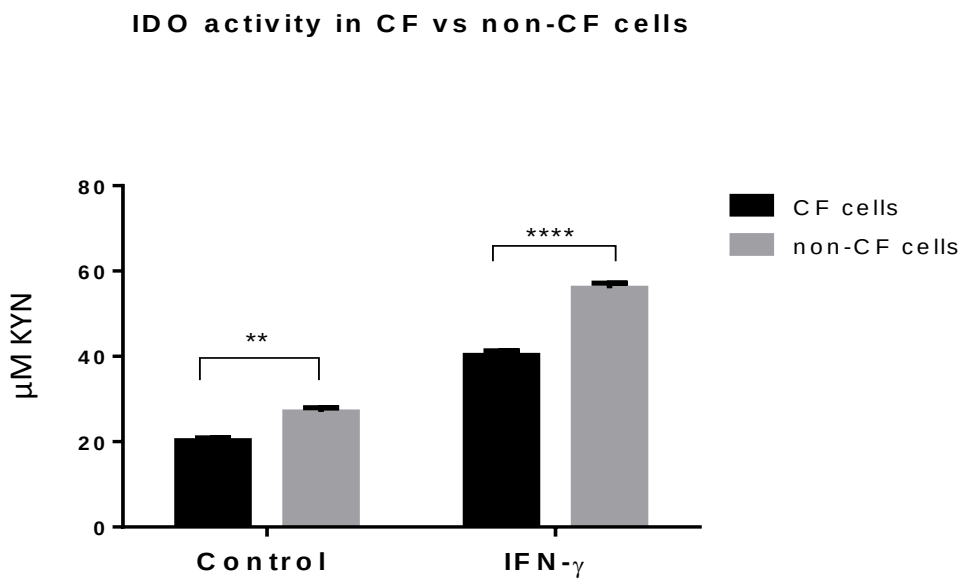


Figure 5.5: Comparison of IDO activity in CF vs. non-CF HBECs. CF patient-derived immortalised HBECs (IB3-1 cells) and their corrected counterparts (S9 cells) were cultured submerged to confluency and then incubated with 10 ng/mL IFN-γ for 24 hours. Supernatants were collected for IDO activity measurements. Data represent three independent experiments. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, and $^{****}p \leq 0.0001$.

5.3.4- Influence of diseased fibroblasts on IDO activity in epithelial cells

After investigating the basal levels and differential regulation of IDO activity in healthy and diseased ECs (asthmatic and CF cells), the effects of diseased fibroblasts on IDO activity in the epithelium were investigated. Here I hypothesised that stromal fibroblasts could modulate the IDO activity in ECs. Human

primary fibroblasts of healthy and COPD donors were thus co-cultured with Calu-3 ECs on TWI before being stimulated with TLR ligands including 2 μg/mL LPS, 10 μg/mL CpG, and 10 μg/mL poly I:C for 24 hours. Interestingly, Figure 5.6 shows higher basal KYN levels in both normal and COPD fibroblasts compared with Calu-3 cells and this level is down regulated in co-cultures. These data suggest that ECs could downregulate IDO activity in fibroblasts. It was previously shown that IDO activity is significantly reduced in the sputum of COPD patients, resulting in chronic airway neutrophilic inflammation (302). However, in the experimental setting reported on here, the levels of IDO activity do not differ markedly between normal and COPD fibroblasts; and the pattern of IDO activity regulation in response to TLRs in these cells was comparable to that of control samples. It is therefore reasonable to suggest that despite high levels of constitutive IDO expression in fibroblasts, the IDO activity in these cells is not changed by the disease status or external stimuli.

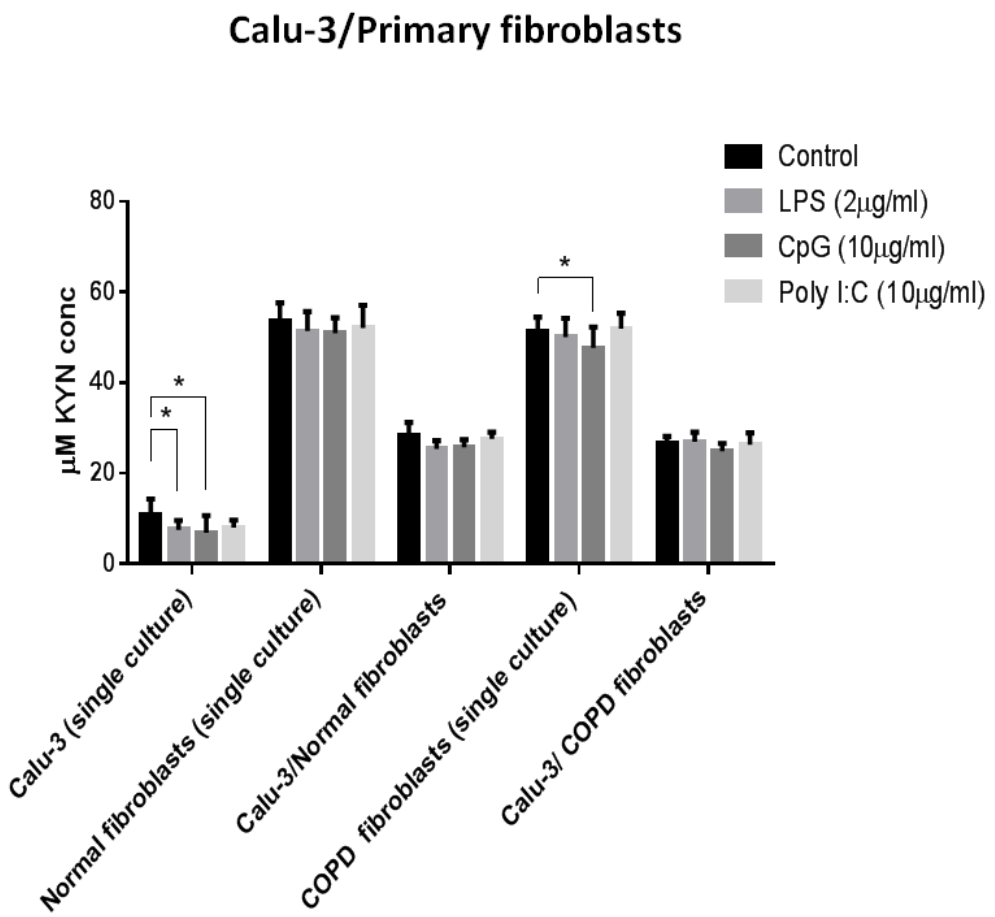


Figure 5.6: Influence of COPD fibroblasts in IDO activity of ECs. Calu-3 ECs were cultured submerged to confluency either as single cultures or by co-culture with COPD or normal fibroblasts. Co-culture was carried out on TWI. Cells were incubated with different TLR ligands for 24 hours after which supernatants were collected for IDO activity measurements. Data represent three independent experiments; *$p \leq 0.05$.

5.4- Discussion

Respiratory infections are thought to be major exacerbating factors in several airway diseases including asthma, COPD, and chronic rhinosinusitis (37, 308, 311). Due to their exposed position, airway ECs are the primary target of respiratory pathogens and allergen-induced lung inflammation. The airway ECs are furthermore the frontline activators of DCs and other immune cells; and the activation of ECs therefore results in the production of cytokines, chemokines (including TSLP, GM-CSF, IL-33, CCL2, and CCL20), and antimicrobial mediators such as prostaglandins and defensins that recruit and activate innate and adaptive immune cells (37).

The IDO is a key immune-regulatory enzyme that is produced by ECs and plays an important role in modulating immune responses through TRP depletion and the generation of kynurenine metabolites (149, 174). It is widely recognised that myeloid cells including APCs have high levels of IDO activity that suppress T cell proliferation and/or responses (121, 161, 176); however, it must be noted that ECs in the context of inflammation are a leading source of IDO activity too (156, 312). Despite the abovementioned information, relatively little is known about the effect of disease status on IDO activity in the epithelium. Accordingly, this *in vitro* study was carried out to investigate the regulation of IDO activity

and expression in diseased and healthy ECs under steady-state and stimulated conditions. Although the small number (n=3) of healthy or diseased subjects in each experiment provided in this study, a better understanding of IDO expression and regulation in various cell types in health and disease, it would provide a clearer comprehensible about its potential role in inflammatory responses in diverse pathology, and this could effectively enable the development of novel IDO-targeted therapeutic strategies in the future.

