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Effect of Proinflammatory Cytokines on Lung and Intestinal Mucosal Permeability *in Vitro*

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Thesis submitted to the University of Nottingham for the degree of Master of Research

September 2012

Abstract

Transport of therapeutics across mucosal barriers provides an attractive route for non-invasive drug delivery, if sufficient drug permeability can be achieved. In inflammatory conditions of the epithelial mucosa, the barrier characteristics were observed to be modified with the permeability of noxious molecules increased significantly due to, at least in part, elevated levels of proinflammatory cytokines leading to tight junction disruption. This work aims to investigate the effect of selected cytokines on the barrier characteristics of airway and intestinal cell cultures *in vitro* in order to improve the understanding of transport features across inflamed epithelial cell layers. Data indicated that a three-four days short-term treatment with tumor necrosis factors-alpha (TNF-α) produced a significant effect on Calu-3 cell layers and some effect on Caco-2 cells, as shown by decreased transepithelial resistance (TEER values and increased permeability of model permeant (fluorescein isothiocyanate dextran with molecular weight of 10kDa, FD10). On the other hand, short-term treatments with proinflammatory cytokines interleukin-4 and interleukin-13 IL-4 and IL-13 did not show significant effects on the tested cell lines. Combined effect of cytokines was shown to cause a significant effect on Calu-3 apparent permeability coefficient (P_{app}) when the combination contains TNF-α, while the P_{app} across Caco-2 layers was observed to be influenced by IL-4/IL-13 combination; the effect being reduced when TNF-α was present. In the situation of long-term treatment (for the duration of cell culture), IL-4 and IL-13 did not produce a significant effect on TEER and P_{app} for both cell lines when incubated for 21 days. TNF-α however produced a significant effect on FD10 permeability across layers of both cell lines. Finally, the work examined the expression features of tight junction proteins (TJP2 and TJP3) and endocytosis pathway components (LAMP1, RAB4A, and RAB5A) in the cell layers following a prolonged exposure to the proinflammatory mediator TNF-α. Results

demonstrated that expression of the tested TJ proteins was downregulated, though endocytosis related proteins did not show alteration in their expression. These results therefore indicated that the presence of proinflammatory cytokines could be involved in the improvement in the transport of macromolecules through epithelial mucosa by affecting a TJ opening.

Acknowledgements

In the name of Allah, the Most Gracious and the Most Merciful. All praises to Allah for the strengths and His blessing in completing this thesis.

First of all, I would like to express my deepest gratefulness to my supervisor, Dr Snow Stolnik-Trenkic, for her supervision, outstanding guidance from the very early stage of this research and providing me with an excellent atmosphere for doing research. Above all and the most needed, she provided me unlimited encouragement and support.

I gratefully acknowledge to my co-supervisor, Dr Driton Vllasaliu, for his advice, supervision, crucial contribution and patiently corrected my writing. His invaluable help of constructive comments and suggestions throughout the experimental and thesis works have contributed to the success of this research.

I would like to express my appreciation Dr Franco Falcone for his support and knowledge regarding gene expression work. In addition, I wish to extend my appreciation to Dr Ian Sayers for his help in the analysis of microarray gene expression data. Not forget, great appreciation go to the rest of Snow's group members that help me from time to time during the lab work.

I wish to express my deepest appreciation to my government in the Kingdom of Saudi Arabia, especially King Abdulaziz City for Science and Technology (KACST) for their financial support.

My parents deserve special mention for their inseparable support and prayers. My Father, Ali, in the first place is the person who put the fundament my learning character and showing me

the joy of intellectual pursuit ever since I was a child. My Mother, Zinah, is the one who sincerely raised me with her caring and gently love.

Finally, words fail to express my appreciation to my wife, Amani. She was always there cheering me up and stood by me through the good times and bad.

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List of Abbreviations

%	Percentage
°	Degree
°C	Degree Celsius
Å	Angstrom
AB/AM	Antibiotic/Antimycotic
AHR	Airway Hyper Reactivity
AIC	Air-Interfaced Culture
ATCC	American Type Culture Collection
bp	Base Pair
BSA	Bovine Serum Albumin
cDNA	Complementary Deoxyribonucleic Acid
CAV1	Caveolin 1 protein
CDH1	E-cadherin
CLTC	Clathrin Heavy Chain
CLDN	Claudin
cm^2	Square Centimetre
CO2	Carbon dioxide
CUBN	Intrinsic Factor-cobalamin Receptor

Da	Dalton
DABCO	1,4-Diazabicyclo-octane
DNA	Deoxyribonucleic Acid
DMEM	Dulbeco’s Modified Eagles Medium
DMSO	Dimethylsulphoxide
ECACC	European Collection of Cell Cultures
EDTA	Ethylene Diamine Tetraacetic Acid
EEA1	Early Endosome Antigen 1
EMEM	Essential Minimum Eagle’s Medium
EVOM	Epithelial Voltohmmeter
FBS	Foetal Bovine Serum
FCGRT	Fc Fragment of IgG Receptor Transporter Alpha
FD10	Fluorescein Isothiocyanate-dextran 10 kilodalton
FDA	Food and Drug Administration
FITC	Fluorescein Isothiocyanate
FPR1	Formyl Peptide Receptor 1
FOLR1	Folate Receptor 1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GEO	Gene Expression Omnibus

GI	Gastro Intestinal
HBSS	Hank's Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IBD	Irritable Bowel Disease
IL-4	Interleukin-4
IL-4Rα	Interleukin-4 Receptor Alpha
IL-13	Interleukin-13
IL-13Rα	Interleukin-13 Receptor Alpha
JAM	Junctional Adhesion Molecules
kDa	Kilodalton
LCC	Liquid-covered Culture
M6PR	Mannose-6-phosphate Receptor
M	Molar
mM	Millimoles
ml	Millilitre
MLCK	Myosin Light-chain Kinase
mRNA	Messenger **Ribonucleic acid**
MW	Molecular weight
NCBI	National Centre for Biotechnology Information
NF-kB	Nuclear Factor Kappa B

nm	Nanometer
LAMP1	Lysosomal-associated Membrane Protein 1
OCLN	Occludin
P_{app}	Apparent permeability coefficient
PCR	Polymerase Chain Reaction
PBS	Phosphate Buffered Saline
RAB	RAS Oncogene Family
SD	Standard deviation
TBE	Tris-Borate-EDTA
TEER	Transepithelial Electrical Resistance
Th-cell	T Helper Cell
TJ	Tight Junction
TJP	Tight Junction Protein
TNF-α	Tumor Necrosis Factor-alpha
v/v	Volume per unit Volume
w/v	Weight per unit Volume
ZO-1	Zonula occludens-1
µl	Microlitre
µm	Micrometer
Ω	Ohm (electrical resistance unit)

Chapter 1

Introduction

1.1.1 Mucosal Drug Delivery

In the field of drug delivery, mucosal delivery is considered a very attractive option for the administration of therapeutic drugs [1]. Currently, considerable effort in pharmaceutical research is focusing on the epithelial drug transport mechanisms involved in mucosal drug delivery [1]. One of the main advantages of mucosal delivery is patients` compliance when using non-invasive methods [2]. Furthermore, mucosally-delivered therapeutics have the capacity to escape the hepatic first-pass mechanism [2]. The mucosal epithelia considered for drug delivery includes several routes: oral, buccal, pulmonary, vaginal, nasal and ocular [1].

1.1.2 Transport Pathways across Epithelial Mucosa

Macromolecules and nanoparticles have been shown to be transported across epithelial mucosa using different mechanisms [3]. The paracellular pathway is one of the major potential mechanisms of transmucosal transport, particularly for hydrophilic and relatively smaller macromolecules [4]. In this route, hydrophilic macromolecules and, according to some authors, nanoparticles pass through restrictive complexes between adjacent cells called tight junctions [4][5]. Hydrophobic and bigger macromolecules, and nanoparticles can be also able to cross the mucosal cell layer through a transcellular pathway [6]. Transcellular transport may occur by transcytosis (carrier-mediated mechanism) [4][7]. In this pathway, the material binds to specific receptors and is captured in vesicles that move the macromolecules from the apical to the basolateral side [7][8]. Figure 1.1 shows a summary of the potential transport pathways.

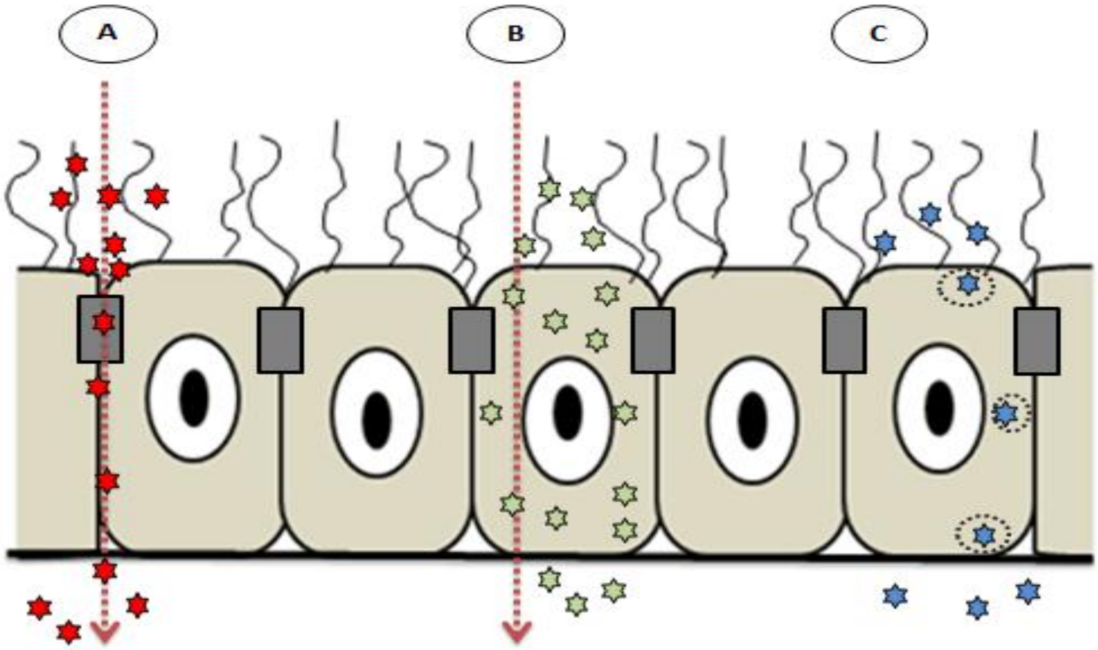

Figure 1.1 Transport mechanisms across epithelial mucosa.
A) Paracellular pathway (red). B) Transcellular pathway (green). C) Transcytosis pathway (carrier-mediated) (blue).

1.1.2 Mucosal Barriers

Understanding the physiology of mucosal tissue is important in the field of drug delivery, because it may lead to improvements in the absorption properties of new therapeutic agents. To achieve delivery, different barriers that mucosa present need to overcome successfully. In the following sections, some of these barriers will be described in more detail.

1.1.2.1 Tight Junctions Barrier

Typically cells in mucosal tissue are connected apically by a strong paracellular complex of proteins, known as tight junctions (TJ). This makes the mucosal barrier very restrictive against the free diffusion of solutes [9][10]. Moreover, the transport selectivity of water, substances and ions could be controlled by the tight junction complex [9]. Several protein components have been reported to play a key role in tight junction function and structure [11], as shown in figure 1.2. Tight junction proteins (zonula occludens - ZO-1, ZO-2, ZO-3), trans membrane proteins (claudins, occludin) and the junctional adhesion molecule (JAM) have been documented to contribute to the TJ structure [9].

It has been suggested that the molecules with molecular weights of more than 3.5 kDa would not be able to cross the paracellular route in healthy barriers because the space width of a TJ is about 15 Å [12][13]. When the tight junction function has been disrupted, the free diffusion of noxious substances would be increased, and several diseases could be significantly stimulated [10]. It has been shown in a number of inflammation studies that intestinal and respiratory inflammatory conditions are essentially associated with defectiveness in tight junctions [14][15][16]. This observation will be described later in this chapter.

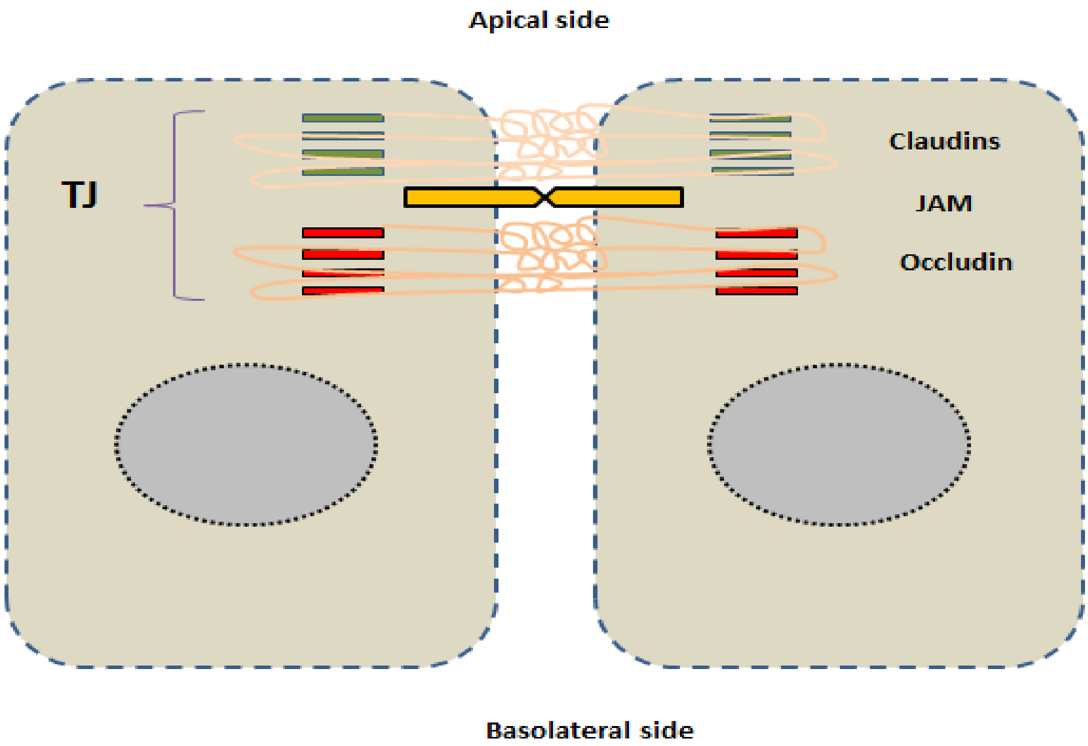

Figure 1.2 The tight junction complex composed of several proteins such as claudins, occludin and junctional adhesion molecule (JAM), which 'seal' the intercellular space tightly.

1.1.2.2 Mucus Barrier

The mucus barrier that covers most epithelial membranes plays an important physiological function *in vivo* [17][18]. It is secreted by mucosal gland or goblet cells [7]. Intestinal and respiratory tissues, for example, are known to secrete mucus to protect their mucosa from the free diffusion of harmful agents from the external environment [19][12]. Moreover, other functions have been observed with mucus in the human body, such as lubrication [20], anti-microbial effects, and water balance [21]. Drug absorption was also documented to be affected by the mucus layers [21].

Mucus constituents are usually dependent on the tissue type, but it is generally composed of water, glycoproteins and lipids, in addition to other components present in low amounts, such as minerals [21]. One of the most common components of mucus is mucin. This macromolecular compound forms a very viscous layer on epithelium, which has a profound effect on the transport of molecules [21][22]. The transport of molecules across the mucus layer depends on several factors, such as the molecular size and affinity for mucus components [21]. It must be noted that a good understanding of mucus production in normal and diseased tissues would improve drug delivery efficiency across mucosa [21]. The alteration in mucus properties has been reported in different disease conditions, including mucus over-secretion or changes in mucus viscosity or thickness [23][24].

1.1.3 The Role of Epithelial Membrane Proteins on the Regulation of Transport Processes

A number of investigations have demonstrated that several membrane proteins play an essential function in the regulation of transport processes across mucosal membranes in humans. TJP2 and TJP3 proteins were reported to be significantly involved in the permeability of substances across intercellular route [25][26][27]. Furthermore, several other proteins were suggested to contribute in the regulation of transport processes involved in the transcytosis pathway. These include, for example, RAB4A, RAB5A and LAMP1 [28][29][30].

TJP2 (ZO-2) and TJP3 (ZO-3) are major components of the tight junction complex regulation and formation which controls the transport across paracellular route of epithelial monolayers [31][5][32]. In healthy epithelial tissue, TJP2 and TJP3 contribute to the prevention of the transport of noxious elements across epithelium into the systemic circulation, while in inflamed tissue the tight junctions were reported to be damaged in inflamed tissue and characterised by an increased permeability of substances using the paracellular route [33].

RAB4A has been documented to regulate the endocytosis pathway of transport and cell-cell adhesions of epithelial cells [29][34]. Moreover, RAB5A and LAMP1 contribute in early and late endosomal process, respectively [30][35]. A study reported that LAMP1 plays multiple functions in transport across cells *via* endocytosis [36]. RAB4A and RAB5A are important members of the RAB family and have a role in the regulation of early and recycling endosomes processes, respectively, and also the endocytosis pathway of transport in a wide range of cells [34][37][38][39].

It might be worth examining the expression features of these proteins in normal and inflammatory conditions in order to establish an understanding of their involvement in transport process regulation. Also, a study on the expression of these proteins will determine whether the inflammation reaction influences the transport across the mucosal tissue by remodelling the tight junction complex (the paracellular route) or by affecting the transcytosis mechanism.

1.2 Effect of Cytokines on Mucosal Barriers Characteristics in Airway and Intestinal Inflammatory Diseases

1.2.1 Proinflammatory Cytokines

Cytokines are relatively small endogenous proteins with low molecular weights between 8 and 40 kDa [40], which are mainly released by immune cells [41]. In terms of the site of production, cytokines can be categorised into two groups: (i) T-helper 1 cytokines, such as TNF-α, and (ii) T-helper 2 cytokines, such as IL-4 and IL-13 [42]. Cytokines play an essential physiological function in the human body by providing a connection between the immune system and the (inflamed) tissues [43] in order to stimulate a host defence mechanism [40][44]. One study, for instance, demonstrated that the human immune system can be induced by cytokines during inflammation reactions [42].

Nowadays, numerous laboratories use cytokines to examine the immune reactions of human mucosa *in vitro* [42]. Moreover, proinflammatory cytokines have been shown to have a significant effect on the structure and function of epithelial cell layers of *in vitro* models [18]. An experimental *in vitro* study on epithelial models has, for example, demonstrated that proinflammatory cytokines play a key role in tight junction dysfunction [18]. Recent research stated that a wide array of proinflammatory cytokines play a role in tight junction remodelling through the disruption of occludin and claudin regulation [45][46][10]. The regulation of tight junction proteins by proinflammatory cytokines is believed to be one of the major mechanisms behind the development of inflammatory disorders [45]. At present, several cytokine antagonist agents are under clinical investigation due to the possible benefit of using these agents when treating inflammatory disorders of airways and the intestines [18][47]. In the experimental chapters in this thesis, three proinflammatory cytokines, IL-4, IL-13, and TNF-α, were used to investigate their effect on the barrier characteristics of airway and intestinal epithelia.

1.2.1.1 Interleukin 4 and 13 (IL-4 and IL-13)

The proinflammatory cytokines that were selected for use in this project are interleukin 4 and 13. Interleukins are a group of cytokines that include several cytokines, such as IL-4 and IL-13, which play a significant function in the regulation of the epithelial paracellular pathway [45]. Both IL-4 and IL-13 are released endogenously from T-helper 2 cells [18]. An inflamed epithelial membrane can be considered as one of the main sources of these interleukins in such inflammation reactions [18][16]. It has been reported that both IL-4 and IL-13 play an essential role in increasing the permeability of mucosa during inflammatory disorders [48]. With regard to the effect of combined cytokines, synergetic influence has been reported with IL-4 and IL-13 when combined with TNF-α [49].

Figure 1.3 illustrates that IL-4 and IL-13 share the receptors; therefore, numerous investigations have stated that these interleukins furthermore share a similar physiological function [48]. Both IL-4 and IL-13 have the ability to bind the IL-4Rα/IL-13Rα1 complex, which then activates the inflammation response in several diseases [50].

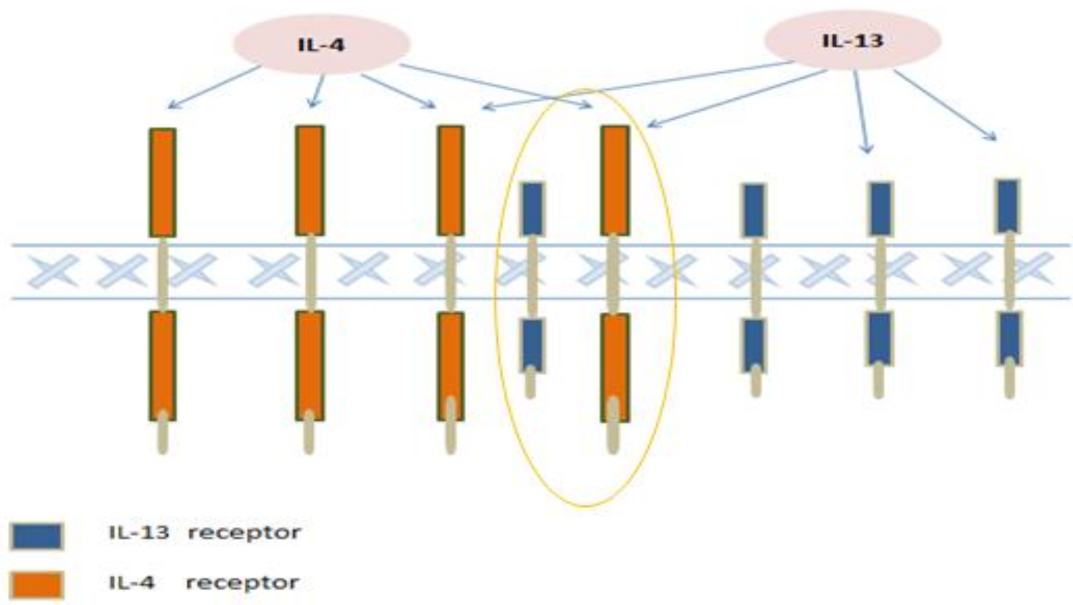

Figure 1.3 Schematic representation of IL-4 and IL-13 receptors to show that these interleukins share the same receptors (IL-4Rα/IL-13Rα1) [50].

IL-4 and IL-13 have been shown to play important functions in the development of asthma [51] and some intestinal disorders [52]. Several studies have documented that the treatment of respiratory and intestinal epithelium with IL-4 could increase the permeability of the cell layer [45]. IL-4 appears to induce paracellular permeability via the downregulation of tight junction proteins, particularly ZO-1 and occludin [45]. A similar effect has been observed with IL-13, showing an increase in the permeability of the paracellular route and down regulation of tight junction proteins [45]. IL-4 and IL-13 play a significant role in the progression of intestinal [52] and pulmonary inflammation reactions [53] by remodelling the tight junction complex structure and function [52]. Interestingly, both IL-4 and IL-13 have dual inflammatory properties by showing anti-inflammatory effects in some cases and proinflammatory actions in other conditions [40].