Asthma is an allergen-induced chronic airway inflammatory disorder that is characterised by the pathogenic role of Th2 cells which produce IL-4, IL-5, and IL-13; leading to accumulation of eosinophils and mast cells; and production of IgE (37, 62). It has been shown that IDO is induced during inflammation by IFN-γ and is suppressed by Th2 cytokines (147, 157, 313). Several studies have emphasised the immunosuppressive role of IDO in allergic airway inflammation. Hayashi *et al*. for example, demonstrated that the activation of innate immunity may inhibit adaptive Th cell responses by showing that the administration of the immunostimulatory sequence oligodeoxynucleotide (analogs TLR9 and TLR4) in mice induced IDO activity within lung epithelial cells, resulting in the inhibition of both Th2-driven experimental asthma and Th1-mediated inflammatory

responses. The observed IDO enhancement was furthermore reversed by the IDO-inhibitor 1-methyl-DL-tryptophan (156). This observation was in agreement with another study in which it was shown that IDO metabolites induce immune tolerance during allergen immunotherapy related to Th2-dependent allergic airway inflammation (155). Moreover, it was found that IDO activity played a role in the pathogenesis of atopy and was significantly lower in atopic than in non-atopic individuals (314). Another study, however, showed that the systemic IDO activity and IL-10 production are elevated in asymptomatic atopy during seasonal allergen exposure (315). In this study I initially investigated the basal levels of IDO in ECs from healthy donors and asthmatic individuals in steady-state conditions or after stimulation with a range of TLR ligands or an allergen extract. Basal and stimulated IDO activities were shown to differ between asthmatic and healthy subjects as well between ECs cultured submerged or differentiated at ALI. Thus, basal IDO levels were found to be significantly higher in pHBECs of asthmatic individuals compared with those of healthy individuals either in submerged or differentiated cells. The IDO levels were furthermore always significantly higher in ALI-differentiated ECs compared with submerged ECs. The findings reported here support previous observations regarding higher levels of

systemic downstream IDO metabolites in allergic asthmatic individuals compared with control individuals (316), and may therefore have physiological consequences on T cell responses. Since ECs are the primary target for respiratory pathogens and allergens and are thus likely to play a pivotal role in allergen-induced lung inflammation, it is interesting to assess the differences in pro-inflammatory cytokine production between asthmatic and healthy subjects. Pro-inflammatory cytokines have been proposed to play a crucial role in the induction of an inflammatory cascade in allergic asthma and thus the severity of asthma is associated with the degree of the inflammation (317). It has been shown that airway ECs stimulated with Th2 cytokines (i.e. IL-4, IL-5, and IL-13) result in GM-CSF induction and IL-8 production, thereby attracting immune cells (37, 318). Viral infection induces the release of IL-8 and other chemokines that attract eosinophils, Th2 cells, and DCs and promote Th2 immunity that drives sustained allergic inflammation (37, 319). Moreover, HDM induces the production of TSLP, GM-CSF, and IL-25 by ECs via uric acid-activated Th2 responses (37). Dente *et al.* demonstrated significantly higher levels of IL-8, GM-CSF, and IL-5 in the sputum of severe asthmatic subjects compared to the mild asthmatic or healthy subjects (320). Increased GM-CSF production by airway ECs has thus been associated with

eosinophilia and the severity of allergic asthmatic responses (321, 322). Correlations have been made between cytokines and IDO regulation. An example of this was demonstrated in an *in vitro* study that showed that TRP depletion by IDO in ECs induces inflammatory mediators including IL-6 and IL-8 (309). Moreover, IDO can undergo proteasomal degradation in the presence of IL-6 in DCs (160). To this end, the initial observations with regard to cytokine production in this study showed differential levels between healthy and asthmatic submerged and ALI-differentiated primary ECs: higher levels of IL-6 and IL-8 were observed in differentiated ECs from asthmatic subjects compared with the control subjects with no production of GM-CSF primary ECs from both groups. There were, however, higher levels of pro-inflammatory cytokines in submerged cells compared with polarised cells. Therefore, given that most of the asthmatic donors used in this study are non-allergic asthmatic subjects, it is speculated that the production of GM-CSF and other pro-inflammatory cytokines can thus be mainly associated with allergic asthma than non-allergic asthma. Although only a handful of healthy and asthmatic subjects has been studied in regard to IDO activity and production of pro-inflammatory cytokines, more associations could be observed when increasing the biological replicates for future studies.

The IDO pathway has been shown to play a potential role in controlling airway inflammation including asthma and COPD; and it has been shown that IDO activity is reduced in the sputum of COPD subjects, most likely due to the suppression of IDO expression in macrophages (301, 302). These studies also confirmed the correlation between reduced IDO activity and an IL-10/IL-17 imbalance in which IL-10 and IL-17 production is reduced and increased, respectively, in COPD patients, depending on the severity of the disease. The induction of IDO has also been shown to regulate Th1 and Th17 accumulation at the site of inflammation and, therefore, to attenuate the degree of inflammation (323). There is little data on IDO expression or activity in COPD, it was therefore of great interest to investigate IDO regulation in COPD in response to TLR ligands in this study and to examine the effects of diseased fibroblasts on the IDO activity of epithelial cells. This *in vitro* study demonstrated high baseline levels of IDO in both normal and COPD fibroblasts; however, co-culture of normal and COPD fibroblasts with ECs resulted in significantly reduced IDO levels which raises the possibility of that IDO activity of ECs could modulated by fibroblasts. However, the same high levels of IDO observed in both healthy and COPD subjects with the same pattern of IDO regulation in response to TLR ligands showing that IDO activity

in these fibroblasts does not changed by the disease status or external stimuli although their elevated level of constitutive IDO expression. This observation certainly warrants further investigation by elucidating IDO protein and gene expression in both normal and COPD cells with increasing the number of donors. Furthermore, to determine how different stages of COPD (non-severe, moderate, severe) could affect the regulation of IDO activity in steady state condition and in response to TLR ligands.

Mutation of CFTR has been shown to influence the innate immunity of the lung epithelium, resulting in exaggerated airway inflammation (123, 303). Moreover, Iannitti *et al.* showed that disrupted IDO activity and expression results in reduced TRP/KYN metabolism and may ultimately contribute to the inflammatory process in murine CF via an imbalance of Th17/Tregs. In this study, ECs with CFTR mutation were assessed for their IDO activity in steady state condition and in response to TLR ligands, allergen extracts, and IFN-γ. This comparison demonstrated comparable patterns of IDO activity and mRNA expression in both CF and control cells. A comparison of the basal IDO levels between CF and non-CF ECs revealed significantly higher baseline levels of IDO in non-CF ECs compared with CF ECs which raises the possibility of CFTR gene contribution in the regulation of IDO activity. This

observation is clearly in line with previous findings in which HBECs derived from CF patients exhibit lower levels of IDO activity and protein expression than control cells (303). These data suggest that CF may express defective IDO which results in a loss of immunosuppressive activity, which in turn leads to damaging inflammatory disorders and increases the severity of the disease. However, evaluating whether IDO is defective in ECs from CF subjects via western blotting and immunofluorescence staining could be considered for future studies. Generally, while comparisons of KYN (IDO activity) and cytokine production between cells of normal and affected clinical states were made using the same starting cell numbers for all conditions, normalisation of IDO activity using cells' DNA or protein contents will allow more meaningful comparison between different samples.

In conclusion, the findings reported here clarify the regulation of IDO as well as demonstrating differential IDO basal levels between healthy and disease states in response to IFN-γ, TLR ligands, and allergen extracts. In inflammatory responses, IFN-γ is the primary inducer of IDO to limit the exaggerated inflammatory reactions; however, IDO is also a substantial component of a regulatory system that provides self-tolerance effects in the longer term (297). We believe that understanding the role of IDO regulation in pathophysiological

conditions and lung immune homeostasis may allow for manipulation of the KYN pathway as a means of therapeutic intervention (i.e. IDO-memetic drugs) targeting pathogenic inflammation.

Chapter 6: General discussion

The human lung inhales 6-12 litres of air per minute, which constantly exposes the relatively large surface area of the human respiratory tract to numerous microbial pathogens and allergens that must be cleared without inducing inflammation (16, 29). Therefore, the integrity of the respiratory tract critically relies on a tightly regulated host defence system. ECs constitute a part of innate immunity in which they are the first line of defence positioned at the interface between the internal and external environment and can produce various immune-regulatory and antimicrobial factors to orchestrate immune responses (14, 16). Airway ECs can communicate with underlying stromal and immune cells and fulfil diverse functions that protect the airways from exogenous insults and maintain homeostasis (14, 15, 78, 94). IDO is the first enzyme in the KYN metabolic pathway, which can be expressed by professional APCs and by non-immune cells such as ECs, endothelial cells, fibroblasts. IDO produces a group of metabolites known as kynurenines that have well-established immune-regulatory functions, through TRP catabolism (121, 159). Additionally, the induction of IDO expression and activity in host cells inhibits the proliferation of intracellular pathogens (anti-microbial mechanism), suppresses T-cell proliferation and responses (135, 166, 175), hence playing a crucial

immunomodulatory role in health and disease (123, 160). The expression of IDO in response to pro-inflammatory stimuli, such as IFN-γ, protects against infection by reducing the bioavailability of TRP, which pathogens require (324).

Elucidating the regulatory mechanisms of IDO produced by ECs particularly in more physiologically representative *in vitro* models, could provide new insights into one of the first activated innate immunity effector pathways to protect against microbial infections. Thus, the main objective of this project was to evaluate the expression and regulation of IDO in human airway ECs in isolation and in 3D co-culture with fibroblasts and DCs. In particular, this study focused on the initial regulatory mechanisms by which various clinically relevant TLR ligands and allergens modulated IDO expression in airway ECs and in turn T cell activation. In addition, the effect of EC differentiation, cross-talk with neighbouring cells, and disease state on IDO activity and expression were investigated.

A number of different conditions and treatments were applied to cover most of the possible environmental conditions that can affect the study. Thus, in Chapter 2 of this work, IDO expression in human airway ECs was assessed by measuring the level of functional enzymatic activity (via colorimetric assay) as well as protein (via flow cytometry and Western

blotting) and gene levels (via conventional PCR). Although these techniques are powerful tools for immunological studies, other technical approaches including HPLC for measuring IDO activity, and quantitative real time PCR for evaluating mRNA expression would generate more informative data and should be considered for future experiments.

It is widely recognised that IDO is constitutively expressed in several tumour cell lines (325-327). Herein, we showed that compared to primary bronchial ECs, Calu-3 human airway ECs produce higher basal levels of KYN. However, treatment of either EC lines or primary epithelial cells with various TLR ligands, including LPS from *Escherichia coli* (TLR4), polyI:C; TLR3, FSL-1, a synthetic lipoprotein of *Mycoplasma salivarium* (TLR2/6), CpG-ODN2216 (TLR9), and allergen extracts, such as HDM, BGP, PEA, and GC extracts, induced the downregulation of IDO expression and activity. Conversely, IFN-γ induced the upregulation of IDO activity in both airway EC lines and primary cells, suggesting that IDO is involved in reducing deleterious inflammatory responses in the airways (i.e. immunosuppression) but at the same time participates in restricting intracellular pathogen growth.

Several studies previously reported that NO and accelerated proteasomal degradation inhibited IDO expression and activity in different cell types (172, 328, 329). The downregulation of

IDO in ECs observed in this study contrasts with its previously reported upregulation in DCs and macrophages in response to similar stimuli in a previous study (121, 182, 290, 330). Thus, it appears that IDO is regulated in a cell type-dependent manner.

Understanding how IDO expression is differentially regulated in airway epithelial cells in response to different TLRs and allergens might reveal how IDO influences other immunological events (e.g., T cell responses) which are the focus of Chapter 3. Since TRP depletion contributes to immunosuppression and pathogen clearance in diverse human and animal cell types, we hypothesized that IDO is expressed in airway ECs at high levels under steady-state conditions to suppress undesirable immune reactions and to maintain immune homeostasis in the airways. After encountering pathogens (i.e. TLR engagement) and/or allergens (i.e. TLR or C-type lectin engagement), IDO is downregulated in ECs, dampening this immunosuppressive mechanism, which in turn results in increased T cell activity and subsequent immune responses to combat the infection. These findings suggest an important role of airway ECs in modulating immune responses and T cell function.

There was growing evidence with regards to understanding the crucial biological role of IDO in linking innate and adaptive immunity to create local immunosuppression, and to provide systemic tolerance (121). Although, DCs are recognised as the primary APCs that contribute to the activation of naive T cells, inhibition of T cell responses and induction of immune tolerance (192, 193, 331), ECs also contribute to the modulation of T cell responses (95). However, the mechanisms by which ECs regulate immune responses, particularly T cell proliferation, remains elusive. Therefore, we also investigated whether ECs regulate T cell activation, the significance of IDO in this process, and the ability of TLRs to modulate this process.

Several studies have demonstrated that ECs can regulate T cell responses. Cruickshank *et al.* showed that primary murine colonic ECs can suppress CD4 T cell responses in a contact dependent manner (205). Alveolar (218) and intestinal ECs (332) can promote immune tolerance through the secretion of soluble factors that suppress T cell proliferation and induce Foxp3+ regulatory T cells. Murine lung ECs can suppress T cell activation and induce regulatory T cells which mediated by TGF-β (204). In the present study, we have shown that human airway ECs (Calu-3) can inhibit T cell activation (i.e. T cell proliferation and IFN-γ production) in an IDO dependent

manner. This suggests that IDO, expressed in airway ECs, contributes to the maintenance of a local immunosuppressive environment that limits T cell responses, and that dysregulation of this process could contribute to chronic inflammation. Virus-infected lung ECs express more costimulatory molecules of the B7 family, modulating CD8+ T cell responses (333), and lose their capacity to suppress T cells (204). These previous findings are consistent with our results demonstrating increased T cell activation when airway ECs were exposed to different TLR agonists, which were used as proxies for bacterial and viral infection. In fact, adding controls such as a single culture of PBMCs/T cells stimulated with LPS or poly I:C need to be done to confirm the possibility that the inhibitory effects on T cell activation could be dependent on ECs.

To determine whether KYN or 3-HAA is involved in EC-mediated suppression of T cell activation, we tested whether KYN or 3-HAA inhibited T cell proliferation. We found that high concentrations of 3-HAA but not KYN inhibited T cell proliferation by inducing cell death and apoptosis. However, low concentrations of 3-HAA did not suppress T cell proliferation. Therefore, the role of other IDO metabolites, including 3-hydroxykynurenine, anthranilic acid, and quinolinic acid, in this process cannot be ruled out. The effects of IDO

metabolites produced by ECs might not be limited to T cells, and might modulate the function of other immune cells (e.g., basophils, and mast cells), suggesting diverse immunomodulatory functions. Further studies will be required to explore these potential functions. Taken together, our results demonstrate the significance of airway ECs in suppressing T cell proliferation, which is promoted by IDO expression and suppressed by TLR engagement (Fig. 6.1). The physiological importance of these regulatory mechanisms warrants further research in primary epithelial cells instead of cell lines and by using BAL leukocytes instead of PBMCs in the co-culture with ECs.

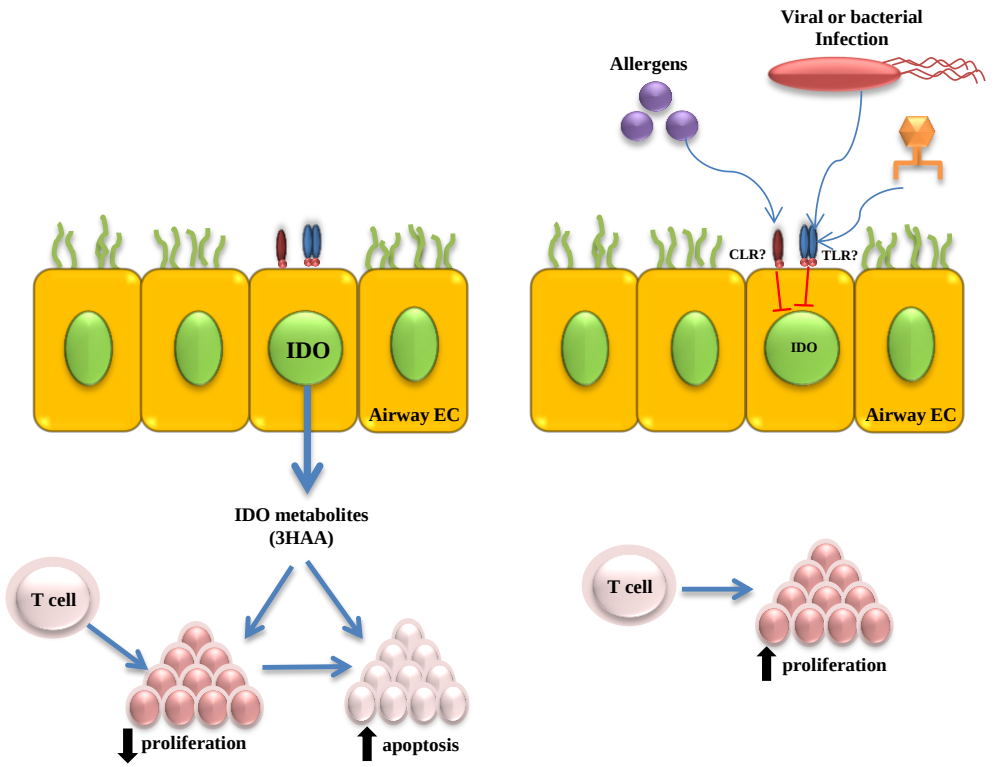


Figure 6.1: Pivotal role of IDO expressed in airway ECs in controlling local immune responses. Airway ECs express high levels of IDO, which is downregulated in response to TLR ligands and allergens. IDO and its metabolites, in particular 3-HAA, inhibit T cell activation (i.e. proliferation and IFN-γ production) by inducing apoptosis. This model suggests that impairment of immunomodulation by ECs might contribute to airway infection or allergic inflammation.

In Chapter 4, the physiological importance of IDO was assessed in differentiated ECs in 2D and 3D culture models and in co-culture conditions, which better simulate physiological conditions. Typical characteristics, including morphological features, TEER, barrier formation, and mucin production, of differentiated EC lines or primary cells at ALI were characterised. In addition, IDO activity was measured in

differentiated ECs and in ECs co-cultured with underlying stromal cells (i.e. fibroblasts) and DCs.