1.2.1.2 Tumour Necrosis Factor-alpha (TNF-α)

The further important proinflammatory cytokine that the project will focus on is TNF-α. TNF-α is a low molecular weight cytokine that is released by Th-1 cells [54]. Several cytokines that are released in inflammation reactions have been shown to be induced initially by TNF-α [55]. TNF-α has been reported to be involved in some intestinal and respiratory inflammatory disorders by causing the dysfunction of tight junctions [45]. The mechanism of TNF-α influence on tight junctions might be via inducing nuclear factor kappa B (NF-kB), which activates the expression of myosin light chain kinase protein (MLCK). This protein, in turn, plays a role in the down regulation of ZO-1 protein [45][56], as shown in figure 1.4. Moreover, treatment of the epithelial cell layers with TNF-α might provide alterations in the function and structure of tight junctions function and structure, and therefore change the permeability properties of the membrane [45]. Most of the effect of TNF-α effect has been seen when combined with other cytokines [57].

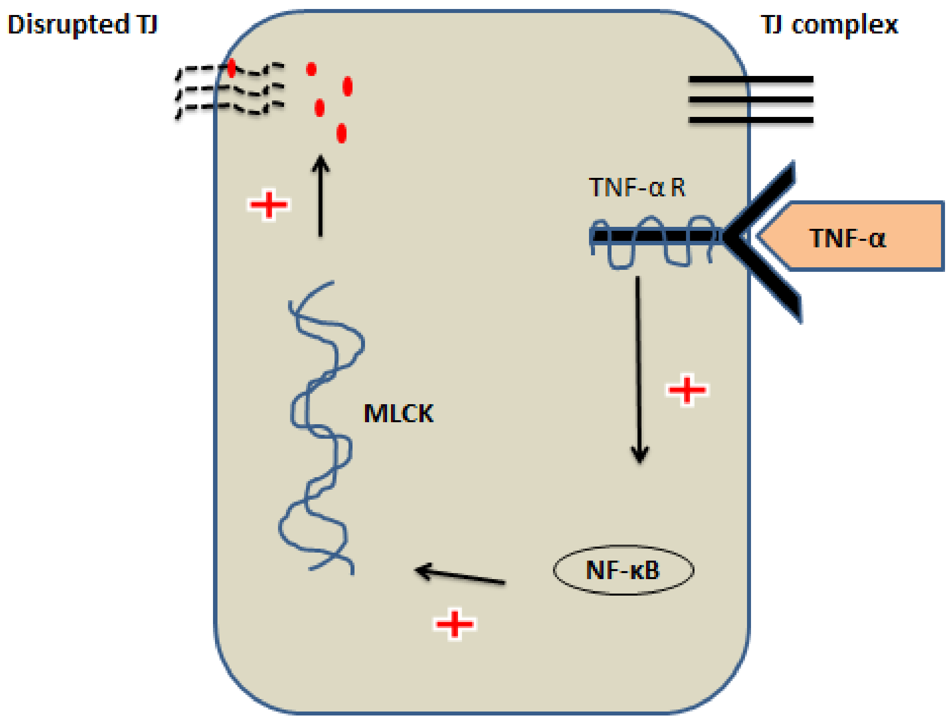

Figure 1.4 Schematic representation of the mechanism of TNF-α effect on tight junction complex during inflammation reaction.

A biopsy from an asthmatic individual demonstrated an increased TNF-α level and suggested that the use of anti-TNF-α could decrease the severity of asthma [15]. The level of TNF-α mRNA was shown to be expressed in asthma [32][15], leading to increased mucus generation, AHR and other features of asthma [33].

A number of strategies have been recommended to control asthma, such as antagonising the effect of multifunctional cytokines, for instance blocking the TNF-α receptor [10][19][58][23]. A recent investigation documented that the treatment of asthmatic patients with anti-TNF-α improved the acute and chronic symptoms significantly [20]. In addition, one of the possible therapeutic approaches that could help in asthma management is to protect the tight junction complex from the disruptive effects of cytokines [10].

1.2.2 Proinflammatory Cytokines and Inflamed Mucosa

A number of research publications have reported that the main component of the paracellular pathway, the tight junction complex, can be controlled by cytokines [54]. In several intestinal and respiratory inflammatory disorders, cytokines have been shown to play an important function in remodelling the tight junction complex, and therefore increasing the permeability of noxious substances through the paracellular space [45]. Alterations in the structure and function of tight junctions structure and function could increase the risk of such inflammatory diseases [54]. It should be noted that inflammation reactions could be induced by the downregulation of anti-inflammatory cytokines or by an increase in the release of pro-inflammatory cytokines [48].

Proinflammatory cytokines could increase the permeability of harmful agents through intercellular pathways by disruption of the tight junction complex, and this might be one of the contributing factors to several inflammatory disorders, such as asthma and inflammatory bowel disease (IBD) [45][59]. The inflamed airway and intestinal cells in asthma and IBD, respectively, have been observed to be damaged due to the significant effect of proinflammatory cytokines [60][61][15]. Recently, increased paracellular permeability and decreased TEER have been shown for treatment of *in vitro* models of epithelium with such cytokines, which suggested the significant effect of pro-inflammatory agents on epithelial layer integrity [48]. Cytokines promote dysfunction of the tight junction barrier by disregulating the ZO proteins [54]. Two of the most common respiratory and intestinal diseases that are significantly stimulated by pro-inflammatory cytokines are asthma and IBD, which will be discussed in the following sections.

1.2.2.1 The Contribution of Pro-inflammatory Cytokines to Inflamed Airway Mucosa

Asthma is a broad term that covers chronic inflammation reactions in the airway epithelium, characterised by various signs such as Airway Hyper Reactivity (AHR), increased mucus secretion and reversible bronchoconstriction [34]. By improving our understanding of inflammatory diseases of the lung, such as asthma, recent efforts have shifted the focus towards studying the biology and physiology of the epithelium [62]. Remodelling of the respiratory epithelium is one of the crucial characteristic features of asthma [63]. Alterations in the structure of the epithelium in such inflammatory processes may, in turn, cause increased mucus secretion by relevant components of the mucosa (goblet cells), dysfunction of epithelial tight junctions and fibrosis [63].

Recent evidence points to an impaired barrier functionality of the epithelium in asthmatic patients, which results in increased permeability to exogenous substances and stimulation of inflammation processes in the lung [62]. Obtaining a full understanding of the pathological mechanisms involved in the regulation of the epithelial barrier is an essential prerequisite for the development of effective therapeutic agents to treat asthma [62].

A significant development in the pathophysiology of asthma in recent years has been the understanding of the involvement of cytokines in the disease [64][57]. Inflammatory and immunological cells have been documented to be the main sites of pro-inflammatory cytokine production in airway inflammation reactions [47]. Derived from Th-2 lymphocytes, cytokines mediate the inflammatory response in asthma [65] and are involved in the structural alterations in the airway epithelium of asthmatic patients [66], as well as in the activation of goblet cells, demonstrated by increased mucus production in asthma [67][65]. Recent studies have reported that cytokines play a key role in the regulation of tight junction function in epithelial cells [54]. For example, work by Relova et al. [68] indicated that the disruptive airway barrier in asthma may occur due to the effect of pro-inflammatory cytokines.

The actual mechanism of morphological and physiological modifications in inflamed airway epithelium by pro-inflammatory cytokines is still poorly understood [47]. The suggested mechanisms of airway remodelling are by (i) stimulating fibroblast cells, as shown with IL-4 and IL-13, or by (ii) hyper secretion of mucus, as shown with TNF-α [10]. Furthermore, eosinophils have been reported to contribute fundamentally to the alteration of airway epithelium structures by the release of numerous proinflammatory cytokines [21][22].

TNF-α and other pro-inflammatory cytokines have been shown to be over-expressed in lung inflammatory diseases [69]. IL-4 was shown to be involved in the pathogenicity of asthma [70], while IL-13, which binds the alpha chain of the IL-4 receptor [70], could produce an even greater pro-inflammatory effect on airway epithelial cells in asthma than IL-4 [51]. Blocking IL-13 binding has shown a significant impact towards decreasing the severity of asthma [22]. To protect the respiratory tissue and suppress inflammation, cytokine inhibition may be a novel therapeutic target which could decrease the severity of asthma [71].

1.2.2.2 Proinflammatory Cytokines Contribution to Inflamed Intestinal Mucosa

Irritable bowel disease (IBD) covers several inflammatory disorders in the GI tract, such as Crohn`s and ulcerative colitis, which are mainly stimulated by cytokines [58]. The disruption of the intestinal tight junction barrier is one of the important features of Crohn`s disease [59][33]. Several inflammatory disorders have shown elevated levels of TNF-α and interleukins [8]. TNF-α plays a significant role in the dysfunction of the intestinal epithelial barrier, which actually presents a major mechanism behind the development of intestinal inflammatory diseases [8][17]. Furthermore, TNF-α was reported to act as stimulator of other proinflammatory cytokines in intestinal inflammatory diseases, including IL-4 and IL-13 [59].

Numerous studies have documented that the intestinal tight junction complex is mainly regulated by the myosin light-chain kinase (MLCK) protein [52][59]. Upregulation of MLCK protein expression, which is associated with TNF-α treatment, was reported in the *in vitro* model of intestinal epithelium [59][56]. Figure 1.4 shows the mechanism of TNF-α involvement in tight junction complex disruption.

The mucus barrier in the intestine plays an important function in protecting the body from the external environment [8]. In Crohn`s disease, the intestinal mucus layer has been reported in several clinical investigations to be thicker than normal condition as a main feature of intestinal inflammation in several clinical investigations [8]. The proinflammatory cytokines have been found to play central roles in inducing mucus production and increasing the thickness of the mucus layer in intestinal inflammatory disorders [8].

1.1.4 Cell Culture Models of Epithelium

Epithelial cell layer is a biophysical barrier which provides a formidable obstacle to the entry of noxious materials into the body [72]. Pharmaceutical laboratories have made considerable efforts to establish a well-characterised *in vitro* model of epithelium in order to (i) examine the cell barrier characteristics, (ii) investigate the drug transport properties in normal and diseased tissue [72][73], and (iii) predict the *in vivo* absorption of new therapeutic molecules [74]. Using primary cells in *in vitro* models would be ideal, but associated difficulties have been reported with primary models such as short lifespan [75] and the high cost of such cell cultures [72].

Nowadays, established and validated *in vitro* models of epithelial cell lines are a common strategy in drug development to evaluate the mechanism of the transport of molecules across the membrane of target epithelium [76][74], and also to examine other important aspects of drug delivery. The majority of research laboratories currently use cell culture models based on established cell lines, due to various advantages, such as cost effectiveness, high reproducibility, a decrease in the use of animal models and the ease of use and maintenance [74][73].

In this project, cell line models of lung and intestinal epithelium were used to examine the effect of proinflammatory cytokines on the barrier characteristics of epithelial cell layers. These cell lines are Calu-3 and Caco-2, which will described in more details in the following sections.

1.3.1 Calu-3 Cell Line

Calu-3 is a well-characterised epithelial cell line, which originated primarily from a bronchial carcinoma of a sub-mucosal gland in a 25 year old Caucasian, and forms confluent monolayers in culture [72][77][78][79][80]. This cell line is widely used in numerous respiratory research studies to represent an *in vitro* model of airway mucosa [81][82]. Currently, Calu-3 is the ‘standard’ layer forming cell line for lung research in pharmaceutical laboratories [83] and has been employed in several different applications, such as drug transport, metabolic property studies and epithelial barrier characteristics of airways [83][84][85].

A number of other of cell lines were examined as *in vitro* models of lung epithelium. However most of these are either difficult to maintain, such as the NHBE cell line [86], unable to form restrictive tight junctions and failed to provide appropriate TEER values, such as A549 [85], or failed to produce a phenotype similar to the relevant tissue [72]. There are several advantages when using Calu-3 cells in studies as a model of lung epithelium *in vitro*. Calu-3 has the ability to produce mucus and tight junction complexes with appropriate TEER values [87] that closely mimic the *in vivo* situation [75][83]. Moreover, the protein components of tight junction structures, such as *Zonula occludens* proteins and the adherin protein E-cadherin, were shown to be expressed in the Calu-3 cell line [75][85]. The formation of tight junction structures has mainly been assessed by TEER measurements [80]. The Calu-3 cell line has been reported to form ciliated columnar cells, mimicking the *in vivo* epithelium phenotype of the upper airway tract lining, as shown in figure 1.5 [72].

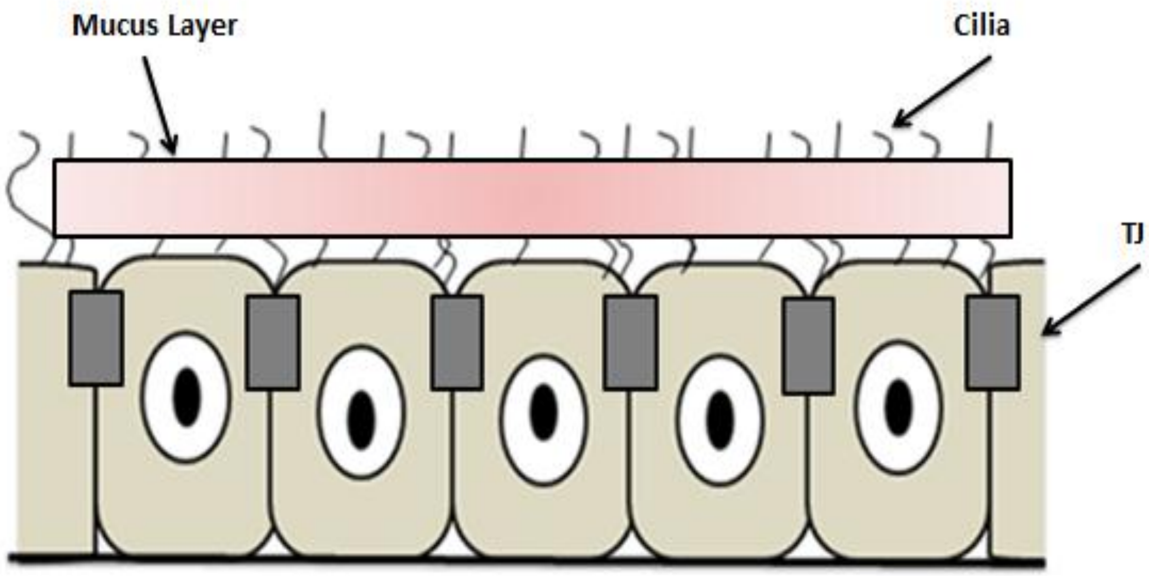

Figure 1.5 Schematic represent the structure of Calu-3 monolayers (ciliated columnar cells, mucus and tight junctions).

One of the key factors that influence epithelial cells culture in general, and Calu-3 in particular, is the culture system [80]. Calu-3 cells are usually cultured using two conditions: (i) liquid-liquid culture (LLC), where the culture medium covers both the apical and basolateral cell sides, and (ii) air-interface culture (AIC), where the culture medium is present only on the basolateral side [72]. AIC culture creates conditions where cells demonstrate more cilia formation and mucus covering the cell surface [72][76]. Furthermore, the apical side of AIC cultures cells is exposed to air that mimics the *in vivo* conditions of the airway mucosa [79]. Also, TEER measurements in AIC were shown to correlate better to the *in vivo* conditions than LLC, showing physiological values of ~300 $\Omega.cm^2$ [83]. Microscopic investigations moreover confirmed that in the AIC system cellular appearance approximates the *in vivo* conditions more than in LLC [83].

It must be noted that numerous factors could affect the quality of Calu-3 culture, such as the culture medium components and the number of passages [72]. Moreover, the number of cells seeded per cm^2 of Transwell® filters also influences the time needed by cells to reach confluence; the usual seeding density used with Calu-3 is 100.000 cells/cm^2 [86][80]. With such a seeding density, Calu-3 cells cultured on Transwell® filters will typically form polarised confluent monolayers within approximately 14 days of culture [80]. As has been shown in numerous studies, the relationship between the TEER value and the apparent permeability coefficient is inversely proportional [85][88]. This observation is due to the restrictiveness on tight junction that control the transport of molecules across epithelial membrane [88].

1.3.2 Caco-2 Cell Line

Caco-2 is a well-characterised epithelial cell line that was originally isolated from a human colo-rectal adenocarcinoma, which is used widely in intestinal research [89][17][90][74]. The Caco-2 cell line is the most commonly used tool for predicting drug transport across the intestinal system [89][6]. Caco-2 cells cultures have been shown to produce realistic TEER values and have the ability to form confluent monolayers when cultured on Transwell® filters [48]. The inter-laboratory protocols and facilities have an essential influence on Caco-2 cells development [4][91]. Also, several further factors could have a significant impact on Caco-2 cultures, such as medium components, the number of passages and the surface of the culture [4]. As illustrated in figure 1.6, Caco-2 cell line grown on Transwell support forms non-ciliated simple columnar cells, which are phenotypically similar to the *in vivo* situation of the colon [17].

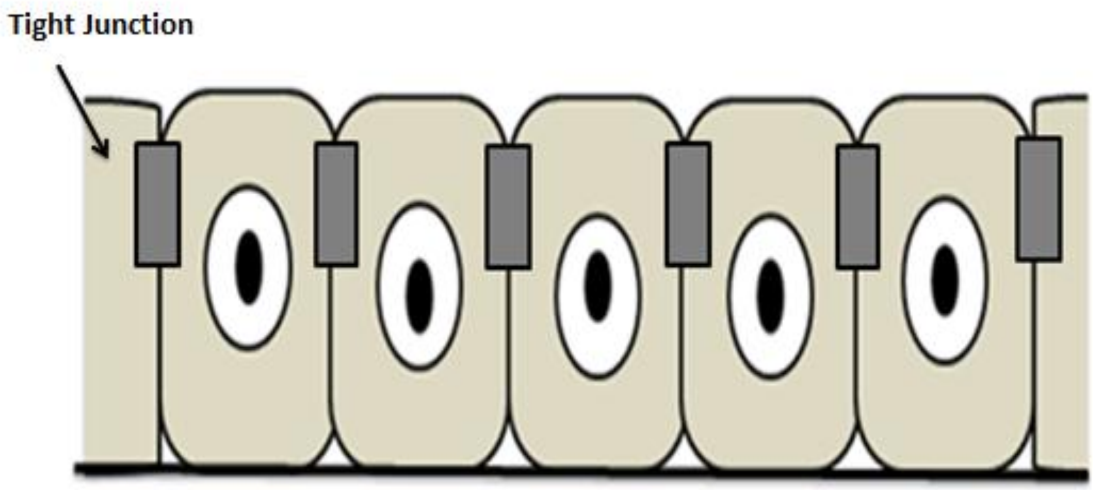

Figure 1.6 Schematic represent the structure of Caco-2 monolayers (simple columnar non-ciliated cells and tight junctions).

TEER measurements confirmed that Caco-2 cells have the capacity to form restrictive intercellular tight junction complexes *in vitro,* with TEER values > 400 $\Omega.cm^2$ [17][91][92]. Moreover, the tight junction proteins which play a key function in TJ formation were shown to be expressed in Caco-2 cell line [17][92]. When Caco-2 cells are cultured on Transwell®

filters with a seeding density of approximately 100.000 cells/cm^2, cells will typically form confluent monolayers after 3 weeks [17]. Unlike the Calu-3 cell line, no mucus has been reported to be produced in Caco-2 cells [76]. Moreover, the paracellular space in Caco-2 cells has been found to be larger than in Calu-3 cells [76]. A study documented that transport through the passive transcellular pathway substantially contributes to the transport of molecules across the Caco-2 cell layer , which mimics the *in vivo* system [19][6]. As shown in Calu-3 cell line, an inverse relationship was observed between TEER and the apparent permeability coefficient (P_{app}) in Caco-2 cells [19][6].

1.4 Conclusion

In the area of drug delivery, studies on the further characterisation of epithelial cell barriers would be useful to expand the understanding of drug transport mechanisms across healthy and diseased (inflamed) mucosal tissue. It should be noted that inflammation reactions in lung and intestine mucosal tissues are mainly induced through structural and functional modification of the mucosal barrier (tight junctions and mucus layer), which are regulated by proinflammatory cytokines. Potential progress associated with this project might lead to an improved understanding of drug transport across epithelium in inflammatory respiratory and intestinal disorders. The epithelial cell layer in diseased conditions is found to be more permeable, relative to normal tissue. This could lead to increased transport across diseased tissue of biological or nanotechnology products, issues which are important for the pharmaceutical industry (drug delivery) and nanotoxicology research. To this end, the current project will assess the effects of proinflammatory cytokines of the permeability of epithelial cell lines in vitro.

1.5 Project Aims

As mentioned in the introduction, the upregulation of pro-inflammatory cytokines is believed to be involved in the remodelling of mucosal tissue, and its barrier properties, during respiratory and intestinal inflammatory diseases. The aim of this project is to study the effects of selected pro-inflammatory cytokines on the barrier characteristics of *in vitro* models of intestinal and airway epithelium. For this purpose, Calu-3 and Caco-2 polarised monolayers will be employed to serve as *in vitro* models of airway and intestinal epithelium, respectively. The initial part of the project will focus on barrier characteristics of Calu-3 and Caco-2 cells with short-term cytokine treatments. After that, the work will examine the effects of long-term treatment (exposure) of cytokines on these cell lines in order to compare them with short-term conditions. Thereafter, the work will explore the gene expression data available from published studies and make comparisons with experimental data obtained in the project.

1.6 References

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Chapter 2

Materials and General Methods

2.1 Materials

2.1.1 Cells and Cell Culture Consumables

Calu-3 cells were purchased from the American Type Culture Collection (ATCC number; HTB-55) and used between passages 27-38. Calu-3 cells were routinely cultured in Eagle`s Minimum Essential Medium (EMEM). Caco-2 cells were purchased from the European Collection of Cell Cultures (ECACC number; 86010202) and used between passages 46-78. Caco-2 cells were cultured using Dulbecco's Modified Eagles Medium (DMEM). Both EMEM and DMEM were purchased from Sigma-Aldrich (UK) and were supplemented with antibiotic/antimycotic solution (100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.25 µg/ml amphotericin B), L-glutamine (200 mM), non-essential amino acids (100%), and foetal bovine serum (FBS, 10%). All media supplements were purchased from Sigma-Aldrich (UK).

Hank's Balanced Salt Solution (HBSS) supplemented with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) to maintain a pH of 7.4 was used as a drug transport medium; both of these materials were purchased from Sigma-Aldrich (UK), as was trypsin/EDTA solution (used to detach adherent cells in the process of cell 'splitting' or 'passaging'). Phosphate buffered saline (PBS) tablets were obtained from Oxoid (UK).

Polystyrene permeable inserts (Transwell®) for cell culture were supplied by Corning Life Sciences (USA). The Transwell® inserts were of 12 mm diameter and 0.4 μm pore size. Cell culture flasks (75 cm^2, canted neck with vented caps), black 96-well plates, and sterile pipettes were all purchased from Corning Life Sciences (USA). Sterile centrifuge tubes were supplied by Grainer (USA). Cells were routinely counted (e.g. prior to seeding on multiwell plates) using an improved Neubauer haemocytometer, which was purchased from Scientific Laboratory Supplies (SLS, UK).

2.1.2 Biological Agents

Recombinant human cytokines, interleukin-4 (IL-4), interleukin-13 (IL-13) and tumour necrosis factor-alpha (TNF-α) (catalogue numbers 204-IL, 213-IL, and 210-TA, respectively) were purchased from R&D systems (UK). Mouse anti-human *Zonula Occludens*-1 (ZO-1; tight junction protein) antibody was obtained from Zymed (part of Invitrogen). FITC-labelled goat anti-mouse IgG was purchased from Sigma-Aldrich (UK).

2.1.3 Model Macromolecular Drug

Fluorescein isothiocyanate (FITC)-labelled dextran of approximately 10 kDa in molecular weight (FD10) was purchased from Sigma-Aldrich (UK).

2.1.4 PCR

OneStep cDNA synthesis kit was purchased from Miltenyi Biotec (UK). JumpStart Taq DNA polymerase, 2% agarose gel, Tris-Borate-EDTA (TBE) buffer, ethidium bromide, and desalted primers were all purchased from Sigma-Aldrich (UK). SynGene Genius equipment and GeneSNAP software were used in this work for expression bands imaging.

2.2 Methods

2.2.1 Cell Maintenance

2.2.1.1 Maintenance of Cells in Culture Flasks

Calu-3 and Caco-2 cells were cultured in 75 cm^2 flasks in standard cell culture conditions (37°C, 5% CO_2 and 95% humidity) until confluence (point at which cells covered about 80-95% of the surface of the flask). Cell growth was monitored regularly using an optical inverted microscope. Culture medium was changed every 2-3 days by aspirating the old medium and replacing with approximately 13 ml of fresh warm (37°C) medium. When confluent, cells were detached and a proportion of these transferred to a new flask. This process of cell ‘splitting’ or ‘passaging’ was conducted by removing the culture medium, washing adherent cells with approximately 5 ml of warmed (37°C) PBS to remove any residual medium and dead cells, followed by the addition of approximately 3 ml of 2.5% trypsin/EDTA solution. Cells were incubated with trypsin/EDTA solution for ~15 min (Caco-2 cells) and ~20 min (Calu-3 cells) until cells were separated from the flask surface. It was noted that Calu-3 cells are more adherent than Caco-2, therefore requiring longer incubation time with trypsin/EDTA solution. Thereafter, approximately 8 ml of culture medium was added to the suspension of cells in trypsin/EDTA; this was done to inhibit the action of trypsin (which could be damaging to the cells if in contact with the cells for prolonged periods). The cell suspension was then transferred to a sterile 15 ml centrifuge tube and the cells were pelleted by centrifugation at 15,000 rpm for 5 min. The supernatant was discarded and fresh culture medium added to the pelleted cells to produce a cell suspension. Approximately 1/6-1/3 of the resulting cell suspension (i.e. resulting in cell splitting ratios of 1:6 – 1:3) was transferred to a new flask, which contained 12-13 ml of fresh culture medium (warmed to 37°C).

2.2.1.2 Cell Seeding on Transwells®

2.2.1.2.1 Calu-3 Cells

Calu-3 cells were initially cultured in 75 cm^2 flasks until confluence. Cells were then detached from the flask surface according to the method described in section 2.2.1.1 Cells (in the suspension) were counted using a haemocytometer, followed by ‘seeding’ on Transwell® filters. Cell seeding was conducted by transferring a volume of cell suspension containing 100.000 cells into each Transwell® insert (1.1 cm^2 filter area). The wells were filled with culture medium (EMEM), with the overall apical compartment volume of 0.5 ml and basolateral compartment volume of 1.5 ml. Following a 2-day culture period post-seeding in conditions where cells were covered by the culture medium on both apical and basolateral sides (i.e. liquid-covered culture, ‘LCC’), cells were thereafter exposed to the culture medium (0.5 ml) only on their basolateral side. This condition where the cell layers are in contact with the culture medium on their basal side and not on the apical side is termed ‘air-interfaced culture’ (AIC). These conditions were employed in this work for Calu-3 culture as it has been shown that the resulting cell cultures more closely resemble the airways *in vivo* compared to liquid-covered culture (LCC). The culture medium (EMEM) was replaced regularly every 2-3 days. Calu-3 cells were typically cultured on transwells for 3 weeks. Cell confluence and the integrity of the cell layer were confirmed by measurement of transepithelial electrical resistance (TEER), which will be described in section 2.2.2.

2.2.1.2.2 Caco-2 Cells

Caco-2 cells were cultured in 75 cm^2 flasks until confluence. Cells were then detached from the flask surface (by addition of trypsin) according to the method described in section 2.2.1.1 Cells were counted using a haemocytometer, followed by seeding on Transwell® filters at 100.000 cells/well (1.1 cm^2 filter area). 0.5 ml of the culture medium (DMEM) was added to the apical compartment and 1.5 ml to the basolateral compartment. Caco-2 cells were cultured using liquid-covered culture conditions, with the culture media present on both apical and basolateral sides of the cells. The culture medium was replaced regularly, every 2-3 days. Caco-2 cells were typically cultured on transwells for 3 weeks. Cell confluence and the integrity of the cell monolayer were confirmed by measurement of TEER (next section).

2.2.2 Measurement of Transepithelial Electrical Resistance (TEER)

Measurement of transepithelial electrical resistance (TEER) is a technique typically employed in cell culture laboratories as an indication of epithelial cell growth, confluence and the intactness of the cell monolayer. TEER is also a measure of tight junction formation in epithelial cells. Figure 2.1 depicts the system that is typically used in the laboratory to measure TEER.

TEER was measured in the work using an EVOM (World Precision Instruments, USA) voltohmmeter, equipped with a pair of 'chopstick' electrodes (Figure 2.1) measurements to detect monolayer confluence and cell integrity. The electrodes were initially sterilised (in ethanol), followed by washing in PBS. To obtain a reading, the chopstick electrodes were immersed in the culture medium bathing the cells, with the longer electrode immersed in the medium present on the basolateral chamber and the shorter electrode in the medium on the apical side.

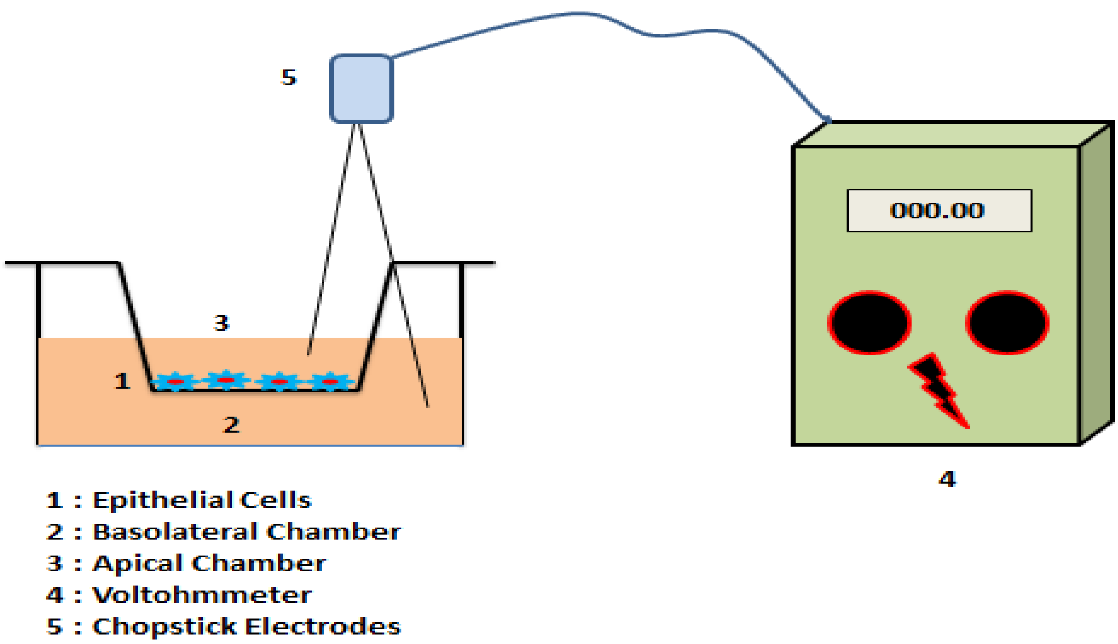


Figure 2.1 Schematic representation of the Voltohmmeter (EVOM, World Precision Instruments) system used to measure transepithelial electrical resistance.

With Calu-3 cells cultured under AIC, culture medium from the basal side was replaced by fresh culture medium, added to both apical and basolateral sides (0.5 ml and 1.5 ml, respectively). Cells were then incubated with the culture medium for approximately 30 min (in normal cell culture conditions), at which point TEER was measured following the procedure described above. The TEER values in this thesis are expressed as ($\Omega.cm^2$) and background resistance due to the filter (~100 $\Omega.cm^2$) was subtracted from the reported values.

2.2.3 Effect of Cytokines on Tight Junctions: Measurement of TEER

Only intact polarised cell layers were included in these experiments. The 'intactness' was determined by measurement of TEER. Only cells expressing a TEER of > 300 and 1000 $\Omega.cm^2$ for Calu-3 and Caco-2 cell layers, respectively, were included in these experiments.

Cytokines (IL-4; 5ng/ml), (IL-13; 5ng/ml) and (TNF-α; 25ng/ml) were added on the apical side of confluent cell layers of Calu-3 and Caco-2, with fresh culture medium applied on the basal side of the cells. Cells were incubated with the cytokines for 3-4 days, with TEER measurements conducted every day.

In another experimental set-up, the cells were treated with the cytokines for a longer duration. Here, cytokines (IL-4; 5ng/ml), (IL-13; 5ng/ml) and (TNF-α; 25ng/ml) were applied after 2 days of culturing the cells in Transwell® Filters and cells were feeding by fresh medium containing the cytokines every 2 days during 21 days of study. TEER modifications are reported as a percentage of the baseline value (i.e. TEER value before cytokines addition, when time is zero). All experiments were conducted with n = 3-4.

2.2.4 Effect of Cytokines on Tight Junctions: Measurement of Permeability

Permeability studies were conducted on polarised cell layers, previously incubated with cytokines (IL-4; 5ng/ml), (IL-13; 5ng/ml) and (TNF-α; 25ng/ml) for 4 days. Cell layer integrity was confirmed by TEER measurements (detailed in the previous section). TEER was also measured after the permeability experiment to ensure that the cell layer integrity was preserved during the experiment. Fluorescein isothiocyanate (FITC)-labelled dextran of ~10-kDa (FD10) was used as a model hydrophilic macromolecule and HEPES-buffered HBSS solution (pH 7.4) as a transport medium.

FD10 sample was prepared by dissolving the FD10 powder in pre-warmed HBSS/HEPES solution (37°C) at 1 mg/ml. Cell culture medium was replaced with the transport medium (0.5 ml apically and 1.5 ml on the basolateral side) and cells incubated with the transport medium (HBSS/HEPES) at 37°C for ~30 min. TEER was measured again to ensure that the cell layers maintained their integrity in the transport medium. HBSS/HEPES was then removed from the apical side of the cell layers and replaced with FD10 (500µg/ml in HBSS/HEPES). The Transwell® plate was covered with aluminium foil to protect it from light and the plate placed in the incubator (37°C). Basolateral solution was sampled periodically by removing 100 µl every 30 min and transferring the solution into a black 96-well plate and replaced. The sampled basolateral solution was replaced with the same volume of the transport medium. Permeability studies were conducted for a period of 3 hours. FD10 was quantified by fluorescence with an MFX microtiter plate fluorometer (Dynex Technologies, USA) using FITC fluorescence parameters (485 nm excitation, 535 nm emission). The resulting fluorescence readings were converted into concentrations and finally amounts using calibration curves, which involved fluorescence measurements of known concentrations of serially diluted FD10.

To determine whether any changes in FD10 permeability following cytokine application result from a tight junction effect, permeability experiments were also conducted at 4°C. In this case, cell layers were treated with the cytokines (IL-4; 5ng/ml), (IL-13; 5ng/ml) and (TNF-α; 25ng/ml) for 4 days. Cells were then incubated with cold HBSS (at 4°C) and following FD10 application cells were transferred to a cold room at 4°C.

FD10 permeability is expressed as apparent permeability coefficient (P_{app}), calculated using the following equation:

$$P_{app} = \left(\frac{\Delta Q}{\Delta t}\right) x \left(\frac{1}{A x C_0}\right)$$

Where*:*

P_{app}: Apparent permeability coefficient (cm/s)

ΔQ/Δt: Permeability rate (µg/s)

A: Diffusion area of the cell layer (cm^2)

Co: Initial concentration of FD10 (µg/ml)

2.2.5 Confocal Microscopy Imaging of Tight Junction Protein, Zonula Occludens (ZO-1)

Initially, Calu-3 cell line was incubated with cytokines (IL-4; 5ng/ml), (IL-13; 5ng/ml) and (TNF-α; 25ng/ml) for 4 days. Confluent Calu-3 cells were then fixed with 3% w/v paraformaldehyde by incubating for approximately 10 min at room temperature. Cells were then washed with PBS and permeabilised by incubating with Triton X-100 (0.1% v/v in PBS) for 10 min. PBS was used again to wash the cells and 1% w/v BSA (in PBS) applied for 1 hour. This solution was then removed and mouse, anti-human ZO-1 (primary) antibody diluted in BSA/PBS at 10µg/ml applied to the cells for one hour. The primary antibody was aspirated and cells washed extensively with PBS. Rabbit, anti-mouse (secondary) antibody diluted according to the manufacturer's instructions in 1% BSA/PBS was then applied to the cells for 1 hour. The secondary antibody was then removed and cells washed several times with PBS. The Transwell® membrane was then cut and placed on glass slides for confocal imaging. Cells nuclei were labelled with Hoechst 33342, whilst 1% 1,4-diazobicyclo-[2,2,2]-octane (DABCO) in 90% glycerol/10% PBS was used as a mounting medium. Confocal imaging was conducted using a Leica TCS SP2 system mounted on a Leica DMIRE2 inverted microscope.

2.2.6 Collection of Gene Microarray Data from GEO

The microarray data of selected genes were collected from Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo). GEO database contains a platform which is the type of array used in the experiment. In total four studies were analysed and the difference in gene expression between healthy samples and asthmatic patients determined. More detailed information regarding the methodology employed for microarray data analysis is included in appendices 8.2 and 8.3. The series numbers of four studies used are listed in Table 2.1:

Study	Series	Platform	Sample number
1	GSE470	GPL8300	12 (6 healthy, 6 asthmatic)
2	GSE18965	GPL96	16 (8 healthy, 8 asthmatic)
3	GSE4302	GPL570	26 (13 healthy, 13 asthmatic)
4	GSE3212	GPL80	30 (15 healthy, 15 asthmatic)

Table 2.1 Summary of four studies examined from GEO database.

2.2.7 Statistical Analysis

Student's t-test was employed to the statistical significance of the data, with p values < 0.05 considered as significant.

Chapter 3

Effect of Cytokines on Barrier Characteristics of Human Airway Epithelial Cell Layers (Calu-3)

3.1 Introduction

The airway mucosa is a biophysical barrier between the external environment (lumen) and submucosal tissues, playing an important role in protecting the lung and other tissues from harmful elements of the external environment by providing selective permeability [1][2][3]. Disruption of the functionality of this epithelial barrier may therefore lead to undesirable effects on the lung [4][5]. To study the influence of airway epithelial barrier disruption on pulmonary disease, or the influence of disease on the airway barrier, researchers have attempted to establish *in vitro* systems that mimic *in vivo* conditions in disease, with limited success [6].

In terms of employing *in vitro* models to study the mucosal barrier of the airways, the use of primary cells would be an ideal choice due to a closer representation of the tissue of interest [7]. However, due to associate disadvantages, as discussed in the Introduction chapter, laboratories currently mainly rely on the use of cancerous cell lines [8].

One of the principal cell lines for *in vitro* investigation of the airway mucosal barrier is the Calu-3 cell line [9][10]. This cell line is derived originally from the human bronchial submucosal glands [1][10]. The Calu-3 cell line is widely used as an *in vitro* model of the upper airways, including the bronchial [10] and nasal [11][12] mucosa, in studies related to investigation of the metabolic and permeability properties of the human airways. The key benefits of using the Calu-3 cell line as an *in vitro* model of the airways lies in the ability of these cells to grow as polarised layers with tight junctions when cultured appropriately [10]. In contrast, several other airway-originated cell lines fail to generate significant transepithelial electrical resistance (TEER, an indication of tight junction formation and 'tightness' of the tight junctions) *in vitro* [10]. Furthermore, Calu-3 cells are capable of producing mucus (when using specific culture conditions), which offers the possibility of studying the effect of mucus on drug permeability [1][13].

Studies determining the usefulness of the Calu-3 cell model for *in vitro* pulmonary drug absorption studies have generally shown that this model is associated with good *in vitro*-*in vivo* correlation [14]. In using the Calu-3 cell line as a model of the airway mucosa, the cells are typically cultured on permeable supports (e.g. Transwell® inserts). Although the use of permeable supports for Calu-3 culture is almost universal in studies employing these cells as a model of the airway barrier, other culture conditions used by different labs vary considerably. Calu-3 cells can be grown *in vitro* under liquid-covered culture (LCC), where cells are bathed by the culture medium on both apical (or luminal) and basolateral (or basal) sides. Calu-3 cells may also be cultured on permeable supports using air-interfaced conditions (AIC), where polarised cells are exposed to the culture medium on the basal side only [14]. Presently there is no consensus on the 'correct' culture conditions that produce *in* *vitro* models that best resemble the *in vivo* tissue. Consequently, examples of Calu-3 culture using both LCC and AIC conditions can be found extensively in the literature. However, there is some evidence that Calu-3 culture using AIC achieves superior representation of the *in vivo* conditions, as compared to LCC [1][14][10]. A study comparing the two systems observed presence of mucus and an apically ciliated surface in the epithelial cell layer resulting from AIC, whilst there was less apparent mucus secretion and cilia presence in cells obtained from LCC conditions [14].

In improving our understanding of the inflammatory diseases of the lung, such as asthma, recent efforts have focused on studying the biology and physiology of the epithelium [2]. Remodelling of the respiratory epithelium is one of the crucial characteristic features of asthma [17]. The alteration in the structure of the epithelium in such inflammatory processes may, in turn, cause increased mucus secretion by relevant components of the mucosa (goblet cells), dysfunction of epithelial tight junctions and fibrosis [17].

As indicated in the Introduction chapter, this chapter seeks to determine the effect of TNF-α, IL-4 and IL-13 proinflammatory cytokines on human airway epithelium in terms of mucosal barrier characteristics and permeability properties.

3.2 Methods

3.2.1 Effect of Site-Specific Cytokine Addition on Epithelial Barrier

3.2.1.1 Effect on TEER

Calu-3 cells were used between passages 27-36. Cells were initially cultured on 75 cm^2 flasks until confluent. Thereafter, cells were detached ('trypsinised') from the flasks, seeded on Transwell® filters and cultured using AIC condition (following the protocols described in sections 2.2.1.1 and 2.2.1.2.1). TEER measurements were conducted every 2 days during a 3-week culture period. A detailed description of the TEER measurement method is included in sections 2.2.2 and 2.2.3.

3.2.1.2 Effect on FD10 Permeability

3.2.1.2.1 FD10 Permeability at 37˚C

Calu-3 cells were cultured on Transwell® filters using AIC conditions. Only the cell layers displaying TEER values >300 $\Omega.cm^2$ were included in this study. Recombinant human IL-4 and IL-13 were applied to the cells at 5 ng/ml, whereas TNF-α at 25 ng/ml in the culture medium. Each cytokine was added to the cell layers on the apical side only, on the basolateral side only, or on both apical and basolateral sides (i.e. bilaterally). A control experiment was conducted where the culture medium (without cytokines) was applied to the cells at the same time. TEER measurements were taken regularly for 3-4 days post sample application. Permeability experiments were conducted on 21 days. For this culture medium was replaced with transport medium (HBSS/HEPES, 37˚C). Cells were incubated for ~45 min to adapt to the new medium, and then TEER was measured again to confirm the adaptation. Half the volume of HBSS/HEPES was removed from the apical side and replaced by the same volume of FD10 dissolved in warm HBSS/HEPES with (500 µg/ml) concentration. The rest of the experiment was described in more detail is section 2.2.4.

3.2.1.2.2 FD10 Permeability at 4˚C

Calu-3 cells were cultured as AIC condition and incubated with (HBSS/HEPES) solution at 4˚C for approximately 45 min. The solution was then replaced with FD10 with concentration (500µg/ml). Cells were placed at 4˚C between sampling intervals.

3.2.2 Effect of Cytokines on Zonula Occludens-1 Protein

Calu-3 cells were grown on filters using AIC condition and imaged by confocal microscope after they formed polarised monolayers. Zonula Occludens-1 (ZO-1) staining was completed as using protocol described in more detail previously in section 2.2.5.

3.3 Results

3.3.1 Effect of Site-Specific Cytokine Application on Calu-3 Barrier for 3 days

3.3.1.1 Effect on TEER

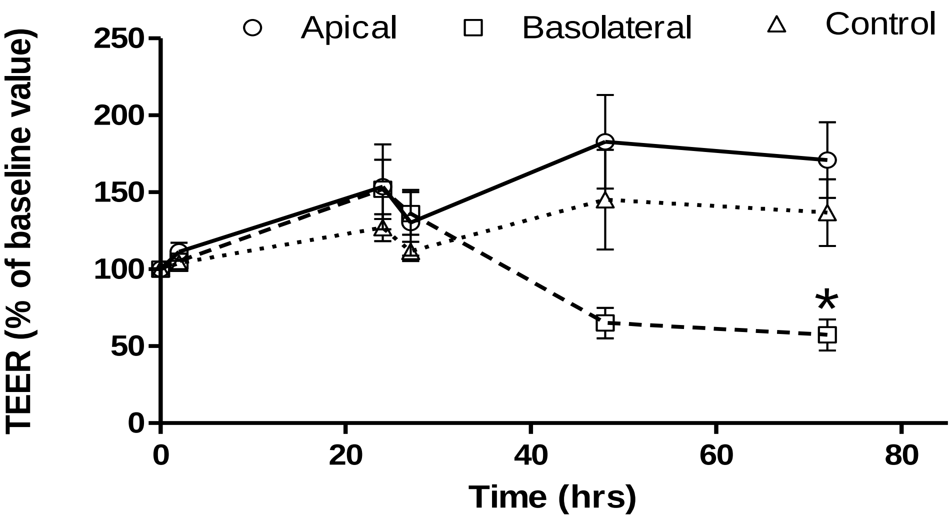
Changes in TEER of Calu-3 cell layers following site-specific addition of TNF-α, IL-4 and IL-13 are presented in Figure 3.1. Application of TNF-α at 25 ng/ml (Figure 3.1a) at the apical side of Calu-3 layers displayed no notable effect, as the changes in TEER were similar to those of control cell layers (where cytokine addition was omitted). In fact, an increase in TEER following apical addition of TNF-α was apparent. The reasons behind this elevation in TEER are not apparent, but control cell layers exhibited a similar trend.

Basolateral application of TNF-α produced an interesting pattern in TEER. A measurement taken 24 hours following its application revealed an increase in TEER at this time point by approximately 50%, as compared to the baseline value. However, TEER recordings at later time points showed that TEER dropped, with values at 48 hours and 72 hours amounting to approximately 65% and approximately 57% of the baseline value, respectively.