Among different human bronchial cell lines, Calu-3 cells are a promising *in vitro* model of airway epithelia, owing to their similarity (e.g. tight junction and cilia formation, and mucus production) to airways epithelial cells isolated from *in vivo* (120, 190, 245, 246). Our data showed that airway ECs had high TEER values (600-800 Ω/cm^2) and expressed the tight junctional protein, ZO-1. ECs co-cultured with fibroblasts in 2D TWI had higher TEER values than ECs cultured alone in 2D TWI, indicating faster differentiation of the former cell population. These results underscore the importance of paracrine interactions and cellular cross-talk in the maintenance of EC barrier functions.

Although TWI system bridge some aspects of simple *in vitro* models and complex animal models, it has some limitation including either small pore size of 2D membrane which limit cell-cell contacts or large pore size which limit the culture of epithelial monolayer. Moreover, two chamber design of the TWI would limit the culture of more than two cell types. Therefore, 3D models, consisting of different cell types, such as ECs (provide first line of defence), fibroblasts (secrete ECM proteins), and DCs (support adaptive immune responses), better represent the complex *in vivo* airway epithelium. 3D

models would also provide an experimentally tractable platform for many applications, including drug discovery and validation applications in the pharmaceutical industry. Therefore, physiologically relevant 3D scaffolds for cell culture were fabricated and characterised in this study. Non-biodegradable PET scaffolds were fabricated in-house. Characterization of PET scaffolds by scanning electron microscopy revealed a porous network structure similar to that of decellularised lung tissue (120), which would facilitate interactions between cells in 3D co-culture models. Calu-3 ECs attached and spread onto PET scaffold surfaces, and expressed ZO-1, indicating the formation of tight junctions. Calu-3 ECs also had higher TEER values when co-cultured with fibroblasts than when cultured alone, indicating that fibroblasts facilitate EC differentiation. Moreover, in response to LPS stimulation, DCs, which were cultured on electro-spun PET scaffolds and sandwiched between two other electro-spun PET scaffolds, migrated through the porous scaffold. These preliminary studies demonstrate the feasibility of using electro-spun PET scaffolds to develop 3D tri-culture models containing DCs, epithelial cells, and fibroblasts (Fig. 6.2). Although EC lines have been used as a substitute for primary cells in pharmaceutical applications, and despite the similarities between Calu-3 cell lines and human primary ECs, EC lines

might not be physiologically relevant. Therefore, it would be desirable to develop 3D co-culture models consisting of only primary cells warrants further investigation.

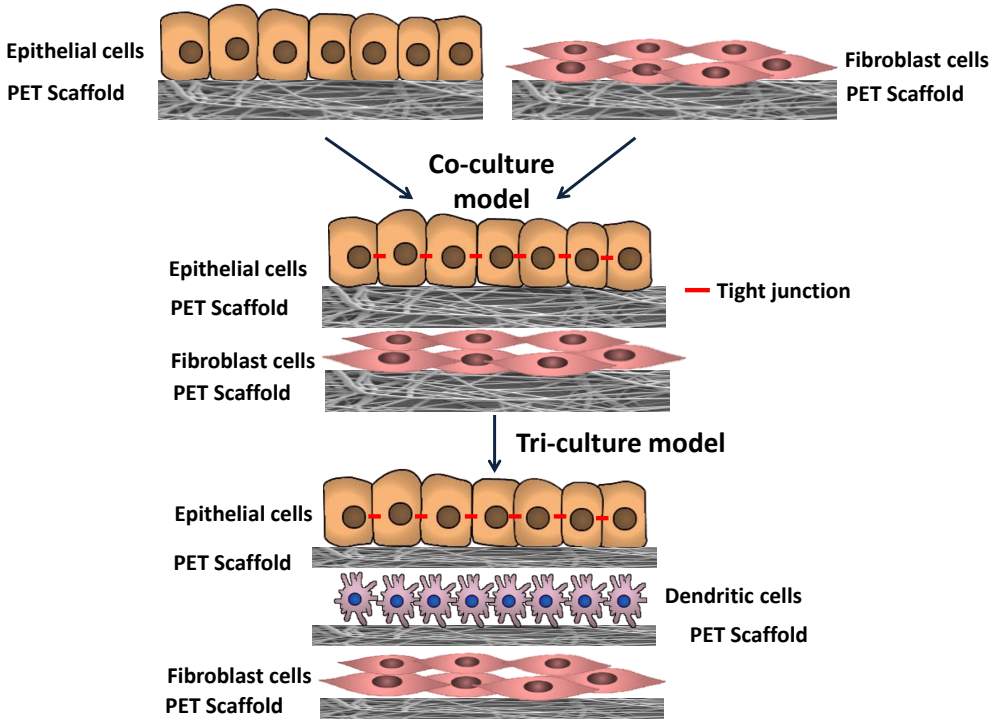


Figure 6.2: Schematic of the 3D model for airway epithelium. 3D tri-culture model including Calu-3 (ECs), MRC-5 (fibroblasts), and DCs (immune cells) were inoculated onto separate electro-spun PET scaffolds. ECs and fibroblasts co-cultured together at ALI for up to two weeks for inducing differentiation and forming tight junctions. Then, the third DCs layer inserted in the middle for tri-culture model. Adapted from (120).

After establishing conditions for EC differentiation, we assessed whether IDO activity was different in ECs, based on the differentiation state. IDO activity was significantly downregulated in differentiated ECs cultured on TWI after LPS treatment, and further inhibited when treated with papain-like cysteine protease. The additional inhibition of IDO activity by papain is likely due to EC barrier disruption, which makes PRRs more accessible. This is consistent with results from previous

studies showing that papain treatment disrupts tight junctional proteins and promotes TLR4 activation (120, 261).

It has been suggested that high doses of TLR ligands (TLR4, and TLR9 ligands) induce pulmonary IDO activity *in vivo* (156, 334). Defects in the induction of IDO activity reduce the ability to protect against experimental asthma (156). In this study, IDO activity was differentially regulated, depending on the EC type and culture conditions. IDO activity and expression were upregulated in ECs co-cultured with DCs. Moreover, IDO activity was not significantly different between LPS-stimulated and unstimulated cells under various culture conditions: in Calu-3 cells cultured alone on 3D substrates, in Calu-3 cells co-cultured with fibroblasts, and in differentiated primary ECs. These differences might be due to changes in the expression pattern of TLR4 that might occur in cells at ALI in 3D cultures compared to in cells in 2D cultures. Given that cells cultured on artificial membranes in 2D monolayers have properties different from those of cells cultured in 3D, including the physicochemical properties of the cell membrane, and that 3D co-cultures permit more physiologically relevant cellular interactions important for cell behaviour, stability, and gene expression, application of our 3D culture model should enhance our understanding of epithelial pathophysiology and may lead to the development of improved therapeutic

strategies (272, 273). However, future research with 3D culture models should focus on replacing EC lines with human primary ECs, which would represent the *in vivo* lung epithelium even better.

Considerable advances in understanding the roles of IDO in physiological and pathophysiological settings have been made. Understanding the role of IDO in specific infectious diseases could benefit the design of novel IDO-based therapeutics to maintain effective host antimicrobial immune responses (123). In the last part of this project, Chapter 5, IDO activities in ECs from clinical states were investigated. To study the effect of disease status on IDO activity in human ECs, we quantified IDO expression and activity in ECs from healthy and asthmatic individuals under steady-state and after stimulation with pathogen-associated ligands. We found that steady-state IDO expression levels were significantly higher in submerged and differentiated pHBECs of asthmatic individuals than in those of healthy individuals. This is consistent with the induction of pulmonary IDO during infection, which would protect the lung from Th-mediated lung inflammation (156). Moreover, our results are consistent with those of previous studies showing a correlation between high levels of IDO in allergic airway inflammation and its crucial immunosuppressive role in such inflammation (123, 155, 315, 316).

Our results revealed high levels of KYN in both healthy and COPD fibroblasts; however, IDO activity was significantly reduced in Calu-3 cells co-cultured with healthy or COPD fibroblasts compared to in Calu-3 cells cultured alone. The downregulation of IDO activity in Calu-3 cells co-cultured with fibroblasts confirms our previous hypothesis that fibroblasts can modulate IDO activity in ECs. However, there was no difference between healthy and COPD fibroblasts in their effect on IDO activity in ECs which warrants further investigation in regards to evaluating IDO protein and mRNA expression in response to different stimuli including pro-inflammatory cytokine (i.e. IFN-γ) and TLR ligands.

Previous studies have shown that CFTR mutations affect innate immunity in the lung epithelium and exacerbate airway inflammation (123, 303). Therefore, we investigated whether IDO activity is different in CF and non-CF ECs. IDO activity and protein expression were lower in HBECs derived from CF patients than in control HBECs derived from normal patients (303). Our results, which also showed lower levels of IDO activity in CF ECs compared to in non-CF ECs, is consistent with lower IDO expression levels in CF ECs reported in the previous study. The severity of CFTR diseases is potentially related to lower levels of IDO activity, which reduces

immunosuppressive activity and in turn exacerbates inflammatory responses.

In addition to expressing IDO, ECs secrete pro-inflammatory cytokines to recruit inflammatory cells to sites of infection, suggesting a major role for ECs in the immune response to infection (335). Pro-inflammatory cytokines have been proposed to play an important role in allergic asthma by inducing inflammatory cascades (317). Herein, we showed that pro-inflammatory cytokines (IL-6, and IL-8) were higher in differentiated ECs derived from asthmatic patients than in differentiated ECs derived from normal patients, which is consistent with the proposed role of pro-inflammatory cytokines in asthma (37, 318, 319). However, we did not observe GM-CSF production from differentiated ECs in either group. Given that most of the donors in this study had non-allergic asthma, the production of GM-CSF and other pro-inflammatory cytokines is likely associated with allergic asthma rather than non-allergic asthma (321, 322).

Collectively, this work advances the hypothesis that IDO expressed in ECs mediates immunosuppressive mechanisms, including inhibiting T cell activation, but this regulatory function is lost in response to pathogenic and allergenic factors. This work covered the evaluating the expression and regulation of IDO in ECs in health and in different clinical

states. In addition, we have established technical approaches that enable the generation of 3D models with greater physiological relevance and cellular complexity than 2D models.

Looking beyond the future work, there are many opportunities could arise for improvement. Investigate the exact mechanism of IDO down-regulation in ECs if it is via NO production or other mechanisms. Improve the physiological relevance by using BAL leukocytes instead of PBMCs in the co-culture with ECs for investigation of the immune-regulatory function of IDO produced by ECs. Furthermore, the focus should be on replacing EC lines with primary ECs to determine the physiological relevance of IDO-mediated immune-regulatory and antimicrobial responses and to develop 3D *in vitro* models for drug discovery applications. Future study also should aim at elucidating the regulatory elements of IDO gene promoters in primary ECs and cell lines. More work on patients' samples would confirm and clarify the possibility of the differences in the IDO activity and expression and may help to explain the role IDO produced by ECs in mentioned clinical states compared with healthy control. Finally, the ultimate goal would be to demonstrate the central role for airway ECs in maintaining the tolerogenic environment of healthy lungs and to construct a fully representative *in vitro* model of lung

epithelium which can pave the way to develop better therapeutic strategies.

Chapter 7: References

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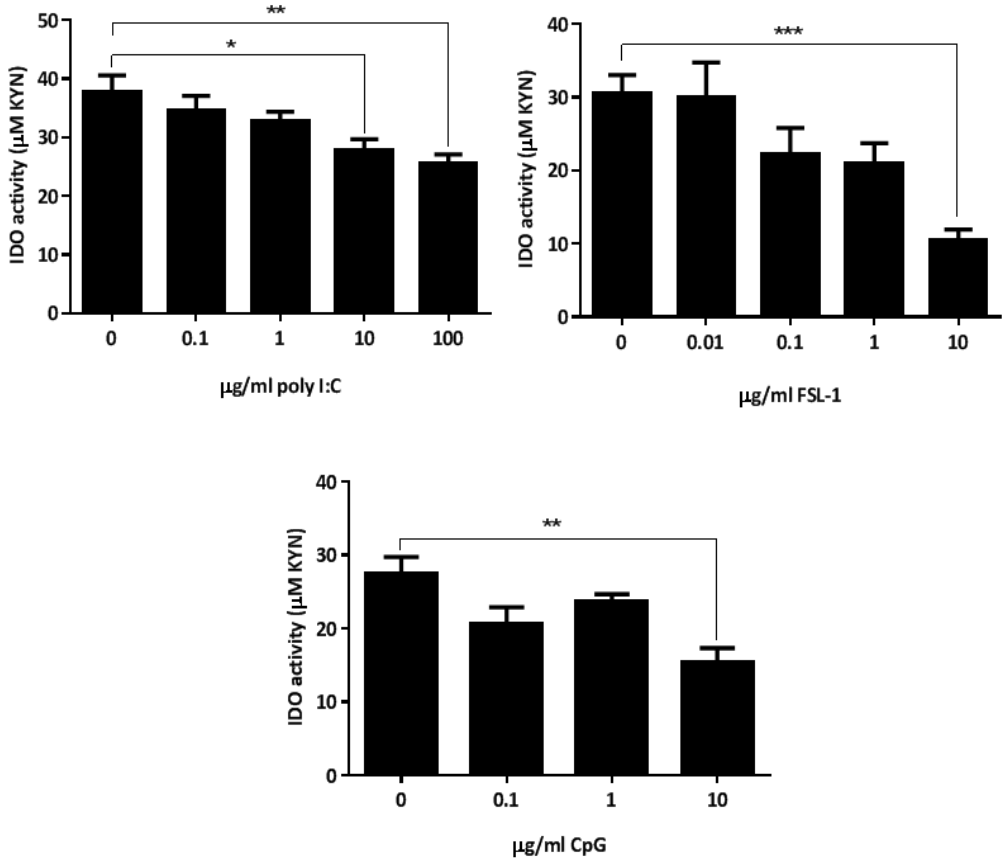
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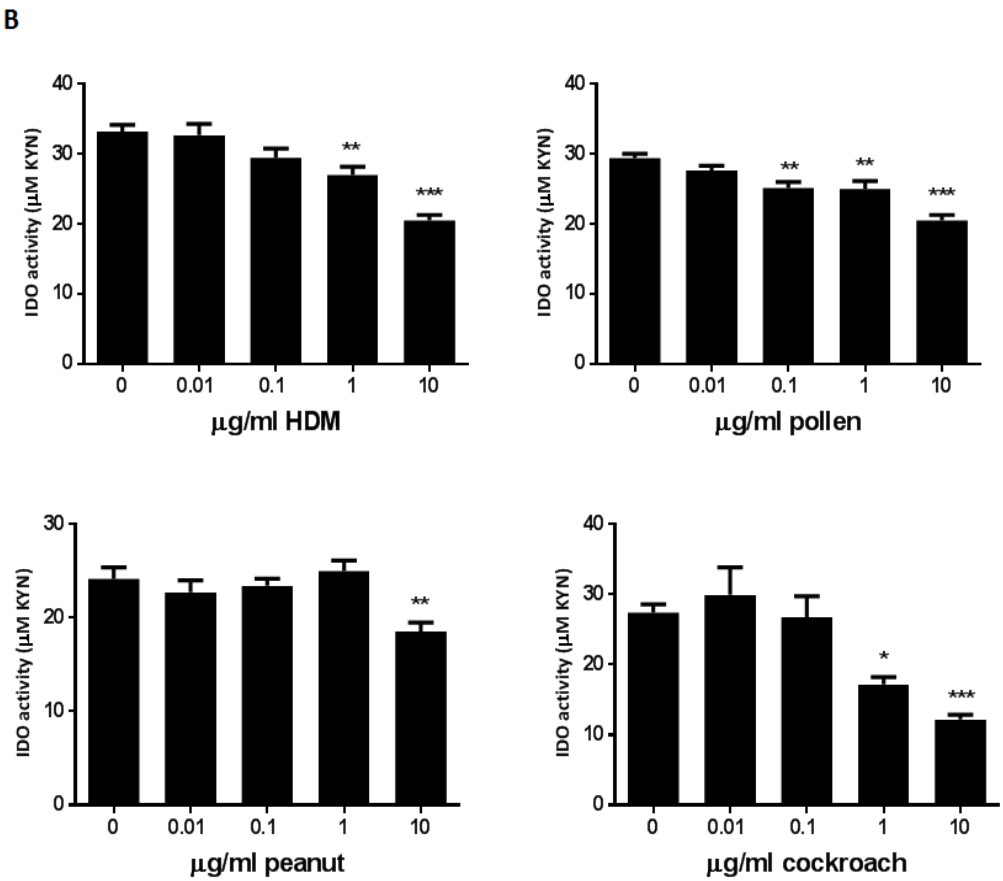
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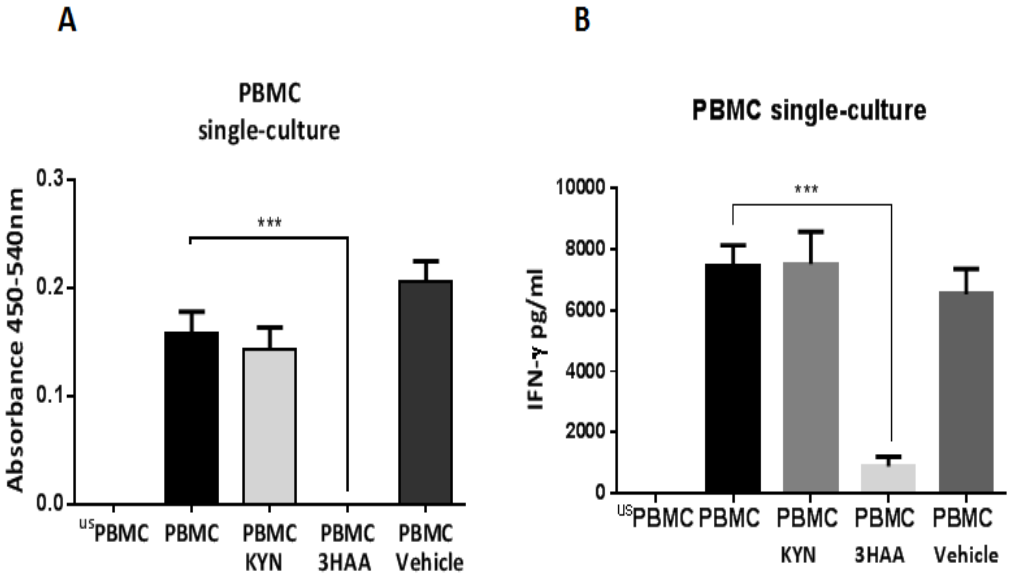
Appendices