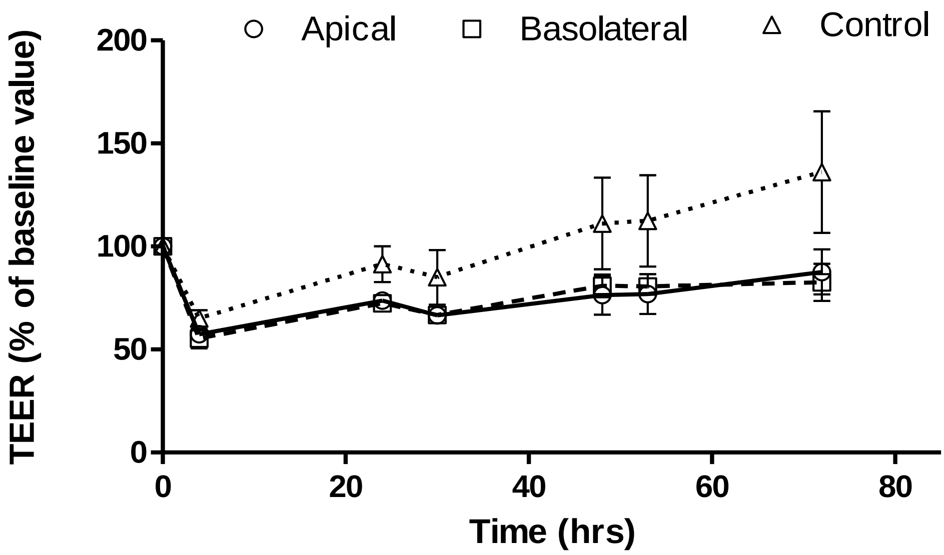
Figure 3.1b shows the TEER pattern – measured over a three-day period – of polarised Calu-3 layers, following their treatment with IL-4 (applied as 5ng/ml). The changes in TEER followed a similar pattern to control cell layers, with an initial decrease to approximately 65% of the baseline value, measured 4 hours after application, followed by a gradual increase. This trend was observed with both apical and basolateral addition of IL-4 and was also apparent with the control cell layers. TEER values at the final measurement point (72 hours) were approximately 87% and 82% of the baseline value with apical and basolateral IL-4, respectively, but these values were not statistically different to control cell layers.

Site-specific treatment of polarised Calu-3 cell layers with the third selected cytokine tested in this work, namely IL-13 (5 ng/ml), produced the effects shown in Figure 3.1c. Both apical and basolateral IL-13 additions resulted in TEER patterns closely resembling the control up to the 48 hours measurement interval. Although at the final measurement point TEER of cell layers treated with IL-13 on the basolateral side was approximately 94% of the baseline TEER, compared to both apical additions of IL-13 and control cell layers (approximately 137% of baseline TEER), the t-test confirmed that TEER values of apical and basolateral sides applications are not statistically significantly different, compared to control (p= 0.1278 and 0.8658, respectively).

a)


Apical
Basolateral
Control
250
200
150
100
50
0
TEER (% of baseline value)
*
0
20
40
60
80
Time (hrs)

b)


Apical
Basolateral
Control
200
150
100
50
0
TEER (% of baseline value)
0
20
40
60
80
Time (hrs)

c)

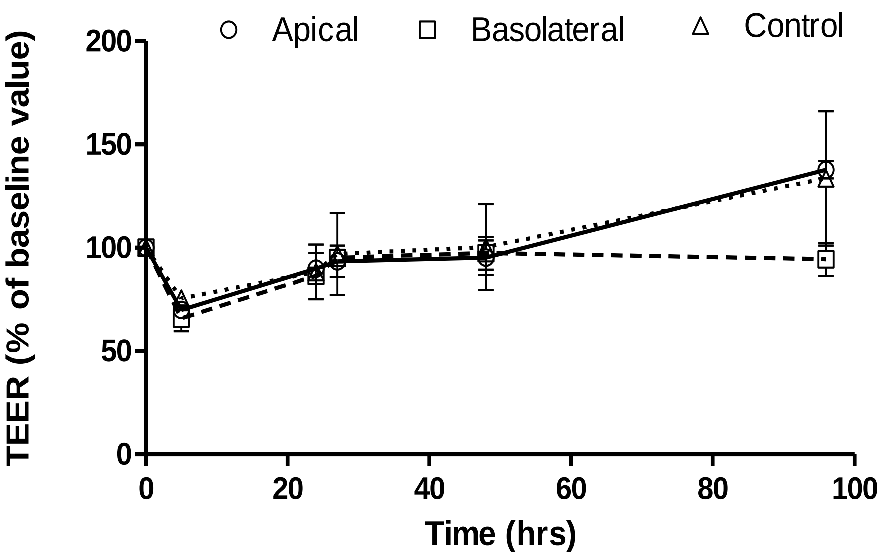


Figure 3.1 TEER measurements of Calu-3 cells cultured on filters using AIC conditions and treated by a) TNF-α (25ng/ml), b) IL-4 (5ng/ml), c) IL-13 (5ng/ml) for 72 hours.

TEER is expressed as % change compared to accumulative value (baseline value). Background TEER due to the filter was subtracted from the reported TEER values. Data presented as the mean ± SD (n=4). Statistical analysis calculated by t-test (P value: ns > 0.05). indicates statistical difference.*

3.3.1.2 Effect on FD10 Permeability

3.3.1.2.1 Permeability at 37^{o}C

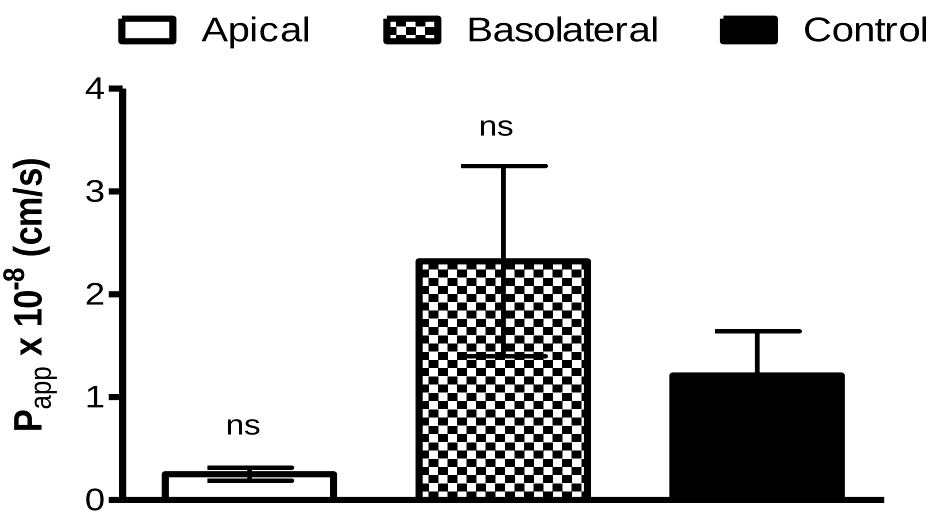
In addition to TEER, changes in the permeability barrier of Calu-3 cell layers following four days exposure to cytokines were also evaluated by measuring the permeability of a macromolecular model compound. Figure 3.2 depicts FD10 permeability across polarised Calu-3 cell layers, after treatment with the cytokines on either the apical or basolateral side. FD10 permeability is expressed as apparent permeability coefficient (P_{app}), which was calculated according to the equation described in section 2.2.4, following regular sampling of the basolateral solution (over 3 hours) and FD10 quantitation by fluorescence. FD10 permeability across the cell layers was measured following treatment of confluent and polarised cell layers (apically or basolaterally) with the cytokines for 4 days.

Figure 3.2a shows that FD10 permeability across the cell layers treated with TNF-α on the apical side was in fact lower, compared to permeability across control cell layers without TNF-α treatment. Regarding FD10 permeability across polarised Calu-3 layers following their exposure to TNF-α on the basolateral side, the P_{app} value reached 2.3 x 10^{-8} cm/s, and with the control permeability of 1.2 x 10^{-8} cm/s, making this increase just outside the statistical significance (p= 1.992 and 2.306, respectively).

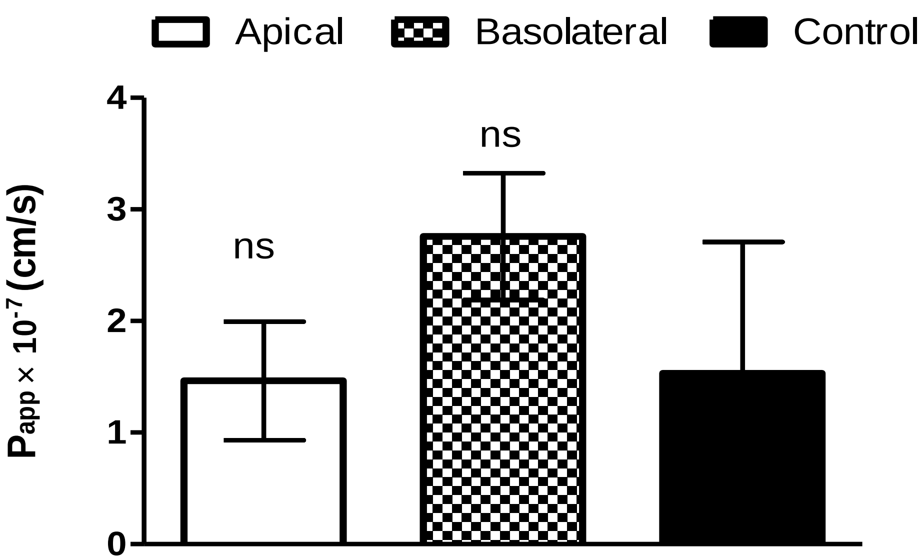
The impact of cell treatment with IL-4, either apically or basolaterally, on their barrier (as measured in terms of FD10 permeability) is highlighted in Figure 3.2b. Prior treatment of cells layers with IL-4 on the apical side did not have an effect on permeability. Cells layers that were previously basolaterally treated with IL-4 displayed permeability of P_{app} 2.75 x 10^{-7} cm/s), relative to control (P_{app}1.53 x 10^{-7} cm/s), but this difference in permeability did not reach a statistical significance (p= 1.864).

FD10 permeability across polarised Calu-3 cell layers previously treated with IL-13 followed a similar pattern to those subjected to IL-4. Figure 3.2c reveals that both apical and basal cell treatment with IL-13 produced cell layers displaying permeability towards FD10 that was not statistically significantly different to control cell layers.

a)


Apical
Basolateral
Control
P_{app} x 10^{-8} (cm/s)
0
1
2
3
4
ns
ns

b)


Apical
Basolateral
Control
P_{app} × 10^{-7} (cm/s)
0
1
2
3
4
ns
ns

c)

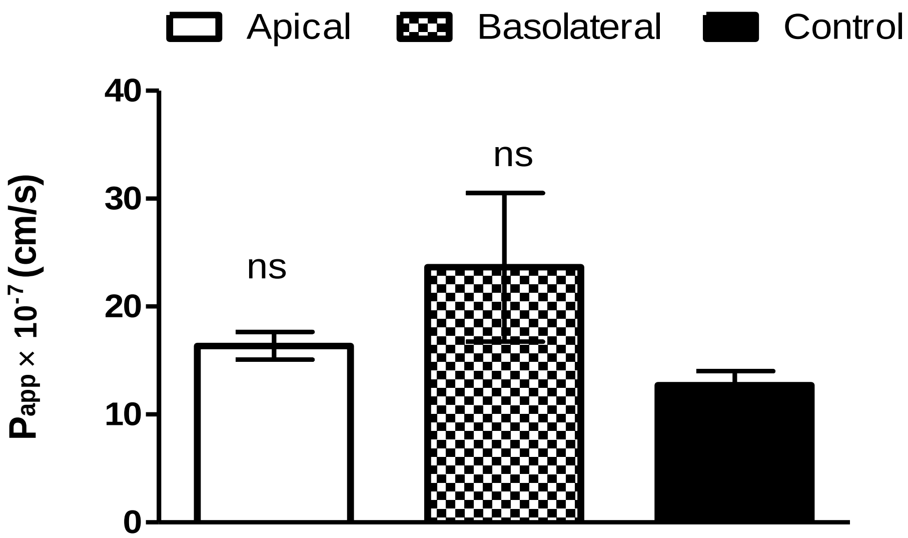


Figure 3.2 Effect of 96 hours cytokine treatment on FD10 permeability across Calu-3 layers at 37˚C. Exposure to a) TNF-α (25ng/ml), b) IL-4 (5 ng/ml) and c) IL-13 (5 ng/ml).

FD10 permeability expressed as P_{app}, calculated using the equation of apparent permeability coefficient which is described in section 2.2.4. Results presented as the mean ± SD (n=4). Statistical analysis calculated by t-test (P value: ns > 0.05).

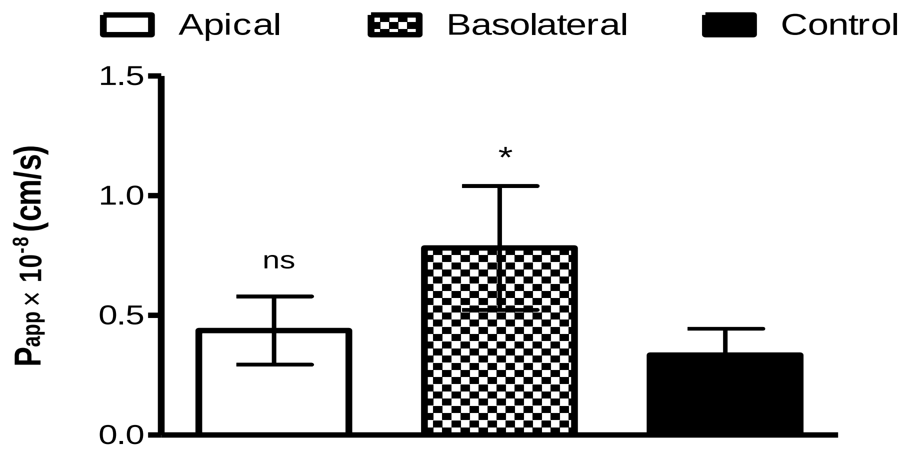
3.3.1.2.2 Permeability at 4°C

To confirm that the change in permeability following cell treatment with the cytokines is due to an effect of cytokines on the tight junctions (and hence the paracellular barrier), FD10 permeability experiments were conducted at 4˚C in addition to normal cell culture conditions (37˚C). In this experimental set-up, the energy-requiring processes, including active transport, would be inhibited and any increase in permeability is likely to result from changes in the passive transport (i.e. the paracellular corridor).

The data in Figure 3.3 shows FD10 permeability across Calu-3 cell layers at 4˚C. In Figure 3.3a the cells were previously treated with TNF-α (at 25 ng/ml) for 4 days and equilibrated at 4˚C before the experiment. The data generally mirror the trends observed at 37˚C, with the cell layers treated with TNF-α displaying a larger permeability compared to control. In this instance, the cell layers exposed to TNF-α on the basal side exhibited a larger FD10 permeability (statistically significant) of 0.78 x 10^{-8} cm/s compared to control 0.33 x 10^{-8} cm/s. In contrast, apical presence of TNF-α did not induce a sufficient increase in cell layer permeability (0.43 x 10^{-8} cm/s), versus control (0.33 x 10^{-8} cm/s), to reach statistical significance.

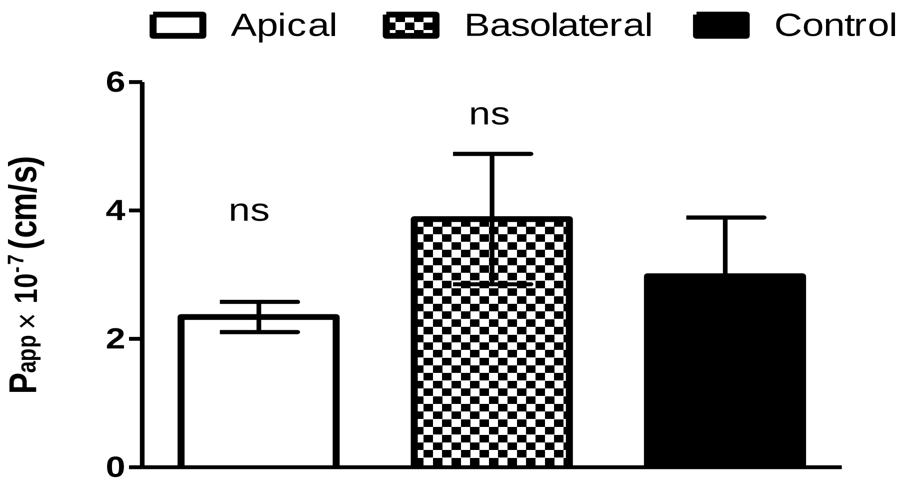
Figure 3.3b shows FD10 permeability of IL-4-treated Calu-3 cell layers, as measured at 4˚C. The data shows no significant increase in permeability, regardless of the site of IL-4 application (P_{app}, apical= 2.34 x 10^{-7} cm/s, basolateral= 3.86 x 10^{-7} cm/s, control= 2.97 x 10^{-7} cm/s). A similar outcome was observed with IL-13 (Figure 3.3c), with no increases in cell layer permeability at 4˚C following IL-13 treatment either on the apical or basal side of the cell layers (P_{app}, apical= 4.12 x 10^{-7} cm/s, basolateral= 3.43 x 10^{-7} cm/s, control= 4.42 x 10^{-7} cm/s).

a)



Apical
Basolateral
Control
1.5
1.0
0.5
0.0
ns
*
$P_{app} \times 10^{-8}$ (cm/s)

b)



Apical
Basolateral
Control
6
4
2
0
ns
ns
$P_{app} \times 10^{-7}$ (cm/s)

c)

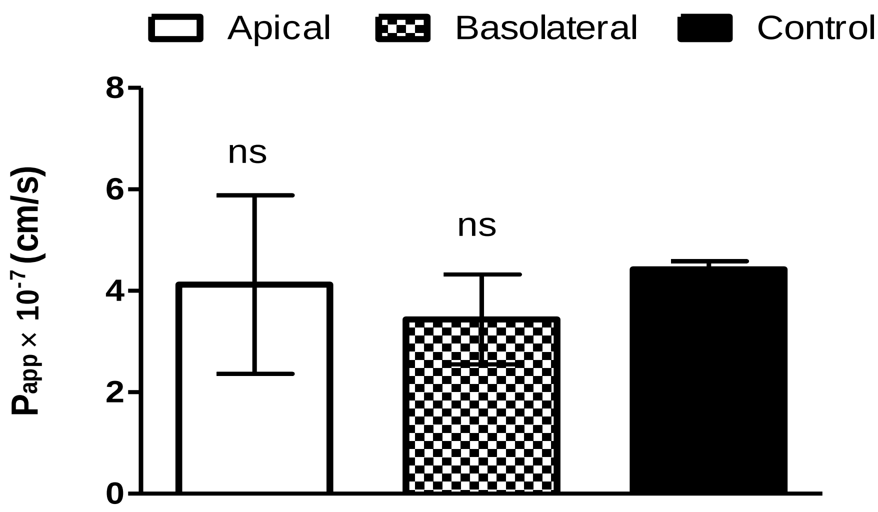


Figure 3.3 Effect of 96 hours cytokine treatment on FD10 permeability across Calu-3 layers at 4˚C. Exposure to a) TNF-α (25ng/ml), b) IL-4 (5 ng/ml) and c) IL-13 (5 ng/ml).

*FD10 permeability expressed as P_{app}, calculated using the equation of apparent permeability coefficient which described in section 2.2.4. Results presented as the mean ± SD (n=4). Statistical analysis calculated by t-test (P value: * < 0.05, ns > 0.05).*

3.3.2 Effect of Basolateral Cytokine Application on Cell Layer Barrier: a Comparison between Different Cytokines

3.3.2.1 Comparison of Effect on TEER

Previous experiments served to establish the experimental conditions that lead to more prominent effects of cytokines on Calu-3 layer barrier. Following the observation that basal application of the cytokines leads to a greater effect on the cell layer barrier (despite this effect not reaching statistical significance), in further experiments, described in the forthcoming sections, cytokines were always applied on the basolateral side of polarised cell layers.

The influence of cytokine application (on the basolateral side of the cells) on TEER is depicted in Figure 3.4. The data compares the effect of the three cytokines by showing TEER values before (baseline) and after cell incubation for four days. The present study highlights that out of the three tested cytokines, only the cell layers treated with TNF-α were found to display a significantly lower TEER, compared to the baseline value before the treatment. When applied on basolateral side, TNF-α showed a decrease in the TEER value from (~131% to ~78%) 96 hours post treatment. Following IL-4 treatment the TEER value changed from (~115% to ~97%) as % of control value. Also, IL-13 provided no effect as TEER values changed from (~106% to ~117%).

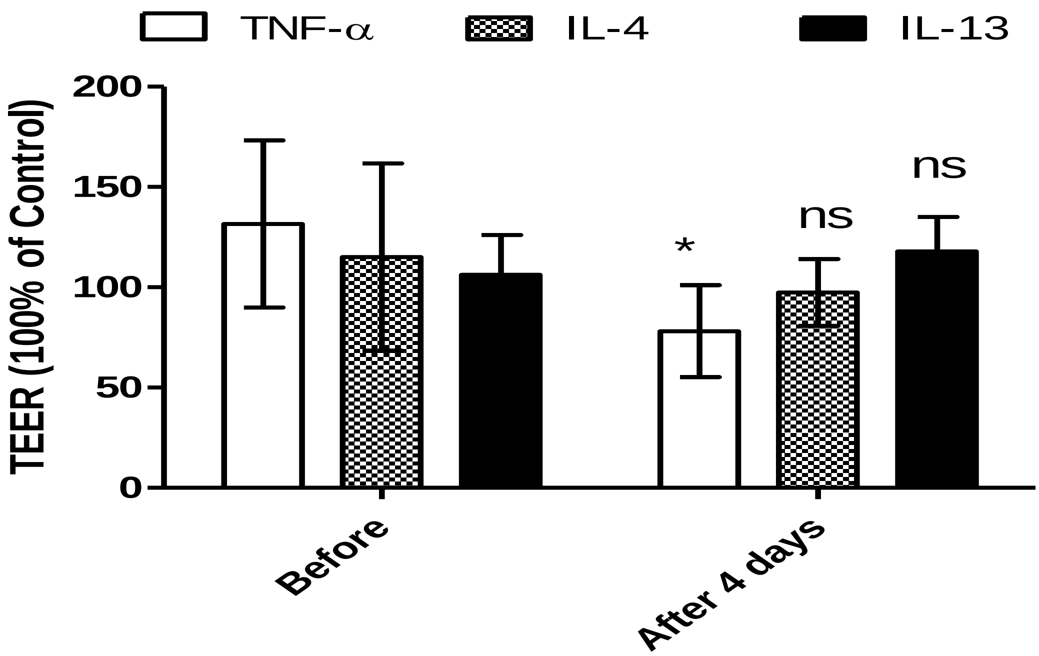


Figure 3.4 TEER measurements of Calu-3 cells cultured on filters using AIC conditions and treated for 96 hours with cytokines (IL-4 and IL-13 at (5 ng/ml) and TNF-α at (25 ng/ml).
*TEER is expressed as % of Control (Calu-3 without cytokines). Background TEER due to the filter was subtracted from the reported TEER values. Data presented as the mean ± SD (n=4). Statistical analysis calculated by t-test (P value: * < 0.05, ns > 0.05).*

3.3.2.2 Comparison of Effect on FD10 Permeability

3.3.2.2.1 Permeability at 37^oC

FD10 permeability across polarised Calu-3 layers treated basolaterally with TNF-α, IL-4 or IL-13 is shown in Figure 3.5. On this occasion, cells subjected to TNF-α or IL-4 incubation exhibited a significantly larger permeability, compared to control ($p < 0.001$ and < 0.05, respectively). The largest effect on permeability was seen in TNF-α-treated cells. Here the cell layers displayed a 3.6-fold larger FD10 permeability compared to control. Dextran permeability across IL-4-treated cell layers was 2.5-fold larger compared to control.

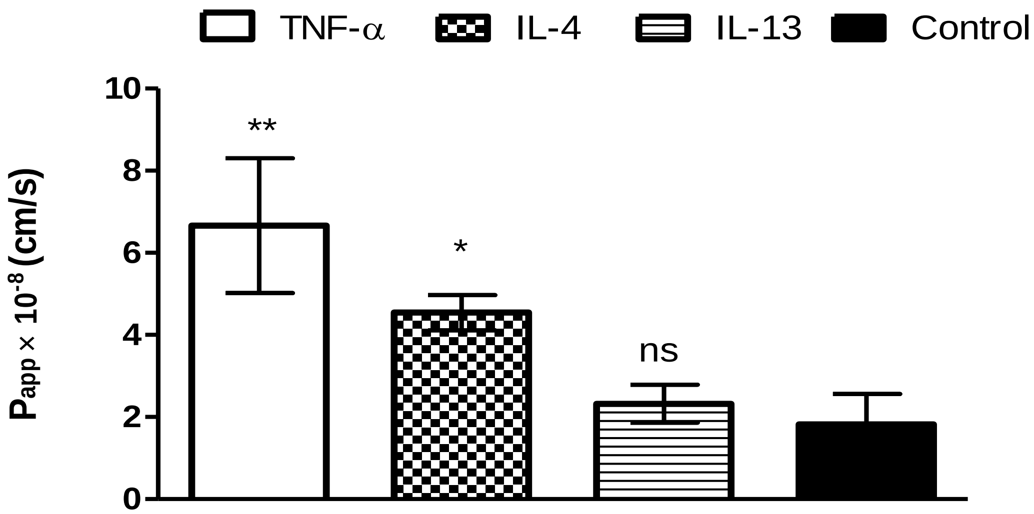


Figure 3.5 Effect of tested cytokines on FD10 permeability across Calu-3 layers at 37˚C. Cytokines treatment for 96 hours.
*FD10 permeability expressed as P_{app}, calculated using the equation of apparent permeability coefficient which described in Chapter 2. Results presented as the mean ± SD (n=4). Statistical analysis calculated by t-test (P value: * < 0.05, ** < 0.001, ns > 0.05).*

3.3.2.2.2 Permeability at 4^oC

A comparison between different cytokines in terms of their effect on cell layer permeability at 4˚C is presented in Figure 3.6. This shows FD10 transport at 4˚C across Calu-3 layers AIC grown and pre-treated with several cytokines for 4 days. The figure below shows that the permeability in cold condition is much lower than in normal condition. At 4^{o}C cell layers treated with TNF-α were found to display a notably higher permeability to FD10, approximately (P_{app}= 1 x 10^{-8} cm/s), amounting to 2-fold, compared to control with P_{app}= 0.45 x 10^{-8} cm/s, ($p <0.0001$). IL-4 and IL-13 treated cells did not exhibit a significantly different permeability relative to control by showing P_{app}= 0.36 and 0.33 x 10^{-8} cm/s, respectively.

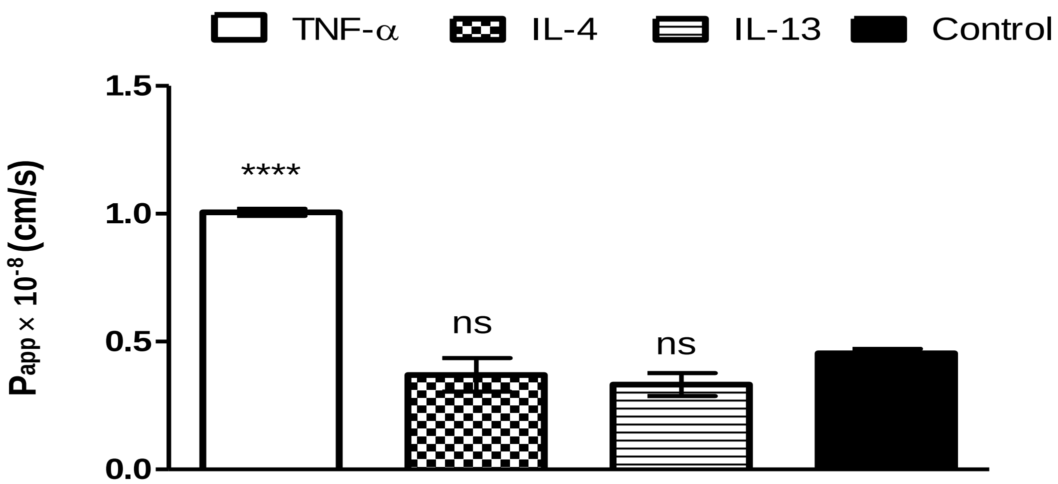


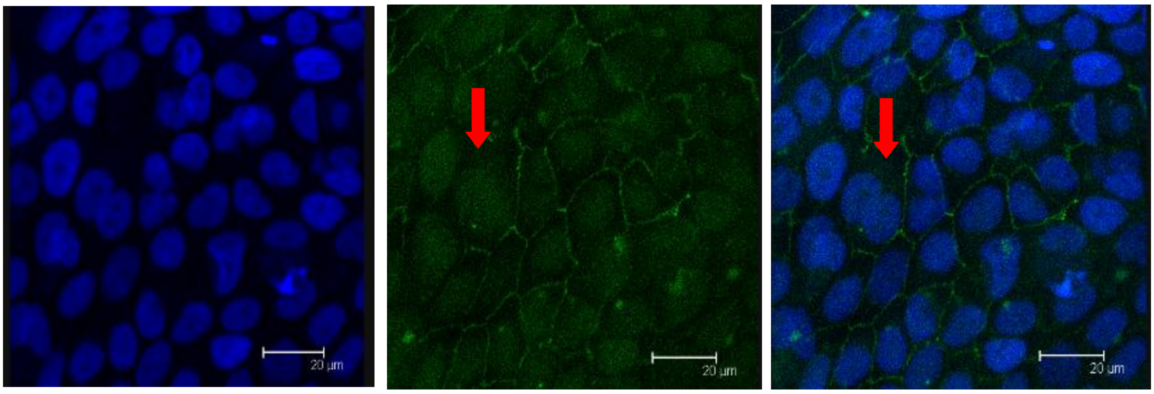
Figure 3.6 Effect of tested cytokines on FD10 permeability across Calu-3 layers at 4˚C. Cytokines treatment for 96 hours.

*FD10 permeability expressed as P_{app}, calculated using the equation of apparent permeability coefficient which described in Chapter 2. Results presented as the mean ± SD (n=4). Statistical analysis calculated by t-test (P value: **** < 0.00001, ns > 0.05).*

3.3.2.3 Effect of Cytokine Application on ZO-1

In establishing the effect of cytokine treatment on Calu-3 layer barrier, changes in cell layer TEER and permeability to a model hydrophilic macromolecule were determined, as described above. In addition to these, immunostaining of a key tight junction protein, called *zonula occludens-1* (ZO-1), was employed as a tool to test the possibility of structural changes in the tight junctions resulting from cytokine treatment. In this instance, only TNF-α-treated cell layers were imaged based on the indications from the above experiments that this cytokine induces greater effects on the epithelial cell barrier. Figure 3.7 shows confocal micrographs of Calu-3 cells, immunostained for ZO-1 following their culture as polarised layers and treatment with TNF-α for four days (3.7a) and control cell layers without cytokine treatment (3.7b). In both subfigures, ZO-1 staining (green) appears in a typical manner of 'belts' surrounding adjacent cells (blue cell nuclei). While the general staining pattern is similar in both conditions, there appears to be a loss of staining intensity in cells treated with TNF-α. Furthermore, under the latter conditions, the ZO-1 'belts' appear somewhat discontinued (arrows), compared to control cells where a more continuous 'chicken-wire'-like network is apparent.

a)



b)

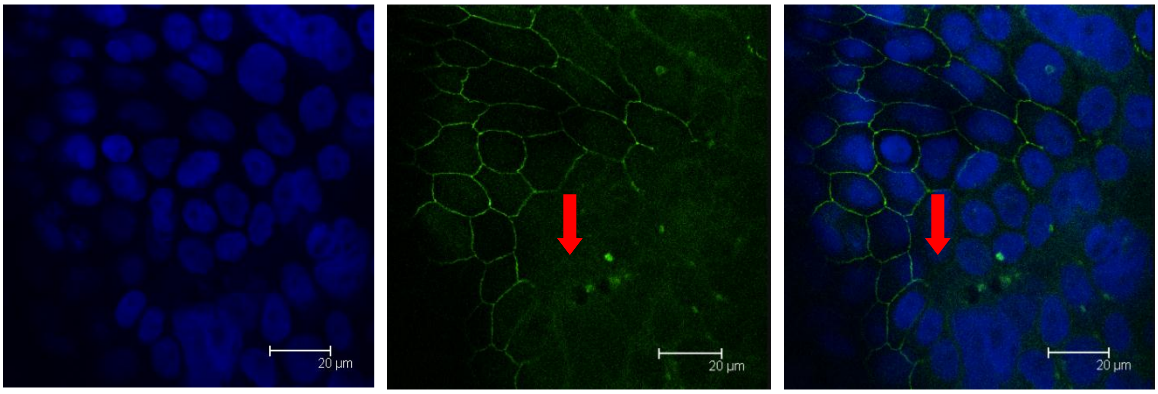


Figure 3.7 a) Immunostaining for ZO-1 tight junction protein in cell layers treated with TNF-α for four days. a) TNF- α treated cell layer and b) control cell layer.

Blue: cell nuclei, Green: tight junction protein (ZO-1) distribution. Protocol described in section 2.2.5. Full image combines blue for cell nuclei and green for ZO-1 staining.

3.3.3 Effect of Combined Cytokine Application on Calu-3 Layer

3.3.3.1 Effect on TEER

In the data presented so far, polarised Calu-3 cell layers were treated with one of the three tested cytokines and the effects on the cell layer barrier measured. To establish whether TNF-α, IL-4 or IL-13 potentially display a synergistic effect on the epithelial barrier, the cytokines were applied to the cell layers in combination and the parameters indicative of the barrier function measured.

Figure 3.8 shows TEER changes after cell layers were incubated with the cytokines, added in combination, for four days. The following combinations of cytokines were added to the cells: TNF-α + IL-4, TNF-α + IL-13, IL-4 + IL-13 and TNF-α + IL-4 + IL-13. The data shows that with the exception of IL-4 + IL-13, all other tested conditions produced a marked reduction of TEER. The reduction in TEER amounted to 28%, 32% and 33% of control cell layers with the combinations of TNF-α + IL-4, IL-4 + IL-13 and TNF-α + IL-4 + IL-13, respectively ($p < 0.0001$ in all instances). It should be noted that a significant decrease in TEER values was associated with presenting of TNF-α in any combination. The combination of IL-4 and IL-13, not including TNF-α, did not show a significant effect on Calu-3 layer TEER.

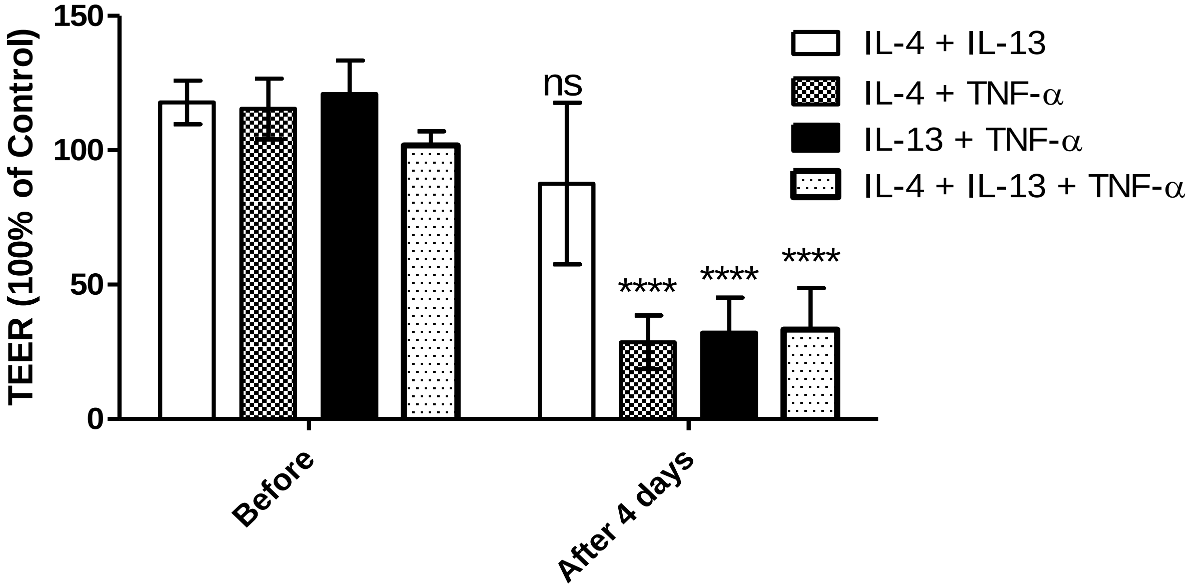


Figure 3.8 TEER measurements of Calu-3 cells cultured on filters using AIC conditions and treated by cytokines combinations (IL-4 and IL-13 at (5ng/ml) and TNF-α at (25ng/ml)) for 4 days.

*TEER is expressed as % of Control (Calu-3 without cytokines). Background TEER due to the filter was subtracted from the reported TEER values. Data presented as the mean ± SD (n=4). Statistical analysis calculated by t-test (P value: * < 0.05, ** < 0.001, *** < 0.0001, ns > 0.05).*

3.3.3.2 Effect on FD10 Permeability

3.3.3.2.1 Permeability at 37°C

Figure 3.9 shows the permeability of FD10 across polarised Calu-3 layers previously treated with the combination of cytokines, as detailed in the previous section. The data shows that the combination of TNF-α with IL-4 exerted the most prominent effect on the cell layer barrier. The increase in FD10 permeability (expressed as P_{app}) amounted to 2.6-fold, from 55 x 10^{-8} cm/s to 143 x 10^{-8} cm/s, which was a statistically significant difference compared to the control cell layers ($p < 0.05$). Other combinations of cytokines did not produce significant increases in FD10 permeability.

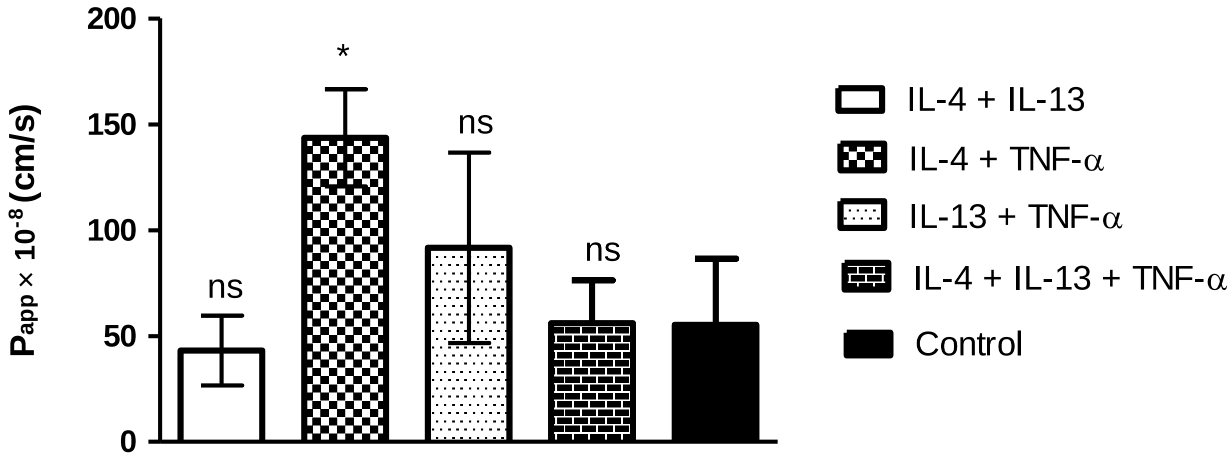


Figure 3.9 Effect of combined cytokines treatment for four days on FD10 permeability across Calu-3 layers at 37˚C.

*FD10 permeability expressed as P_{app}, calculated using the equation of apparent permeability coefficient which described in section 2.2.4. Results presented as the mean ± SD (n=4). Statistical analysis calculated by t-test (P value: * < 0.05, ns > 0.05).*

3.3.3.2.2 Permeability at 4°C

Conducting the permeability experiment at 4˚C following cell treatment with different combinations of cytokines, is shown in Figure 3.10. Under the tested conditions, FD10 permeability was significantly higher (compared to control) across the cell layers subjected to the following combinations of cytokines: IL-4 + TNF-α, IL-13 + TNF-α and IL-4 + IL-13 + TNF-α. The combination of IL-4 and IL-13 displayed no significant effect on permeability.

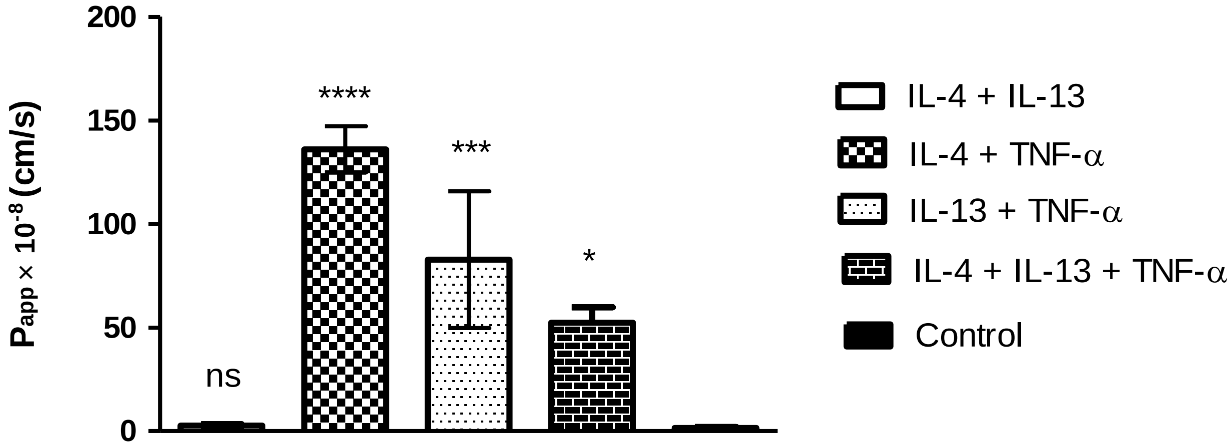


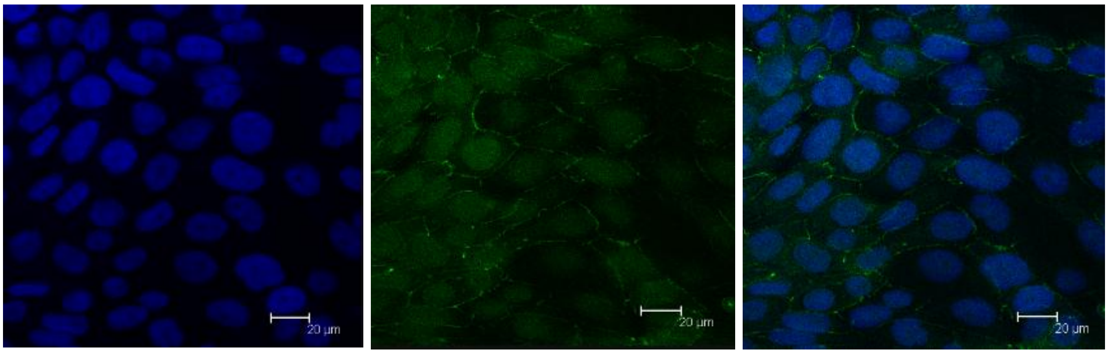
Figure 3.10 Effect of combined cytokines treatment for four days on FD10 permeability across Calu-3 layers at 4˚C.

*FD10 permeability expressed as P_{app}, calculated using the equation of apparent permeability coefficient which described in section 2.2.4. Results presented as the mean ± SD (n=4). Statistical analysis calculated by t-test (P value: * < 0.05, ** < 0.001, *** < 0.0001, ns > 0.05).*

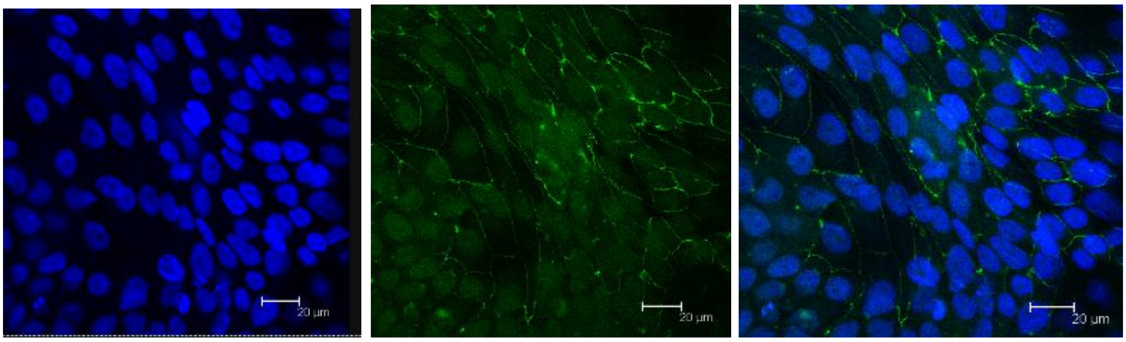
3.3.3.3 Effect of Treatment of Cells with Cytokine Combinations on ZO-1

ZO-1 tight junction protein expression pattern was also determined following incubation of polarised Calu-3 cells with combinations of cytokines. The combinations of cytokines that produced most prominent effects on the cell layer barrier were investigated in the current experiment. These included the combination of TNF-α with IL-4 (Figure 3.11a) and TNF-α with IL-13 (Figure 3.11b). The images reveal a somewhat lower staining intensity, which was also more discontinuous in cells treated with both combinations of cytokines, as compared to control cell layers (no cytokines treatment) (Figure 3.11c, also shown in Figure 3.7b).

a)



b)



c)

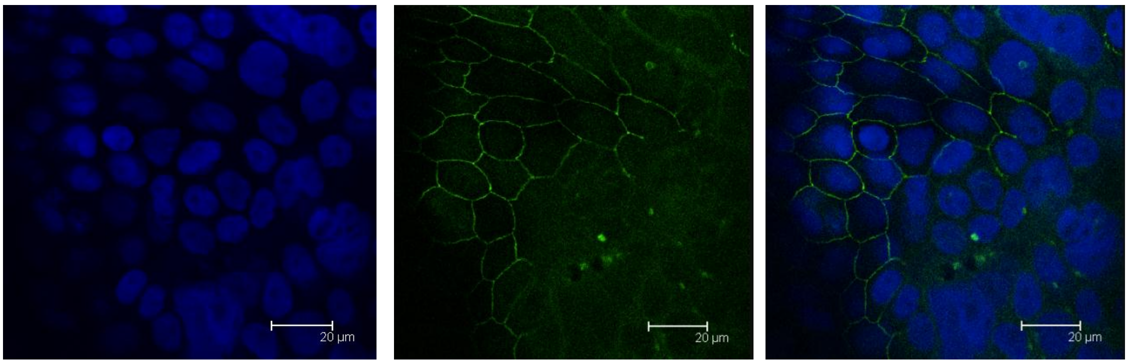


Figure 3.11 Immunostaning of TJs protein (ZO-1) of Calu-3 cells pre-treated with cytokines combinations for 4 days. a) TNF-α (25 ng/ml) + IL-4 (5ng/ml) b) TNF-α (25ng/ml) + IL-13 (5ng/ml) c) Control cell layers not treated with cytokines.