A

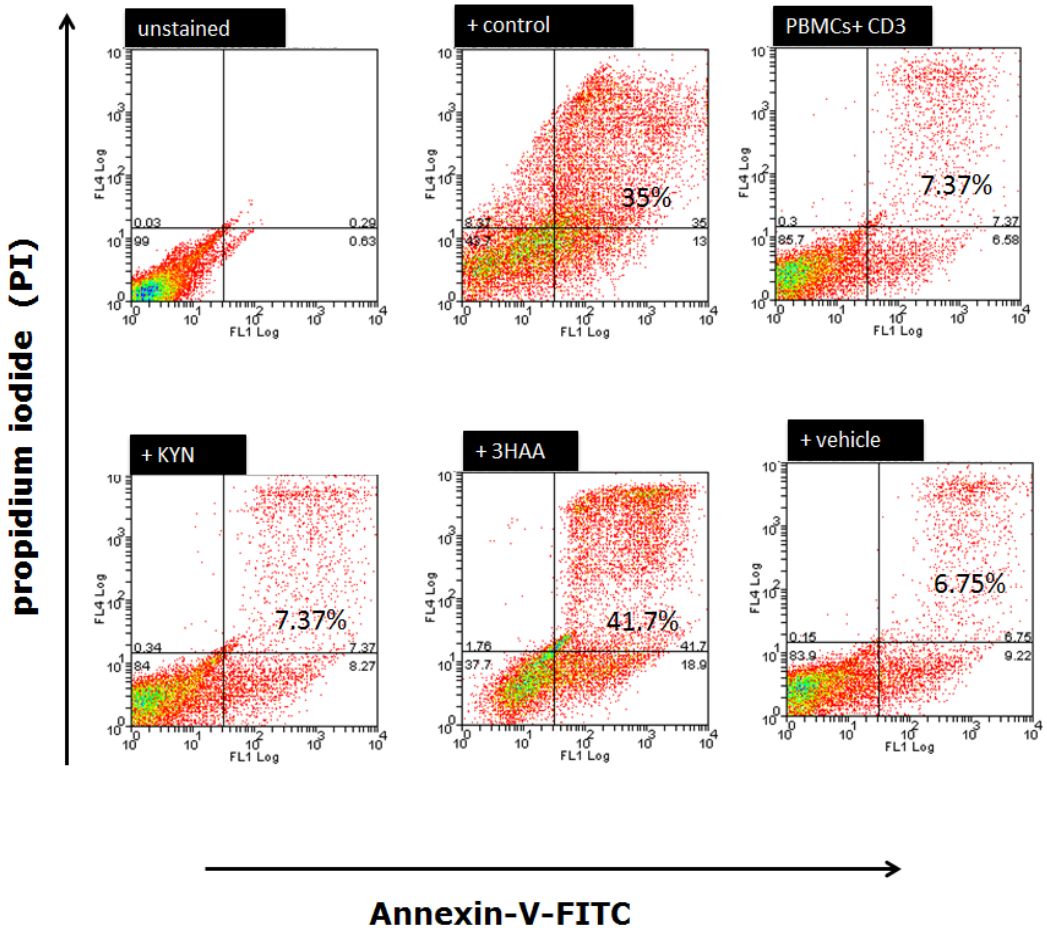




Appendix 1. Dose-dependent stimulation of human airway ECs with TLR ligands and allergen extracts. Calu-3 cells were incubated with (A) TLR3 ligand = poly I:C (n=4), TLR2/6 ligand = FSL-1 (n=3), TLR9 ligand = CpG (n=3) or (B) with allergen extracts (HDM, Pollen, Peanut, Cockroach; n=3) for 24 hours, after which the supernatants were collected for IDO activity measurements. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.


A
PBMC
single-culture
Absorbance 450-540nm
0.3
0.2
0.1
0.0
***
usPBMC
PBMC
PBMC KYN
PBMC 3HAA
PBMC Vehicle
B
PBMC single-culture
IFN-γ pg/ml
10000
8000
6000
4000
2000
0
***
usPBMC
PBMC
PBMC KYN
PBMC 3HAA
PBMC Vehicle

C

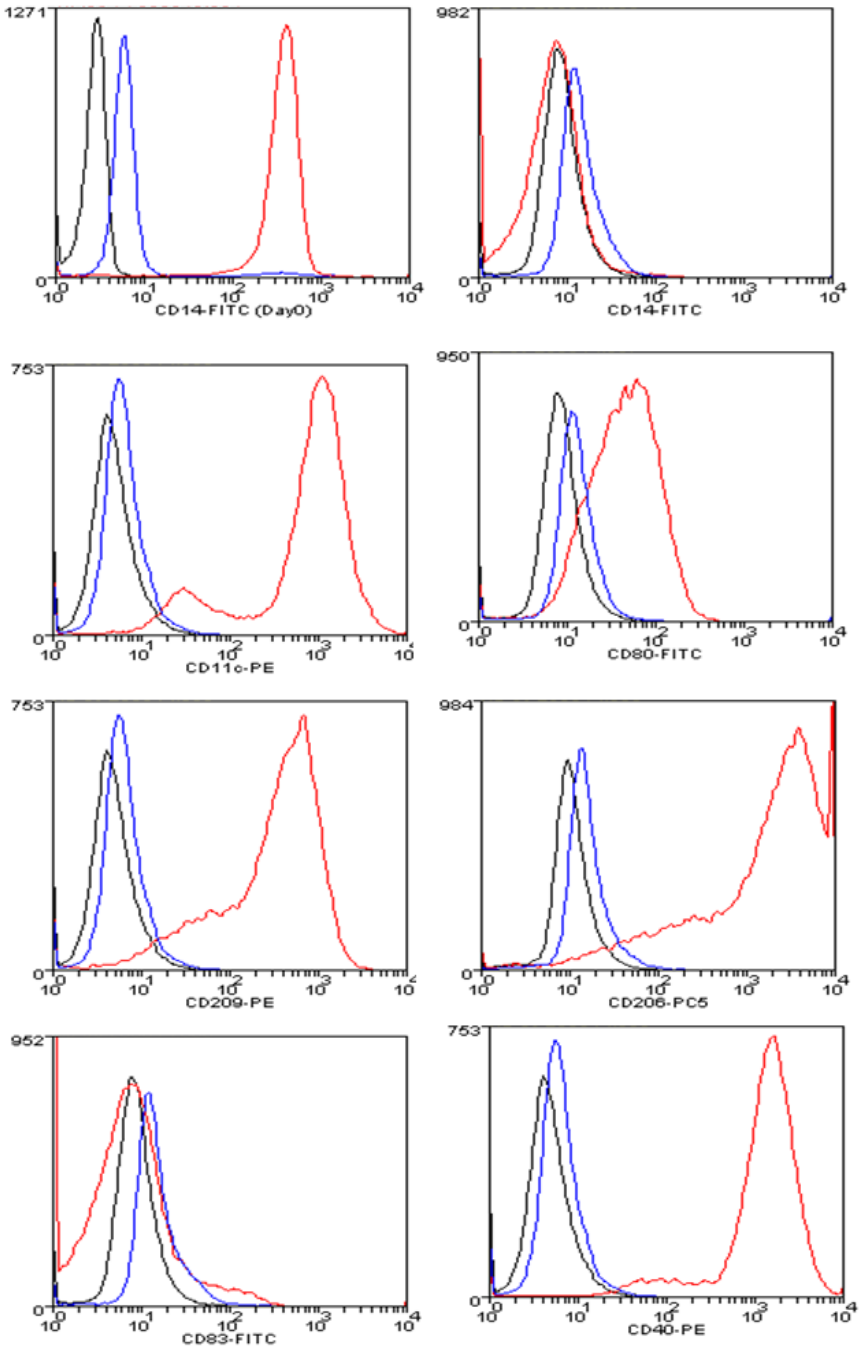


Appendix 2. IDO metabolite 3-HAA, but not KYN, affects PBMC proliferation by inducing apoptosis. Single culture of PBMCs were stimulated with 2 µg/ml of immobilised anti-CD3 antibody for 90 minutes in the presence of 200 µM of IDO metabolites L-KYN and 3-HAA or its vehicle (HCL). After three days of stimulation, (A) proliferation and (B) IFN-γ production were assessed with BrdU assay and ELISA respectively (n=3). (C) Dot plot representations of annexin-V-FITC and PI staining in the presence of 2 µM KYN and 3-HAA. Formaldehyde (3%) was used as a positive control (n=2). ^{us}PBMCs= cells without anti-CD3 antibody. ***$p \leq 0.001$.