Note that 'not treated' images were also shown in Figure 3.7b. Green is ZO-1 staining and blue is nuclear staining. Protocol described in section 2.2.5.

3.4 Discussion

Evidence from recent studies has emerged that cytokines may play a key role in the progression of asthma [1][15] by remodelling the functionality and structure of airway epithelial barrier in asthmatics [16][17]. However, this research area is still at its beginning and there is insufficient knowledge on the mechanisms of how cytokines induce changes in the epithelium and the extent to which the epithelium is compromised. The present study was designed to determine the effect of three proinflammatory cytokines on the airway epithelial barrier. Work employed the Calu-3 cells, based on advantages offered by this cell line when employed as an *in vitro* model of the airways [18][19][20].

Recent work investigating changes in the epithelium in asthma has suggested that the epithelium function as permeability barrier is compromised [21][22] and that this results from changes in the tight junctions. More specifically, down-regulation of tight junction proteins, including ZO-1, has recently been implicated in the epithelial dysfunction in asthma [23]. This disruption in epithelial barrier is thought to be mediated by the action of proinflammatory cytokines [23].

Epithelial tight junctions are complex structures. They are composed of various proteins, such as occludin, claudin, JAM and ZO-1 [24] and have a critical role in maintaining the permeability barrier function of the epithelium [25]. Tight junctions are located at the apical side of epithelial cells and act as a gate, controlling the paracellular permeability [24]. The function of some tight junction components is still poorly understood [25].

Recent studies have shown that pro-inflammatory cytokines induce changes in the composition and structure of epithelial tight junctions and increase paracellular permeability

[26]. In this study to evaluate the effects of proinflammatory cytokines, TNF-α, IL-4 and IL-13, on the airway barrier, formed polarised Calu-3 layers were treated with these cytokines for four days and the barrier properties tested by measuring TEER, macromolecular permeability and structural changes in the tight junctions. Initial experiments tested the conditions under which cell treatment with the cytokines produced most prominent changes in the epithelial barrier. For this the cytokines were applied on the apical, basolateral or both sides of the cell layers. The changes in TEER values and FD10 permeability induced in these conditions in this preliminary set of experiments were not significant, it was noted that basolateral application of the cytokines produced more notable effects compared to their apical addition. This observation can be explained by the presence of TNF-α receptors on the basolateral side of the airway epithelium [27]. It was therefore decided that in the subsequent set of experiments the cytokines were always applied on the basal side of the cell layers.

The concentrations of cytokines employed in this study were initially optimised, with preliminary studies establishing the minimum doses of cytokines that cause maximum response on the epithelial barrier. The use of IL-4 and IL-13 at the concentration employed here (5 ng/ml) to induce inflammation was reported before [2], while TNF-α has been used in such experiments at concentrations ranging from 5 ng/ml up to 100 ng/ml [27][28]. In the present work, TNF-α was used at 25 ng/ml, after initial experiments using TNF-α at 10, 25 and 50 ng/ml established that the epithelial barrier response was similar with 25 and 50 ng/ml (see appendix 8.1).

Observation that TNF-α may exert undesirable effects on mucosal barrier has been reported previously [29] and this work appears to be in agreement It showed that cell layer treatment with TNF-α (applied basolaterally) led to a decrease in TEER and this effect of TNF-α on cell layer TEER was reflected in the FD10 permeability study, where cell layers treated with this cytokine were more permeable to FD10, compared to control. To determine that the compromised barrier of the Calu-3 layers following TNF-α treatment results from an effect on the tight junctions, two further experiments were conducted: measurement of FD10 permeability at 4˚C and imaging the cells for structural changes in a key tight junction protein.

Hydrophilic macromolecules pass across the epithelium by a combination of paracellular route and transcellular active transport processes, such as pinocytosis [30]. To establish that the increase in overall FD10 permeability following cytokine treatment results solely from changes in the paracellular barrier, additional FD10 permeability experiments were conducted at 4˚C. Under these conditions, the active transport route will be inhibited and FD10 will traverse the cell layers paracellularly. Therefore, any increased FD10 permeability following cytokine treatment could be a result of changes in the paracellular barrier. Conducting these experiments was important considering that some groups have reported that transcellular transport of material across the epithelium is upregulated in inflammatory conditions affecting the epithelium (e.g. in Crohn's disease) [31].

The data revealed that at 4˚C FD10 permeability across Calu-3 cell layers treated with TNF-α was significantly higher compared to control. In fact the extent of permeability increase following cytokine treatment at 4˚C is similar to that at 37˚C. This indicates that the TNF-α induced increase in epithelial permeability occurs through an effect on the paracellular pathway (i.e. the tight junctions). Furthermore, TNF-α-treated cells displayed changes in the appearance of ZO-1 tight junction protein. Together, the data may suggest that the increased permeability of Calu-3 cell layers by TNF-α application results from structural changes in the epithelial tight junctions. The data is in agreement with previous reports that TNF-α plays a key role in remodelling the airway epithelium [32] by changing the expression of tight junction proteins such as ZO-1[25].

The effect of IL-4 on the epithelial barrier is less clear. The present data suggest that, apart from a single instance where an increase in FD10 permeability was apparent, other experiments found that the change in permeability was not statistically significantly different, compared to control. IL-4 has been previously shown to affect the airway barrier by decreasing the expression of tight junction components, such as occludin and ZO-1 [2]. Finally, treatment of Calu-3 cell layers with IL-13 produced no significant effect on their properties, as indicated by non-significant changes in TEER and FD10 permeability.

To examine whether the tested cytokines produce synergetic effects on the Calu-3 barrier, the cytokines were added to the cells in combinations. It was noted that all the combinations of cytokines containing TNF-α (TNF-α + IL-4, TNF-α + IL-13 and TNF-α + IL-4 + IL-13) produced significant effects on the Calu-3 barrier, as indicated by notable changes in TEER and FD10 permeability. The combination of cytokines which included TNF-α led to larger effect on the cell layer barrier compared to individual application of the cytokines. The data therefore points to a synergistic effect of the cytokines on the mucosal barrier – a phenomenon that has been reported previously in a study showing that the effects of TNF-α are most prominent when combined with other cytokines [33].

3.5 Conclusions

The work in this chapter demonstrates that some proinflammatory cytokines produce epithelial barrier disruptive effects on airway Calu-3 cell layers and suggests that the mechanism of this disruption is related to actions on the tight junctions, including changes in ZO-1 distribution. The work contributes to our understanding of the role of cytokines on lung inflammatory disease.

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Chapter 4

Effect of Cytokines on Barrier Characteristics of Human Intestinal Epithelial Cell Layers (Caco-2)

4.1 Introduction

The intestinal epithelium is a biophysical wall that controls the absorption of nutrients and has a protective role by selectively limiting the passage of noxious elements from the external environment into the body [1][2][3][4]. In inflammatory bowel conditions such as Crohn`s disease and Ulcerative Colitis, the intestinal barrier has been reported to be defective and associated with paracellular leakiness [5][6]. A number of studies have demonstrated disruption of the tight junction complexes in Crohn`s disease [6][7]. This defective barrier capacity of the intestinal epithelium in inflammatory conditions therefore compromises the physiological function of the epithelium in preventing the passage of potentially harmful substances found in the intestinal lumen into the body [8]. In addition to an increased epithelial membrane permeability [2], abnormally high mucus secretion is another feature of the inflamed intestinal epithelium [9].

Proinflammatory cytokines have been shown to play a key role in remodelling of the intestinal mucosal barrier in inflammatory bowel conditions. In this respect, downregulation of tight junction protein ZO-1, leading to increased paracellular permeability of the intestinal epithelium has been reported [1][10][11]. Proinflammatory cytokines such as TNF-α and interleukins are therefore heavily involved in the progression of inflammatory bowel disorders such as Crohn`s disease and Ulcerative Colitis [1][13]. Clinical investigations have documented that intestinal inflammatory diseases can be characterised by elevated levels of proinflammatory cytokines [10], which mediate remodelling leading to dysfunctional epithelial barrier [13]. Some proinflammatory cytokines were described in detail in sections 1.1 and 3.1.

The Caco-2 cell line used as a model of the intestinal epithelium is used widely in pharmaceutical research to study or predict the absorption of pharmaceutical agents and study intestinal disease [14][15][16][17]. The Caco-2 cell line expresses functional and morphological characteristics of human intestine [12] and it is used extensively due to the good *in vitro*-*in vivo* correlation provided by this model [17][18]. When cultured *in vitro*, the Caco-2 cell line has the ability to form tight junctions and express relevant components (found *in vivo*) such as transport and efflux proteins and brush border digestive enzymes [19][4].

This chapter assesses the effect of select cytokines on the epithelial barrier of the Caco-2 monolayers, which were used as a model of the intestinal epithelium. Specifically, the influence of TNF-α, IL-4 and IL-13 on cell monolayer transepithelial electrical resistance and macromolecular permeability were investigated.

4.2 Methods

4.2.1 Effect of Site-Specific Cytokine Addition on Epithelial Barrier

4.2.1.1 Effect on TEER

Caco-2 cells were used in this work between passages 46-76. Cells were routinely cultured on flasks (75 cm^2, canted neck with vented caps) until confluence (cell coverage of flask surface by approximately 80-90%). Next, cells were detached from the flasks (by incubation with trypsin), seeded and subsequently cultured on Transwell® filters using liquid-covered culture conditions (following the protocols described in sections 2.2.1.1 and 2.2.1.2.1).

TNF-α, IL-4 or IL-13 were applied to confluent Caco-2 monolayers at day 21 of culture on Transwell® supports. The cytokines were only applied to the cell monolayers expressing a TEER >1000 $\Omega.cm^2$, which was confirmed by measurement using an epithelial voltohmmeter (a detailed description of TEER measurement method is included in sections 2.2.2 and 2.2.4). TNF-α (25 ng/mL), IL-4 (5 ng/mL) or IL-13 (5 ng/mL) were applied either on the apical side or the basolateral side of the cell monolayers. TEER was subsequently measured periodically during the period that the cell monolayers were treated with the cytokines, which was 3-4 days.

4.2.1.2 Effect on FD10 Permeability

4.2.1.2.1 FD10 Permeability at 37ºC

Caco-2 cells were cultured on Transwell® filters, as described previously. Only cell monolayers displaying TEER values >1000 $\Omega.cm^2$ were used in this study. Recombinant human IL-4 and IL-13 were applied to the cells at 5 ng/ml, whereas TNF-α at 25 ng/ml in the culture medium. The cytokines were added to the cells apically only, or on the basolateral side. A control experiment was conducted where the culture medium without the cytokines was applied to the cells at the same time. Permeability experiments were conducted following a 4-day cell treatment with cytokines and 21 day culture on Transwell® supports. FD10 permeability experiments were conducted using HBSS/HEPES as the transport medium. Cells were initially equilibrated in HBSS/HEPES (for approximately 45 min) at 37ºC, followed by the addition of FD10 (in HBSS/HEPES at 500 µg/ml). Cells were placed in an incubator at 37ºC in between sampling periods. The general protocol for permeability experiment was described in section 2.2.4.

4.2.1.2.2 FD10 permeability at 4ºC

FD10 permeability at 4ºC was conducted in a similar way as at 37ºC. However, polarised Caco-2 monolayers (displaying TEER > 1000 $\Omega.cm^2$) were initially equilibrated with HBSS/HEPES at 4˚C for 45 min. FD10 was then applied in HBSS/HEPES at 4˚C (500 µg/ml) and the cells were kept at 4˚C between sampling intervals.

4.3 Results

4.3.1 Effect of Site-Specific Cytokine Application on Caco-2 Barrier

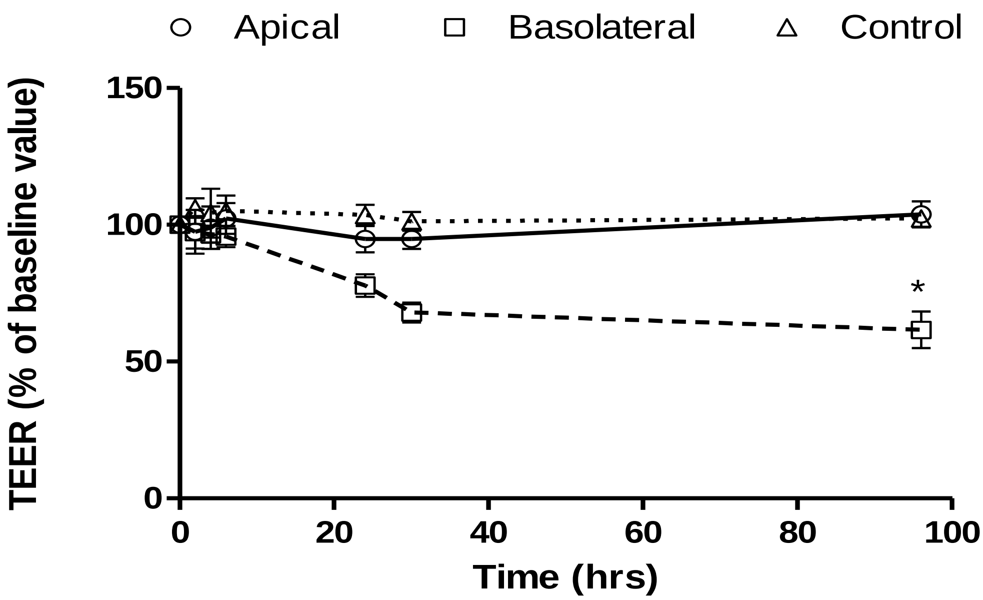
4.3.1.1 Effect on TEER

Figure 4.1 shows the TEER of Caco-2 monolayers following the application of TNF-α, IL-4 or IL-13 at either the apical or basolateral side of the cells. The addition of TNF-α on the apical side of the cell monolayers did not influence the TEER, as suggested by TEER values similar to the control condition (Figure 4.1a). However, following application on the basolateral side of the cells, TNF-α produced a notable decrease in TEER; this equated to approximately 61% of the baseline value after 96 hours of cell treatment. In the control experiment, TEER measurement at 96 hours amounted to approximately 102% of the baseline value. The difference in TEER values between basal TNF-α treatment and control at the final measurement point (96 hours) was statistically significant ($p=0.018$).

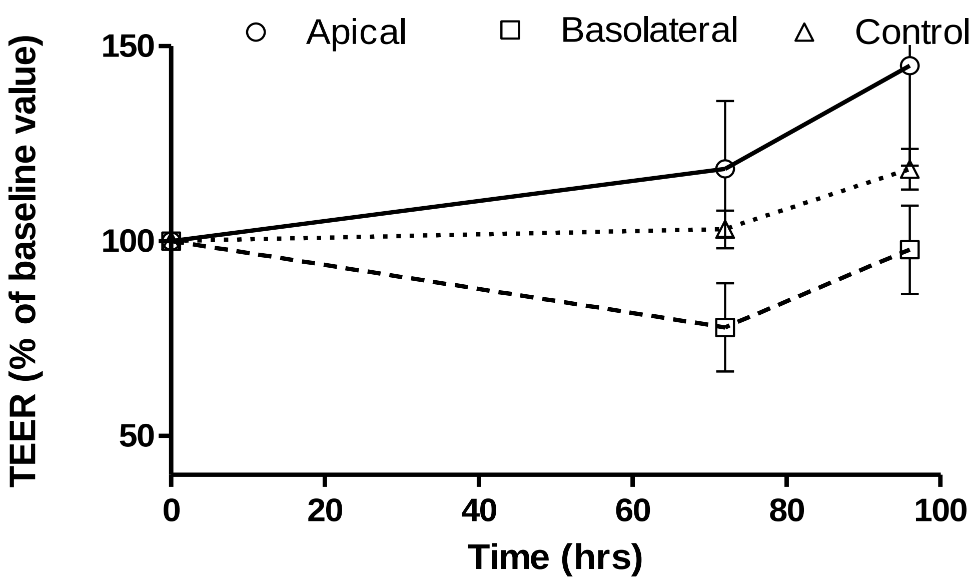
Changes in Caco-2 monolayer TEER after treatment with IL-4 (applied at 5 ng/ml apically or basolaterally) is shown in Figure 4.1b. TEER of cell monolayers treated with IL-4 apically increased by approximately 45% (compared to the baseline value). Basolateral treatment provided a decrease in TEER to approximately 77% of the baseline figure 72 hours after treatment; however, TEER subsequently reversed to approximately 96% (of the baseline value), 96 hours post-IL-4 application. In control cell monolayers (i.e. those not treated with IL-4), TEER after 96 hours amounted to approximately 118% of the baseline value.

Figure 4.1c depicts Caco-2 monolayer TEER over time after cell treatment with IL-13 (5 ng/ml) for 4 days. Both apical or basolateral application of this cytokine showed no decrease in TEER. In fact, cell monolayer TEER increased notably during Caco-2 incubation with IL-13 on the apical or basal side of the cells. A similar pattern was also observed with control cell monolayers.

a)


Apical
Basolateral
Control
150
100
50
0
TEER (% of baseline value)
*
0
20
40
60
80
100
Time (hrs)

b)


Apical
Basolateral
Control
150
100
50
TEER (% of baseline value)
0
20
40
60
80
100
Time (hrs)

c)

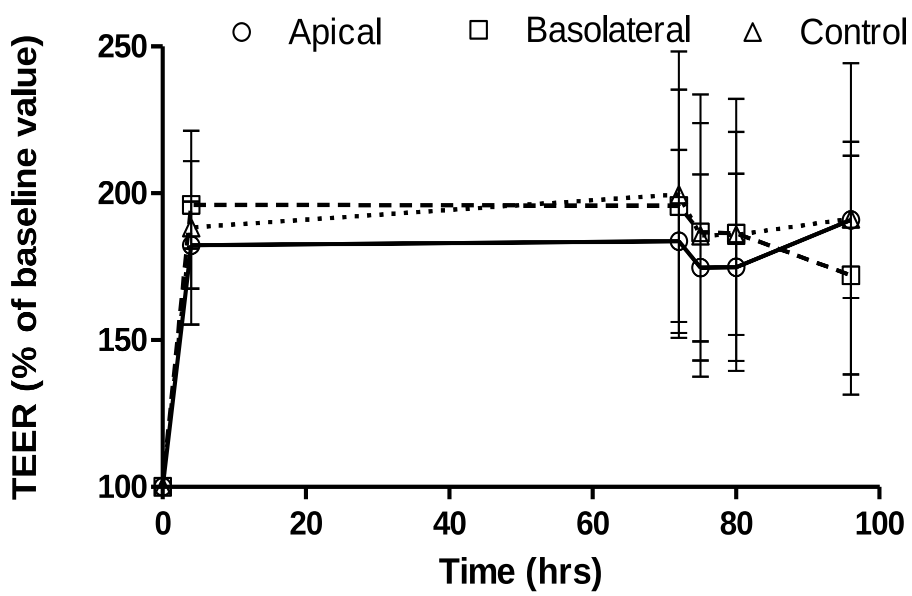


Figure 4.1 TEER measurements of Caco-2 cells cultured on filters and treated by a) TNF-α (25ng/ml), b) IL-4 (5ng/ml), c) IL-13 (5ng/ml) for 96 hours.

*TEER is expressed as % change compared to initial value (baseline value). Background TEER due to the filter was subtracted from the reported TEER values. Data presented as the mean ± SD (n=4). Statistical analysis was calculated by t-test (P value: * < 0.05, ns > 0.05).*

4.3.1.2 Effect on FD10 Permeability

4.3.1.2.1 Permeability at 37°C

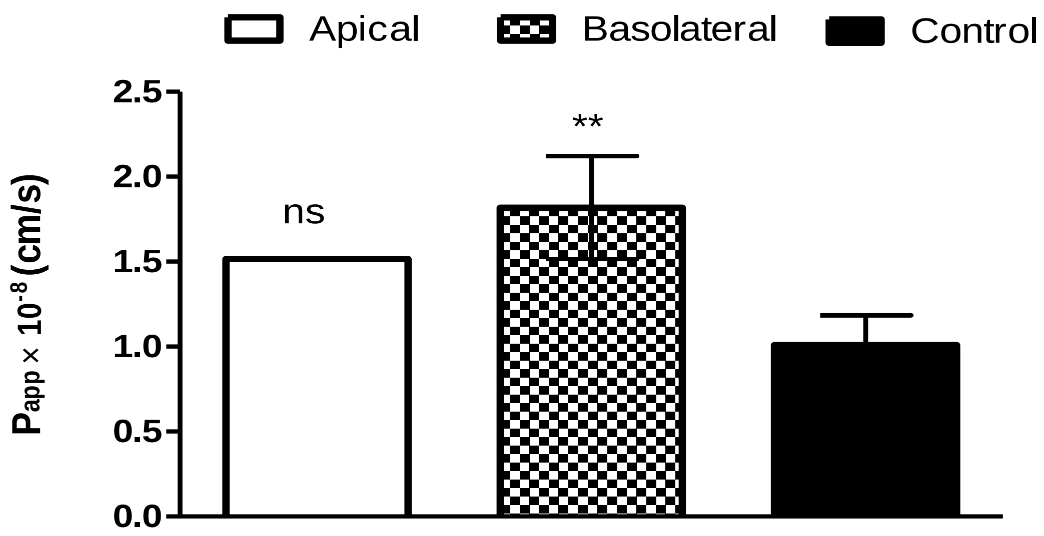
FD10 was measured after Caco-2 monolayers were treated with the cytokines for four days (i.e. under the same conditions as in the TEER study). The permeability of FD10 across the cell monolayers is expressed as apparent permeability coefficient (P_{app}), calculated using the equation described in section 2.2.4.

Figure 4.2a shows FD10 permeability across cell monolayers previously treated with TNF-α. FD10 permeability across the cells treated with TNF-α on the apical side amounted to P_{app} of approximately 1.5 X 10^{-8} cm/s, compared to that across control cell monolayers, which amounted to 1.01 X 10^{-8} cm/s; this difference was not statistically significant ($p > 0.05$). Conversely, Caco-2 monolayers treated with TNF-α on the basolateral side showed a significantly higher FD10 permeability (P_{app} ~1.8 X 10^{-8} cm/s) compared to control ($p = 0.0076$).