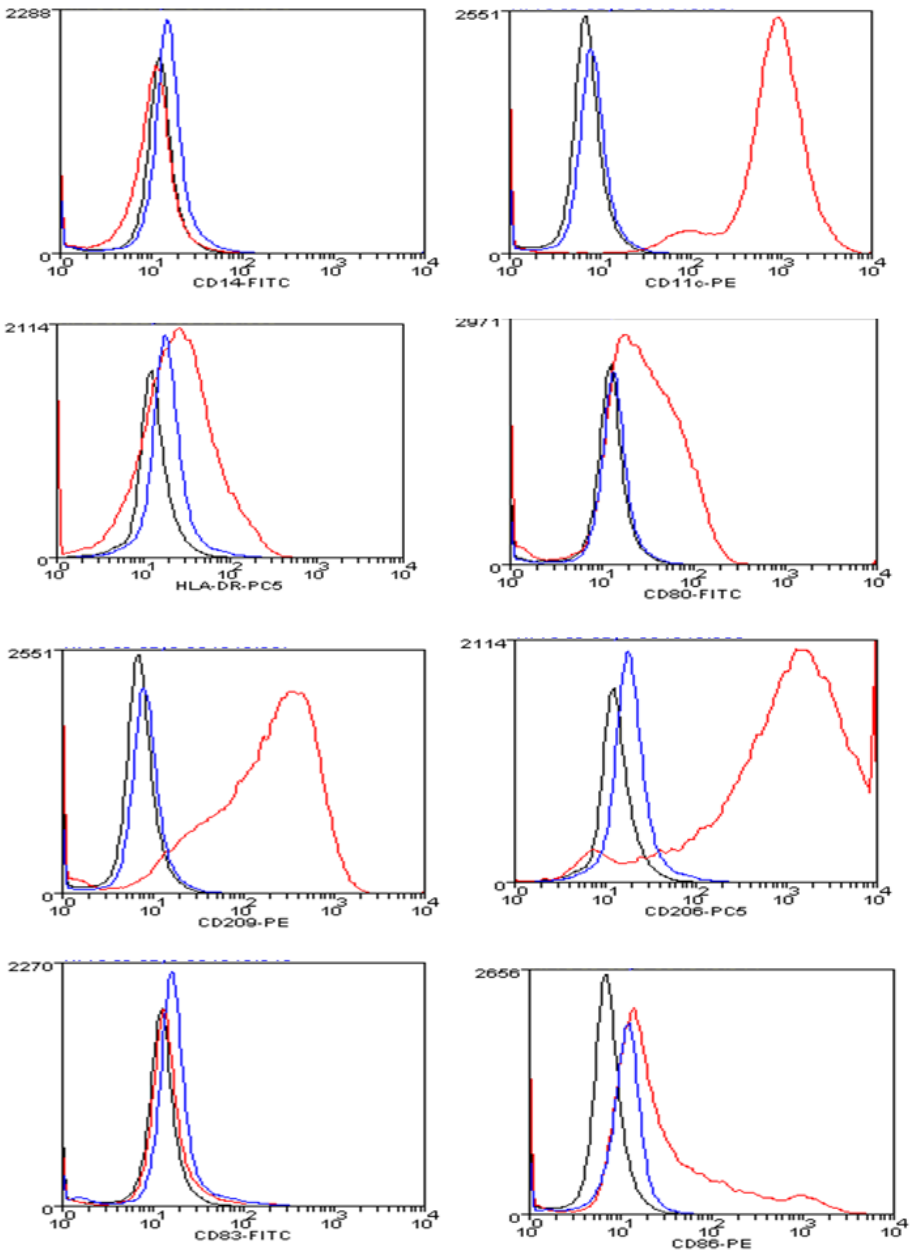
Donor	Ethnicity	smoking	Diagnosed with asthma	Age	Gender	Height cm	Weight kg	Medications	Comorbidities
MMP-1H06	White British	Never smoked	healthy	29	Female	131	56.4	no	non
MMP-1H07	White British	Never smoked	healthy	51	Male	168	90.5	no	non
AZAC02	White British	Never smoked	healthy	22	Male	180	76	no	non
MMP-1A06	South Korean-Asian	never Smoked	> 1Years	22	Male	184	82	no	non
AZAD03	White British	never Smoked	> 5 years	63	Male	177	77.9	no	non
AZAD08	White British	never Smoked	> 4Years	57	Female	168	102	prednisolone, salbutamol, seretide	sinusitis

Appendix 3. The nature and the clinical information of the healthy and asthmatic volunteers for primary HBECs.

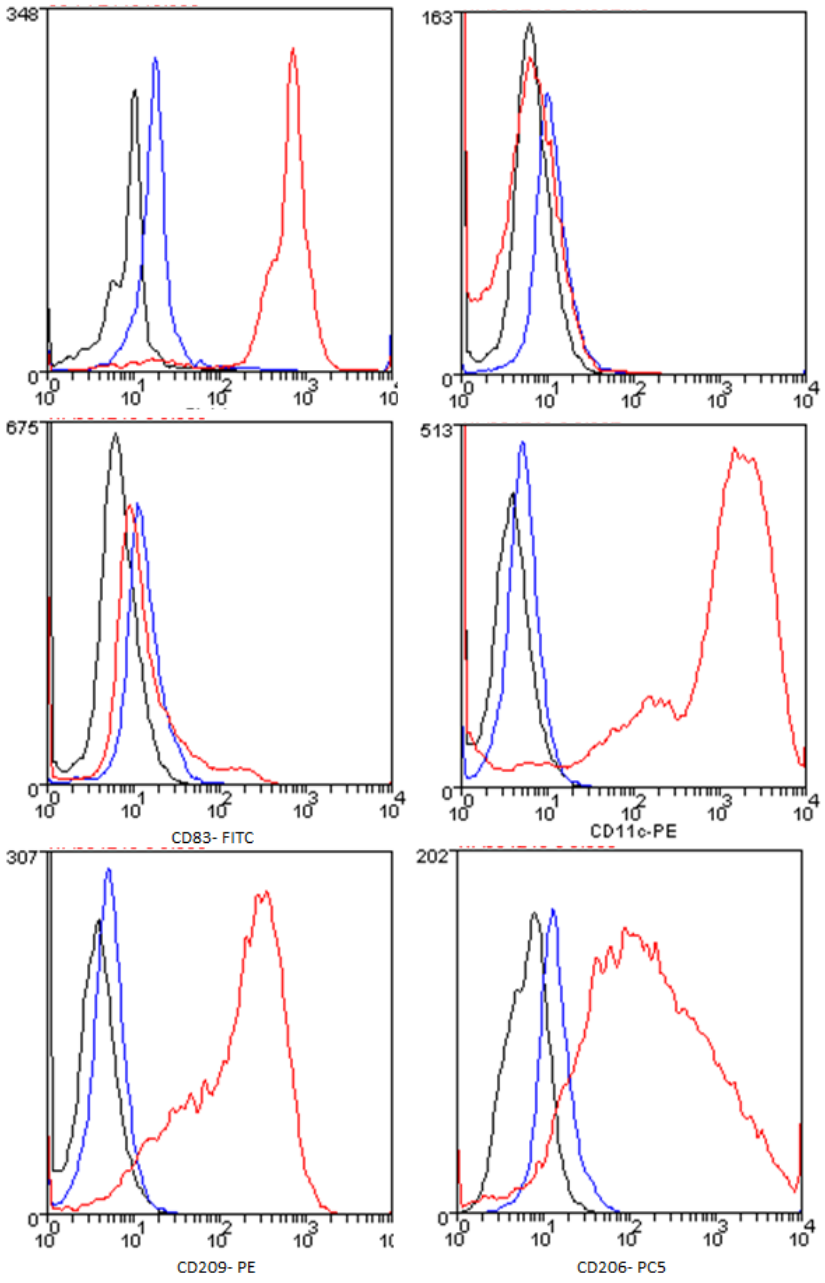
Experiment 1



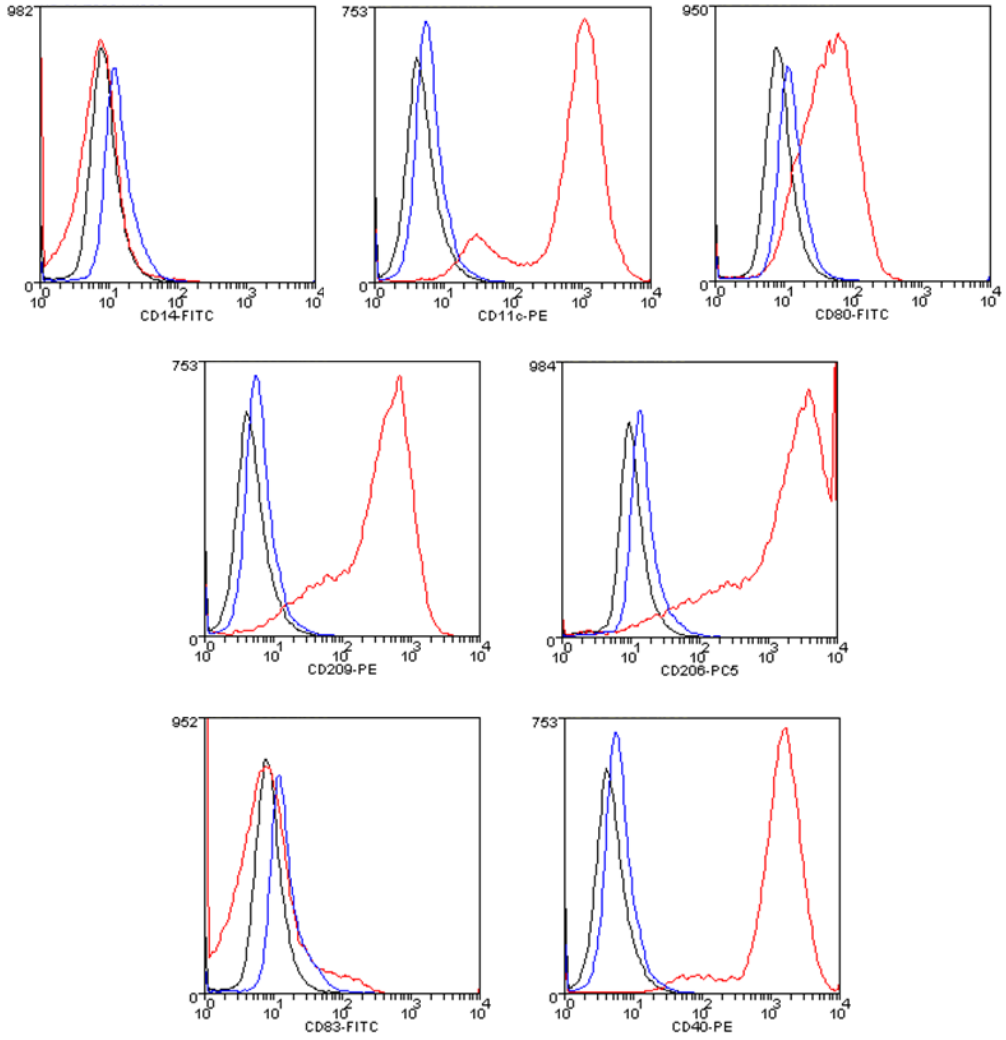
Experiment 2



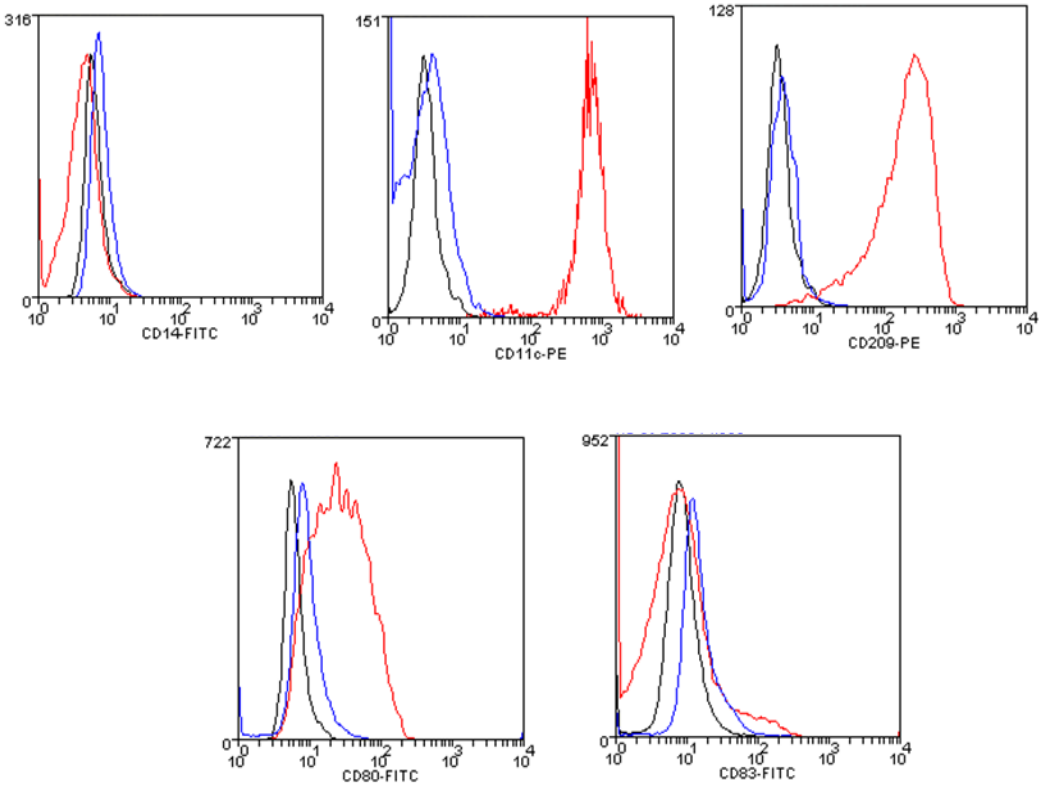
Experiment 3



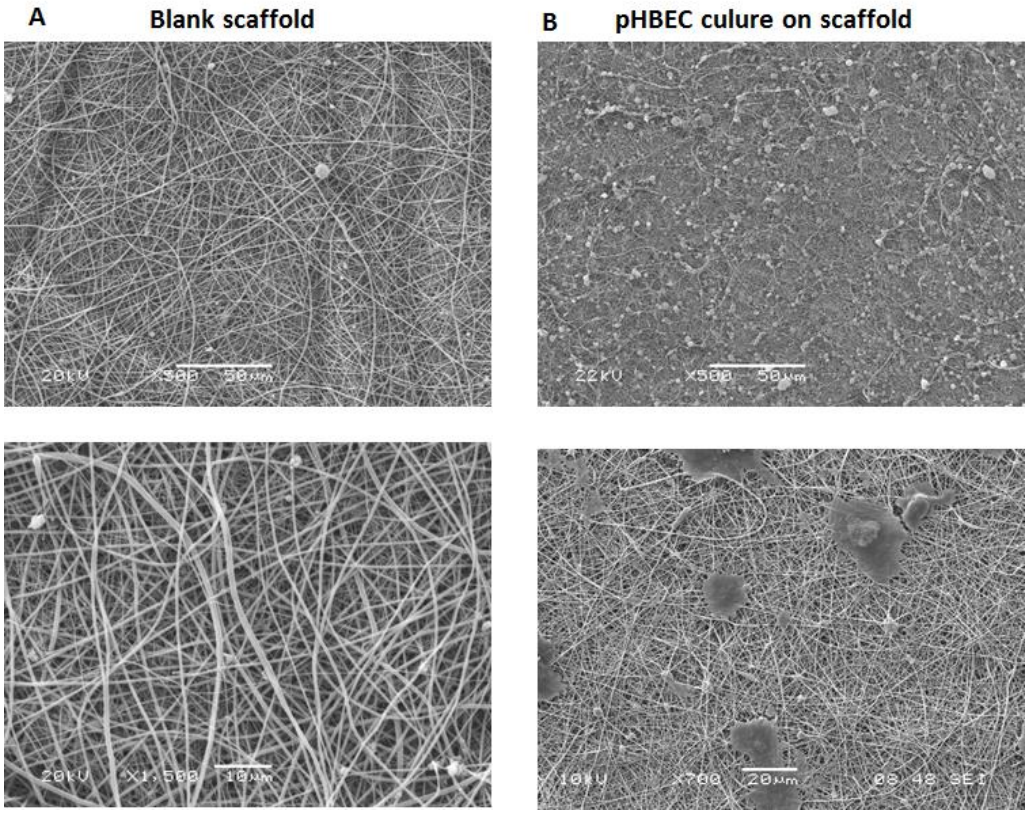
Experiment 4



Experiment 5



Appendix 4. Phenotypic analysis of human monocytes and Mo-DCs. Cells from five different experiments were collected and stained with monoclonal antibodies specific to the indicated cell surface molecules. Histograms depicting the number of cells exhibiting surface expression of different marker. Black lines represent unstained cells (autofluorescence), blue lines represent the isotype control, and red lines represent cells that stained positive for the indicated marker.



Appendix 5. SEM images of single culture of primary human bronchial ECs (pHBECs). (A) Blank PET electro-spun scaffold (without cells) with scale bars of 10 μm and 50 μm. (B) pHBECs were cultured on PET electro-spun scaffold for up to three weeks.

Human Lung Primary Cell Culture

Tissue were collected from the biobank in DMEM.

CM+ = DMEM with 10% FCS, Penicillin (100U/ml) /Streptomycin (100ug/ml), L-Glutamine (4mM) and Amphotericin B 2.5µg/ml.

Fibroblast Outgrowth:

CM+ was warmed to 37°C before use. Lung biopsies were cut into 1mm^3 fragments 10mm apart on surface of 10cm culture dishes label passage number 0 (P0) and allow to adhere for 5 minutes. Then 2ml CM+ as added with pastette and cells were incubated at 37°C for 24 hours. After that, a further 8ml of warm CM+ was added to the dish. Media was changed 1x per week. Once fibroblasts outgrowth is observed (6-8 days), cells were feeded 2x per week. When cells reach 40% confluent, re-plate is recommended to encourage growth.

Appendix 6. Human lung primary fibroblast outgrowth.