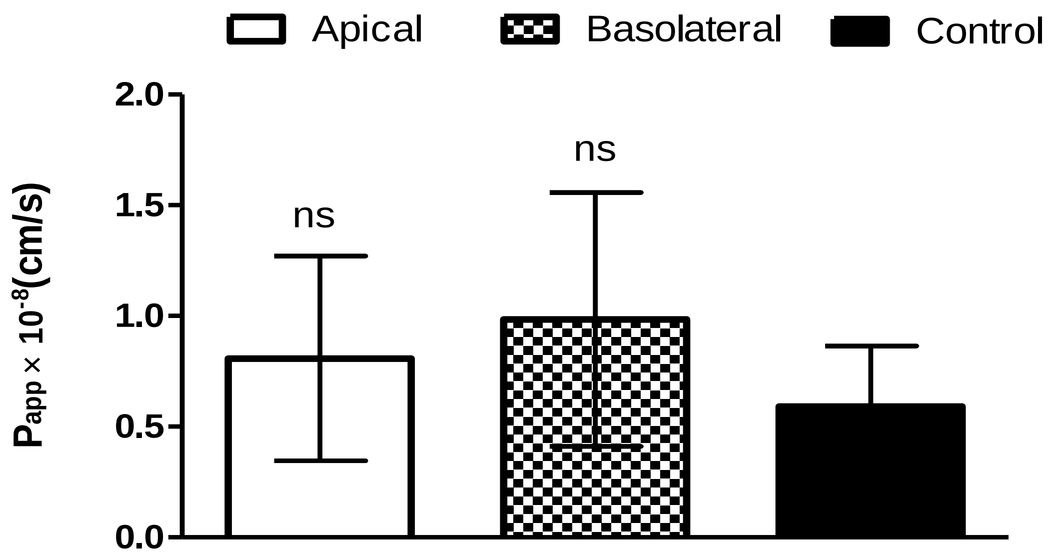
Figure 4.2b shows FD10 permeability across the cell monolayers after their exposure to IL-4 (5 ng/ml) for 4 days. IL-4 treated cell did not show an increase in FD10 permeability compared to control, as suggested by apparent permeability values of approximately 0.80 X 10^{-8} cm/s and 0.98 X 10^{-8} cm/s, for apical and basolateral treatment, respectively, which were not statistically significant (p=0.21 and 0.39, respectively) compared to control.

Likewise, cell monolayer treatment with IL-13 (5 ng/ml for 4 days) did not show notable effects on cell permeability to FD10, regardless of the site of application. The data, shown in Figure 4.2c, show that FD10 permeability in cell monolayers treated with IL-13 apically or basolaterally amounted to approximately 2.8 X 10^{-8} cm/s and 3.2 X 10^{-8} cm/s, respectively. In both scenarios, the difference in permeability to control (P_{app} 2.3 X 10^{-8} cm/s) is insignificant ($p = 0.5$ and 0.9 for apical and basolateral IL-13 treatment compared to control, respectively).

a)


Apical
Basolateral
Control
2.5
2.0
1.5
1.0
0.5
0.0
ns
**
P app × 10-8 (cm/s)

b)


Apical
Basolateral
Control
2.0
1.5
1.0
0.5
0.0
ns
ns
P app × 10-8 (cm/s)

c)

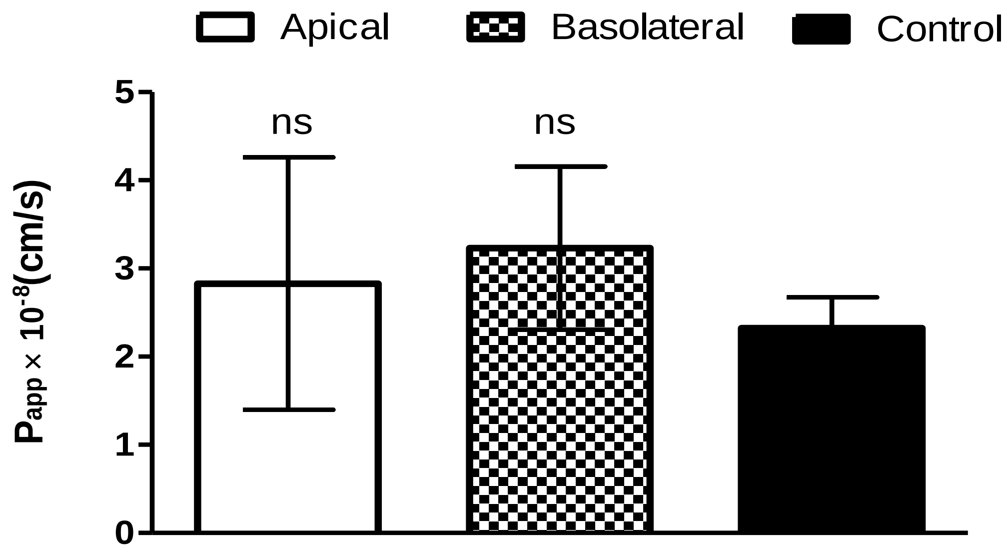


Figure 4.2 Effect of a) TNF-α (25ng/ml), b) IL-4 (5ng/ml), c) IL-13 (5ng/ml) on FD10 permeability across Caco-2 layers at 37˚C.

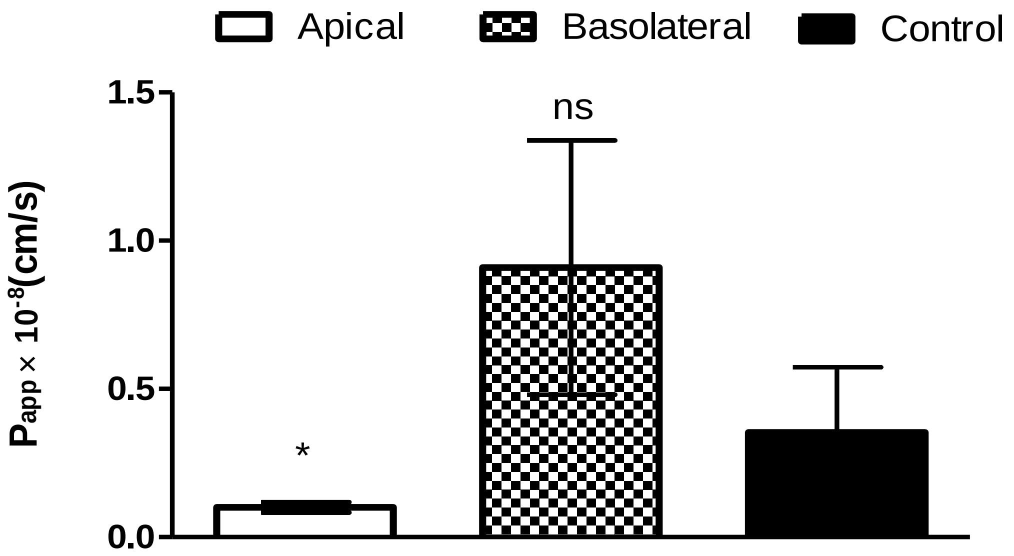
*FD10 permeability expressed as Papp, calculated using the equation of apparent permeability coefficient which described in 2.2.4. Results presented as the mean ± SD (n=4). Statistical analysis was calculated by t-test (P value: ** < 0.001, ns > 0.05).*

4.3.1.2.2 Permeability at 4°C

To determine whether the alteration in permeability after cell treatment with the proinflammatory cytokines is due to an influence of cytokines on the tight junctions, additional FD10 permeability experiments were conducted at 4˚C, where any permeability largely occurs through the paracellular corridor (active transport processes are inhibited). Figure 4.3 shows FD10 permeability across Caco-2 cell monolayers at 4˚C, following prior cytokine treatment at normal cell culture conditions (37˚C; treatment of cell monolayers for 4 days). Figure 4.3a depicts FD10 permeability at 4˚C across the cell monolayers pre-treated with TNF-α (25 ng/ml). Basolateral treatment of the cell monolayers with TNF-α did not significantly alter the paracellular route, as indicated by non-significant difference between FD10 permeability in these conditions (0.91 x 10^{-8} cm/s) and control (0.35 x 10^{-8} cm/s; p = 0.55). Caco-2 monolayers treated with TNF-α on their apical pole displayed a lower FD10 permeability (0.101 x 10^{-8} cm/s) compared to control (p=0.0302).

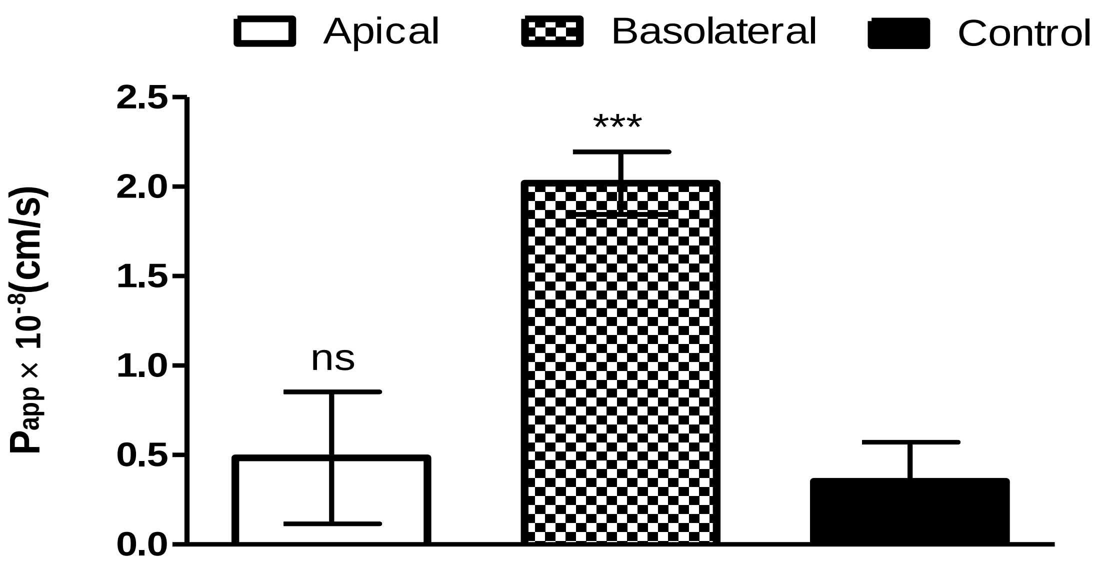
Figure 4.3b shows FD10 permeability in cold conditions (4˚C), measured across Caco-2 monolayers pre-treated with IL-4 (5 ng/ml). Polarised cells subjected to IL-4 basolaterally exhibited a significantly higher permeability (2.02 x 10^{-8} cm/s), as compared to control (0.35 x 10^{-8} cm/s; p= 0.0005). On the other hand, apical treatment did not significantly affect the permeability under these conditions (P_{app} amounted to 0.48 x 10^{-8} cm/s; p= 0.1313, comparison with control). FD10 permeability at 4˚C across Caco-2 monolayers pre-treated with IL-13 (5 ng/ml) is shown in Figure 4.3c. Apically-treated cell monolayers displayed FD10 permeability of 0.42 x 10^{-8} cm/s, whilst basolaterally-treated cells showed the permeability of approximately 1 x 10^{-8} cm/s. However, both these values are not statistically significantly different compared to control (p = 0.07 and 0.65, compared to control for apical and basolateral cytokine treatment, respectively).

a)



Apical
Basolateral
Control
1.5
1.0
0.5
0.0
ns
*
Papp × 10-8(cm/s)

b)



Apical
Basolateral
Control
2.5
2.0
1.5
1.0
0.5
0.0
***
ns
Papp × 10-8(cm/s)

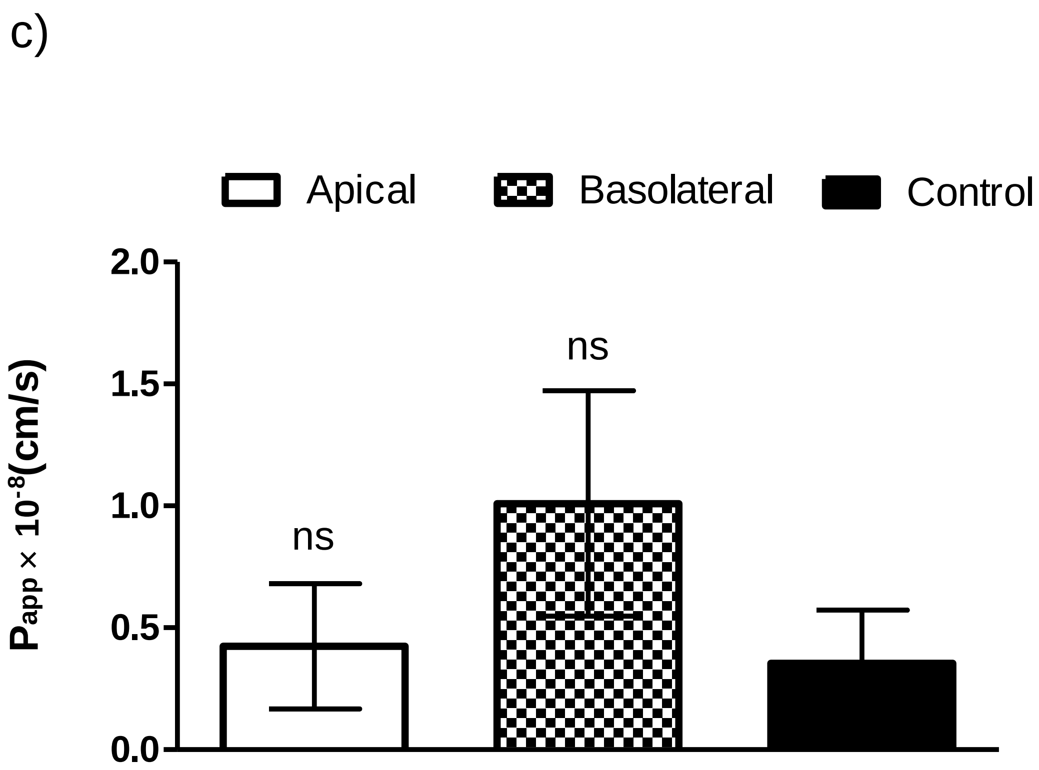


Figure 4.3 Effect of a) TNF-α (25ng/ml), b) IL-4 (5ng/ml), c) IL-13 (5ng/ml) on FD10 permeability across Caco-2 layers at 4˚C.

*FD10 permeability expressed as P_{app}, calculated using the equation of apparent permeability coefficient which described in 2.2.4. Results presented as the mean ± SD (n=4). Statistical analysis was calculated by t-test (P value: * < 0.05, *** < 0.0001, ns > 0.05).*

4.3.2 Effect of Basolateral Cytokine Application on Cell Layer Barrier: a Comparison between Different Cytokines

4.3.2.1 Effects on TEER

Based on the observation that the tested cytokines produce a larger effect on the epithelial barrier when applied on the basolateral side of polarised cells, in the further experiments (described in the subsequent sections of this chapter) cytokines were exclusively applied on the basolateral side of Caco-2 monolayers.

Figure 4.4 compares the effects of different cytokines (when applied to confluent Caco-2 monolayers basally for 4 days) on TEER. The cytokines were used at the same concentrations as in the previous section, namely 25 ng/ml (TNF-α) and 5 ng/ml (IL-4 and IL-13). The data show TEER values before (baseline) and after basolateral cell incubation with the cytokines for four days. In this experiment, all cytokines were found to cause a significant decrease in Caco-2 monolayer TEER. Electrical resistance across the monolayers decreased by approximately 48%, 38% and 37% (compared to baseline values) after cell treatment with TNF-α, IL-4 and IL-13, respectively.

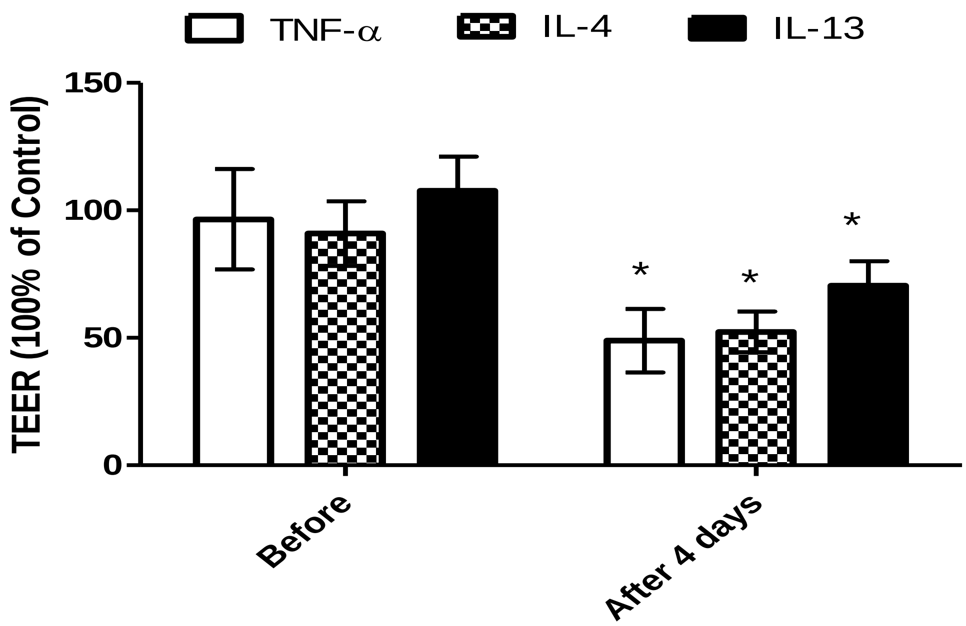


Figure 4.4 TEER measurements of Caco-2 cells cultured on filters and treated by individual cytokines {IL-4 and IL-13 at (5ng/ml) and TNF-α at (25ng/ml)} for 4days.

*TEER is expressed as % of Control (Caco-2 without cytokines). Background TEER due to the filter was subtracted from the reported TEER values. Data presented as the mean ± SD (n=4). Statistical analysis was calculated by t-test (P value: * < 0.05, ns > 0.05).*

4.3.2.2 Effects on FD10 Permeability

4.3.2.2.1 Permeability at 37°C

Figure 4.5 shows FD10 permeability across Caco-2 cells, previously treated with different cytokines basolaterally. Treatment with the cytokines did not produce a notable effect on cell monolayer permeability in this experiment: FD10 permeability was similar in all cases, equating to approximately 2.27, 3.78, and 3.78 X10^{-8} cm/s for TNF-α, IL-4 and IL-13-treated cells, respectively). The difference relative to control samples (whereby P_{app} amounted to 2.65 X 10^{-8} cm/s) was not statistically significant in all cases ($p > 0.05$).

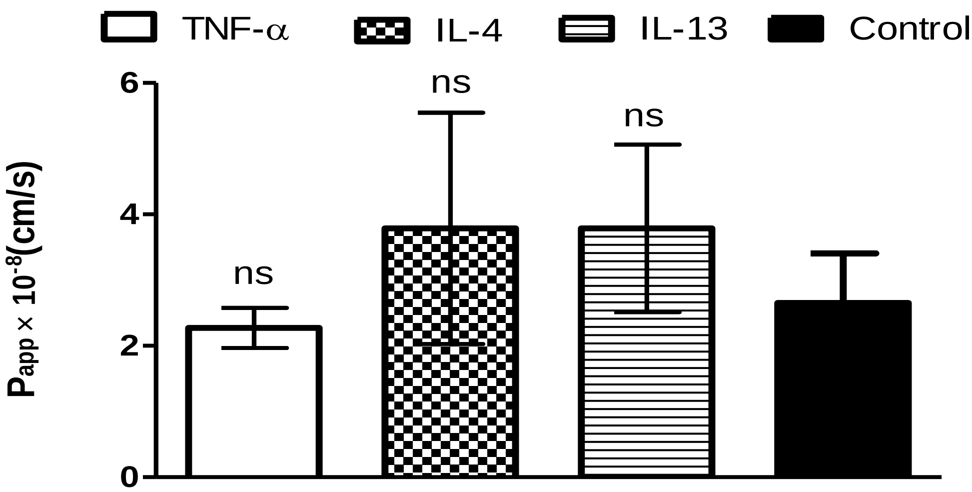


Figure 4.5 Effect of individual cytokines on FD10 permeability across Caco-2 layers at 37˚C.

FD10 permeability is expressed as P_{app}, calculated using the equation of apparent permeability coefficient which described in 2.2.4. Results presented as the mean ± SD (n=4). Statistical analysis was calculated by t-test (P value: ns > 0.05).

4.3.2.2.2 Permeability at 4°C

Figure 4.6 compares FD10 permeability in cold conditions (4˚C) after cell treatment with the tested cytokines for 4 days. In the current experiment, cell monolayers treated with TNF-α, IL-4 or IL-13 gave rise to FD10 permeability of 3.71, 2.12, and 1.11 x 10^{-8} cm/s, respectively. FD10 permeability in control conditions (where cytokines addition was omitted) was 5.37 x 10^{-8} cm/s. The data analysis shows that difference to cytokine treatment was not statistically significant in all cases.

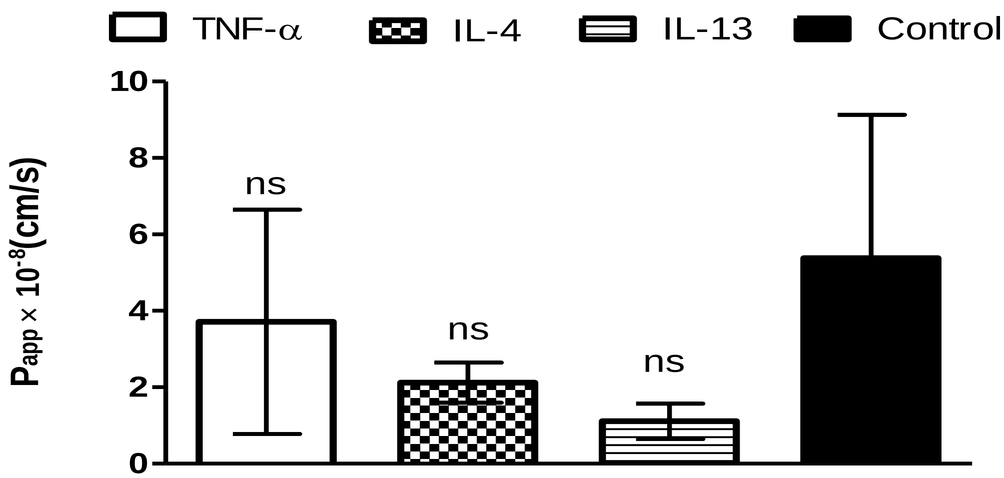


Figure 4.6 Effect of individual cytokines on FD10 permeability across Caco-2 layers at 4˚C.

FD10 permeability expressed as P_{app}, calculated using the equation of apparent permeability coefficient which described in 2.2.4. Results presented as the mean ± SD (n=4). Statistical analysis was calculated by t-test (P value: ns > 0.05).

4.3.3 Effect of Combined Cytokine Application on Caco-2 Barrier

4.3.3.1 Effect on TEER

Experiments presented in this section aimed to establish whether TNF-α, IL-4 or IL-13 exert a synergistic effect on the epithelial barrier of Caco-2 monolayers. The tested cytokines were therefore added to the cells in combination in the current experiments and TEER and FD10 permeability measured. Specifically, the following combinations of cytokines were used: IL-4 + TNF-α, IL-13 + TNF-α, and IL-4 + IL-13 +TNF-α.

Effect of cell treatment with the combinations of cytokines on cell monolayer TEER is shown in Figure 4.7. TEER values were measured twice, once at day 0 before cytokines addition and the second measurement was 4 days post cytokines treatment, and the results expressed as % of control value. The data demonstrate that when applied in any combination, the tested cytokines significantly decreased the cell monolayer TEER. The decrease in TEER with IL-4 + IL-13 combination amounted to approximately 60% ($p <0.05$). Presenting TNF-α in any combination resulted in a marked decrease of TEER. This decrease was approximately 77%, 89% and 104% in cell monolayers exposed to IL-4 + TNF-α, IL-13 + TNF-α, and IL-4 + IL-13 + TNF-α, respectively ($p < 0.001$ in all instances).

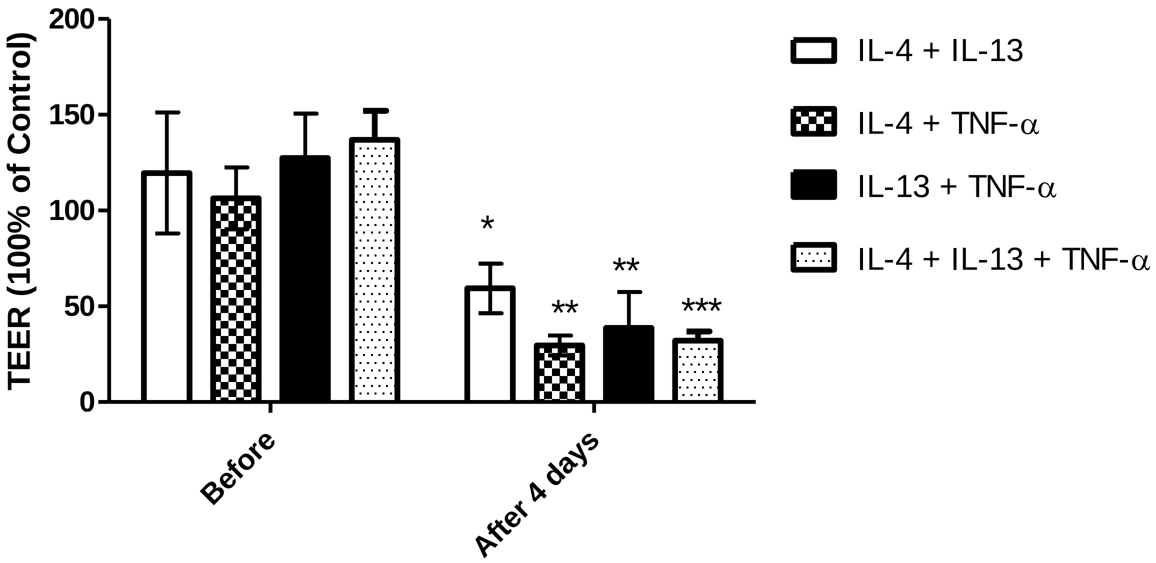


Figure 4.7 TEER measurements of Caco-2 cells cultured on filters and treated by combined cytokines [IL-4 and IL-13 at (5ng/ml) and TNF-α at (25ng/ml)] for 4 days.

*TEER is expressed as % of Control (Caco-2 without cytokines). Background TEER due to the filter was subtracted from the reported TEER values. Data presented as the mean ± SD (n=4). Statistical analysis was calculated by t-test (P value: * < 0.05, ** < 0.001, *** < 0.0001, ns > 0.05).*

4.3.3.2 Effect on FD10 Permeability

4.3.3.2.1 Permeability at 37°C

Caco-2 monolayer permeability (to a macromolecular model, FD10) following treatment with a combination of three cytokines is shown in Figure 4.8. The data shows that the only combination that induced a significant effect on cell monolayer permeability was IL-4 with IL-13. In this instance, FD10 permeability amounted to approximately 3.4 x 10^{-8} cm/s, compared to untreated (control) cell monolayers, which displayed FD10 permeability of approximately 0.8 x 10^{-8} cm/s. The other tested combinations of cytokines failed to significantly influence Caco-2 monolayer permeability, with observed FD10 permeability ranging from 0.9 - 1.2 x 10^{-8} cm/s with other tested combinations.

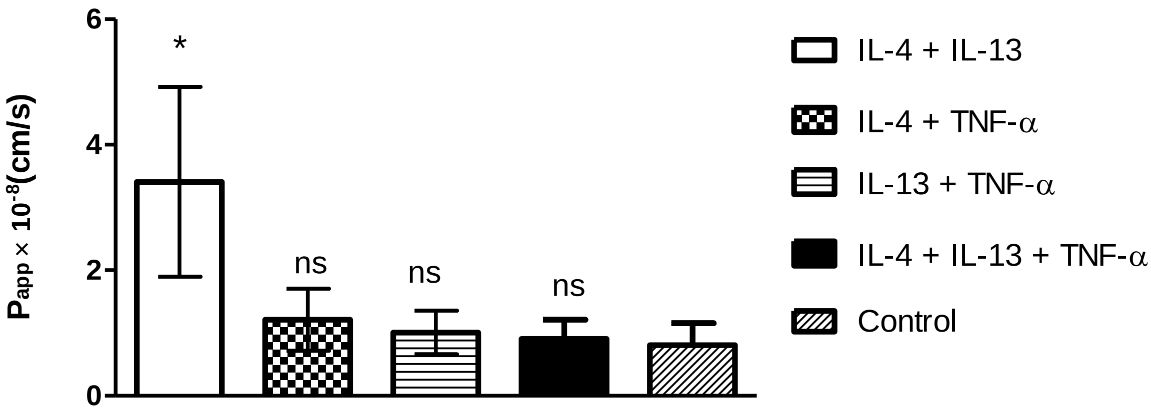


Figure 4.8 Effect of combined cytokines on FD10 permeability across Caco-2 layers at 37˚C.

*FD10 permeability expressed as P_{app}, calculated using the equation of apparent permeability coefficient which described in 2.2.4. Results presented as the mean ± SD (n=4). Statistical analysis was calculated by t-test (P value: * < 0.05, ns > 0.05).*

4.3.3.2.2 Permeability at 4°C

Figure 4.9 shows FD10 permeability across Caco-2 monolayers, as measured at 4˚C following cell treatment with different combinations of cytokines (for 4 days). It is apparent from the figure that FD10 permeability was similar in all the tested conditions and there was no significant increase in permeability following cell treatment with the cytokines.

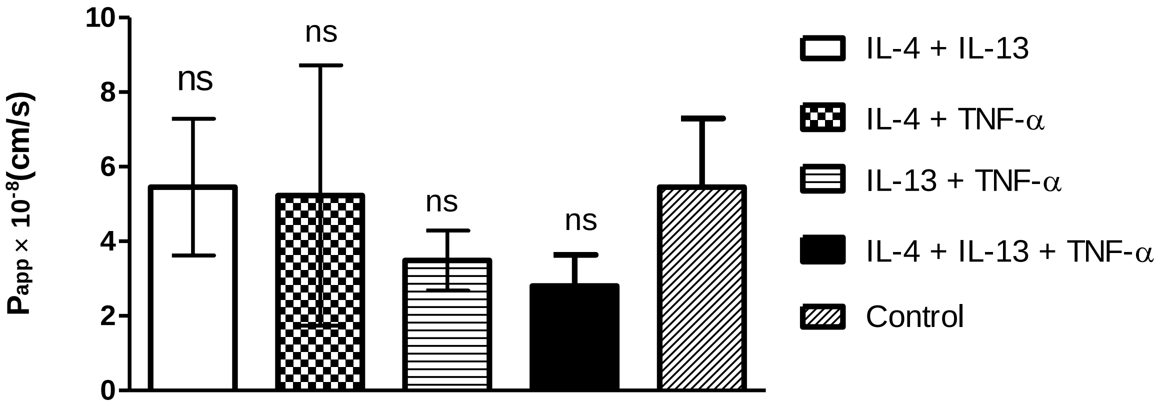


Figure 4.9 Effect of combined cytokines on FD10 permeability across Caco-2 layers at 4˚C.

FD10 permeability expressed as P_{app}, calculated using the equation of apparent permeability coefficient which described in 2.2.4. Results presented as the mean ± SD (n=4). Statistical analysis was calculated by t-test (P value: ns > 0.05).

4.4 Discussion

Inflammatory bowel disease has been linked to an upregulation of proinflammatory cytokines, which play a significant function in mediation of inflammation [20][9]. Furthermore, it has been demonstrated that the intestinal barrier in certain inflammatory conditions is disrupted, whereby the permeability of harmful substances from the intestinal lumen across the gut wall [21][22]. The extent to which the intestinal barrier is compromised in inflammation is not known and was the focus of this chapter. Proinflammatory cytokines, IL-4, IL-13, and TNF-α were used as ‘inducers’ of inflammation in intestinal Caco-2 monolayers and the effect on their barrier determined.

In normal non-pathological state, the intestinal epithelium provides a robust biophysical gate, enabled by structural features such as the tight junctions, preventing non selective absorption of material, including potentially toxic elements, across the gut wall [23][8]. However, one of the main features of some intestinal inflammatory diseases is modulation of tight junction structure and function, leading to increased passage of noxious substances across the epithelium [24]. The intestinal epithelium has been shown to be 'leaky' in inflammatory disease states such as Crohn`s disease [23]. Recent research has suggested that the changes in epithelial barrier in such conditions arise from dysfunctional tight junctions, which in turn are affected by the action of proinflammatory cytokines, including TNF-α and interleukins [24] [21].

This work assessed the effects of three proinflammatory cytokines, namely TNF-α, IL-4 and IL-13, on the epithelial barrier of intestinal Caco-2 cells. The cells were cultured as polarised monolayers – a popular use of Caco-2 cells. Cell monolayers were treated with these cytokines and the epithelial barrier tested by measuring TEER and macromolecular permeability. Caco-2 monolayer treatment with TNF-α led to a significant decrease in TEER when applied on the basolateral side of the cells, whilst no significant effect was observed when TNF-α was presented on the apical side of polarised cell monolayers. The decrease in TEER after cell treatment with TNF-α is expected to be a result of barrier dysfunction (e.g. an effect on the tight junctions) rather than cell death, which would result in dramatically reduced TEER [21]. A similar influence of TNF-α on Caco-2 monolayer TEER has been shown previously [23][21][22].

The pattern of TEER data was somewhat reflected in the permeability study, whereby FD10 permeability across Caco-2 monolayers following a 4-day treatment with TNF-α, applied *basolaterally,* increased (this increase was significant in one experiment and in another failed to reach statistical significance). The observed inverse relationship between TEER and permeability is expected [23], considering that both parameters measure the 'tightness' of the epithelial barrier. Apical treatment of the cell monolayers with TNF-α did not affect the permeability. The influence of cytokines on the epithelial barrier when added basolaterally and the lack of effect following apical exposure has also been reported previously [21]. This observation may be related to the basolateral presence of cytokine receptors in epithelial cells [21].

Several investigations have implicated TNF-α as a mediator of change in the barrier properties of intestinal epithelium, increasing the permeability of tight junctions in bowel disease [25]. The mechanism of influence of TNF-α on epithelial barrier has been suggested to be the down-regulation of occludin and up-regulation of claudin proteins [26]. For example, the down-regulation of occludin expression has been reported in numerous patients suffering from inflammatory bowel disease [26]. Moreover, a recent study has demonstrated elevated expression of claudin in patients with Crohn`s disease [22]. Another change shown to occur in the inflamed epithelium, which is mediated by TNF-α, relates to the myosin light chain kinase (MLCK) protein, which is heavily involved in tight junction regulation [2]. Up-regulation of TNF-α in inflammatory conditions could stimulate MLCK protein expression, opening the tight junctions [13]. TNF-α has the capacity to induce nuclear factor kappa B (NF-κB), which may act to increase the expression of MLCK [22]. The stimulation of MLCK expression led to increased paracellular permeability in Caco-2 monolayers [23].

Furthermore, TNF-α inhibitors, such as cycloheximide, could decrease MLCK expression and lower the permeability across intestinal epithelium [13].

The two interleukin-based cytokines, IL-4 and IL-13 share the same receptors and studies have shown that they also share similar physiological roles [10]. Both IL-4 and IL-13 have capacity to bind IL-4Rα/IL-13Rα1 complex, which stimulates inflammation reaction in numerous diseases [27]. IL-4 was previously reported to produce an increase in intestinal permeability and produce changes in the tight junctions, including decreased ZO-1 and occludin expression and increased expression of claudin-2 [22]. In this work, IL-4 and IL-13 did not display a significant effect on cell monolayer TEER and FD10 permeability. It is presently unclear why the data in this thesis did not confirm the previous findings with regards to the effect of IL-4 and IL-13 on intestinal barrier, though this work employed a cancerous cell line, which may be more 'resistant' to the application of cytokines, as compared to primary cells or the tissue *in vivo*.

Conducting the permeability studies at 4˚C was performed with the view of obtaining mechanistic information on the action of the cytokines on the intestinal Caco-2 monolayers. Inhibiting the active transport route in these conditions would leave the passive route via the paracellular space as the only means for FD10 to traverse the cell monolayers. Any increase in FD10 permeability following cell treatment with the cytokines therefore would indicate a tight junction effect rather than an upregulated active transport route. This is particularly important as active transport processes such as transcytosis have been shown to occur in inflammatory bowel disease [28]. Permeability studies at 4˚C with individual cytokines were somewhat inconclusive, showing a non-significant effect in FD10 permeability with TNF-α and IL-13, whilst IL-4 demonstrated a significant effect in one study.

The most significant effect on the Caco-2 epithelial barrier was provided by application of cytokines in combination. This method of cell treatment, where epithelial cells are exposed to a 'cocktail' of cytokines probably most closely reflects the *in vivo* conditions in inflammatory bowel disease. All the combination of cytokines produced a significant decrease in Caco-2 monolayer TEER. The level of this decrease was notably larger than that with individual application of the cytokines. This synergetic effect on the epithelium resulting from the combined application of has been shown with IL-4 and IL-13 when combined with TNF-α [29]. Regarding the permeability of FD10 across the cell monolayers exposed to the combination of cytokines, IL-4 with IL-13 was the only combination that significantly increased FD10 permeability. It is not clear why other combinations did not produce a significant effect on permeability. It is also not known why, when measured at 4˚C, there was no significant increase in FD10 permeability with all the combination of cytokines. This is especially the case considering the large effect on TEER. However, it may be that TEER is a more sensitive indicator of tight junction opening compared to permeability, especially considering the relatively large molecular weight of FD10 (approximately 10 kDa).

In addition to changes in tight junction permeability, inflammatory bowel disease is also associated with other tissue abnormalities such as defects in mucin expression [1] and the thickness of epithelium [1][30]. Recent research stated that proinflammatory cytokines also play a crucial function in the regulation mucin secretion and therefore the thickness of mucosal wall [1]. Clinical trials have shown that anti-TNF-α antibodies significantly decrease the progress of inflammatory intestinal disorders such as Crohn`s disease and ulcerative colitis [31][32]. The FDA-approved anti-TNF-α, infliximab, has been shown to enhance the intestinal barrier healing and improve the status of intestinal epithelium in inflammatory disorders [33][34]. Whilst reversing the changes in the epithelium in inflammatory disease states is essential for disease treatment, exploiting the defective barrier function of the intestinal epithelium in inflammation could be useful in delivering macromolecular therapeutic agents such as proteins non-invasively.

4.5 Conclusion

Of the tested cytokines, TNF-α and IL-4 produced some effects on the Caco-2 monolayer barrier when applied on the basal side of the cells, whilst apical treatment had no influence. The largest effect on the cell monolayer barrier was seen when the cytokines were added to the cells in combination, showing synergy.

4.6 References

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Chapter 5

Effect of Long-Term Cytokines Treatment on Barrier Characteristics of Human Airway and Intestinal Epithelial Cell Layers

5.1 Introduction

Inflammatory disorders, such as asthma and irritable bowel disease, are commonly characterised by the presence of chronic lesions in the epithelial tissue [1][2][3]. In conjunction with the present inflammation reaction, the 'leakiness' of epithelial mucosa has been reported as one of the symptoms [4][3][5]. The epithelial lesions are believed to mainly be a result of the effects of inflammatory mediators which induce remodelling of epithelial structure and its function [6][7]. It has been shown that endogenous inflammatory mediators play an important function in stimulating the inflammation responses in different tissues of the human body, including pulmonary and intestinal epithelium [8][9]. The presence, and intensity, of inflammatory reaction can cause permanent damage of epithelium [2][7][10][11]. The recent papers attribute a disruption in epithelial barrier properties (the 'leakiness') to be a consequence of damages to the tight junctions, as the essential component of paracellular transport pathway [12][13][14]. With regards to described inflamed cells behaviour *in vivo*, damaged tight junction complex was observed in several respiratory [15] and intestinal studies [16].

Pro-inflammatory cytokines have been found involved as key players in the mechanism of inflammation reaction [7][17][18]. Cytokines are proteins released by wide varieties of cells, and they contribute to the intracellular communication and immunological responses [19][20]. For this project it is important to note that pro-inflammatory cytokines such as interleukin-4 (IL-4) [17], interleukin-13 (IL-13) [21] and tumour necrosis factor-alpha (TNF-α) [22] were found to stimulate *in vivo* dysfunction of epithelium [12]. In several inflammatory disorders (e.g. asthma and Crohn`s disease) cytokine exposure was reported to result in modification of epithelial properties [7][12][18].

In previous Chapters 3 and 4, formed Calu-3 and Caco-2 cell layers were treated for a short-time period with cytokines (for 4 days), to potentially represent acute inflammation conditions. These were assessed in terms of epithelial permeability and tight junction structure. The transepithelial resistance (TEER) and permeability results demonstrated the influences of cytokines tested on epithelial mucosa, particularly tight junctions. The results shown in previous chapters were obtained following a 4 days treatment of formed cell layers with IL-4, IL-13, TNF-α or their combinations. However, these studies did not probe the effect that a prolonged exposure to cytokines has on epithelial cell layers *in vitro*. Therefore, effects of prolonged exposure time of the mucosal cells to the cytokines on epithelial layers properties would be investigated in this chapter. This is aimed to better represent chronic inflammation conditions.

Currently, a number of publications from research laboratories are attempting to design well-characterised *in vitro* model that closely represent inflamed epithelial tissue [2]. Due to the difficulties of using *in vivo* models to study the effect of cytokines on epithelium [2][23][24], designing representative *in vitro* models would be a good approach. Since the remodelling of epithelial structure in lung [25] and intestinal diseases [7] is assumed to be due to chronic inflammation reaction, the effect of chronic / prolong presence of pro-inflammatory cytokines with the epithelial monolayers seem necessary to study. Prolonged treatment of Calu-3 and Caco-2 cells with cytokines could lead to a design of practical *in vitro* model for respiratory and intestinal inflammatory disorders.

Therefore, this chapter examines the effect of long-term pro-inflammatory cytokines treatment (21 days) on epithelial cell layer formation and properties with the aim to expand our understanding of a contribution of cytokines on inflammatory responses in epithelium and to investigate the chronic effect of cytokines on epithelium.

5.2 Methods

5.2.1 Effect of Cytokines on Calu-3 and Caco-2 TEER

Calu-3 and Caco-2 cell lines were plated in 75 cm^2 flasks with passage numbers 37 and 76, respectively. After confluence, cells were seeded on Transwell® (12 mm diameter, 0.4µm pore size) at 100.000 cells/cm^2 seeding density. Calu-3 cells were typically used as air-interfaced culture (AIC), whereas Caco-2 cells were cultured normally as liquid-covered culture (LCC). EMEM medium was used for Calu-3 cells, while Caco-2 was cultured in DMEM medium, and the medium replaced every 2 days. Cell polarised layers integrity was identified by measuring the transepithelial electrical resistance (TEER). A detailed description of TEER measurement method is included in sections 2.2.2 and 2.2.4.

5.2.2 Effect of Cytokines on Calu-3 and Caco-2 Permeability

Recombinant human pro-inflammatory cytokines IL-4, IL-13 (5ng/ml), and TNF-α (25ng/ml) were applied on the basolateral side of Calu-3 and Caco-2 cells. Only the cell layers displaying TEER values >300 and >1000 Ω.cm^{2,} for Calu-3 and Caco-2 respectively, were included in this study. Cells were exposed to cytokines for 21 days (the time needed by cells to form polarised monolayers). In further experiments, cells were treated with TNF-α (the cytokine that provided the most effect on epithelial barrier as shown in earlier chapters) for short-term (4 days) to compare with long-term condition. Control cells were conducted without cytokine treatment. FD10 transport permeability studies were conducted on day 21 in culture after TEER confirmed that cells formed confluent membranes. Cells were firstly equilibrated in warm (HBSS/HEPES, 37˚C) solution, then cells incubated for ~45min to adapt with transport medium and TEER was measured again to confirm cells situation. FD10 solution was replaced with the half-apical medium at concentration (500µg/ml). The rest of experiment was described properly and in more information in 2.2.4.

5.3 Results

5.3.1 Effect of Long-Term Cytokines Application on Calu-3 and Caco-2 Barrier Properties

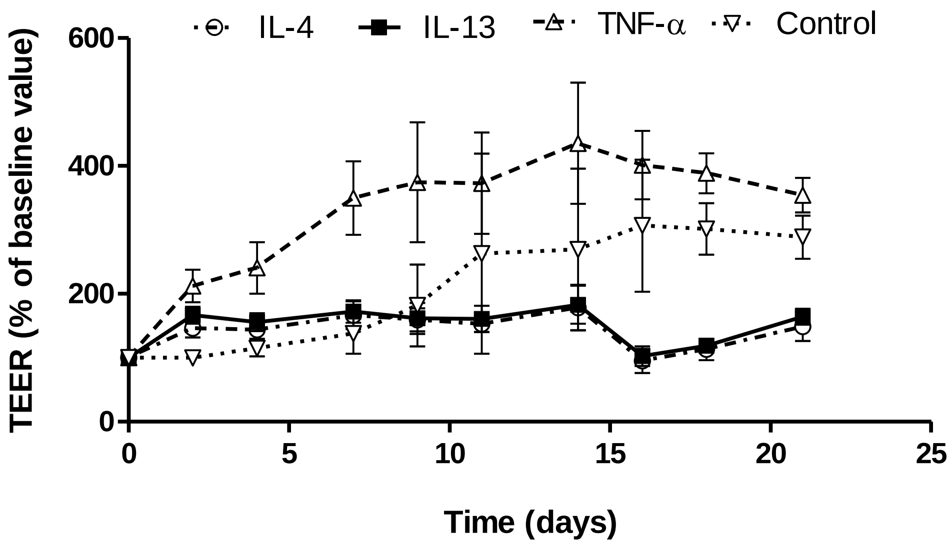
5.3.1.1 Effect on TEER

The effect of long-term cytokines treatment on TEER of Calu-3 and Caco-2 cell layers following the basolateral side addition of TNF-α, IL-4 and IL-13 is presented in Figure 5.1. Figure 5.1a shows the effect of long-term cytokines treatment on TEER of Calu-3 cells grown at air-liquid interface. From the results for control cells, it should be noted that the

TEER during the culture gradually increased to approximately 288% of the initial/baseline value (average TEER value at day 0 before cytokines addition and taken as 100%). During 21 days of TNF-α exposure, TEER values gradually increased to approximately 354% of the baseline value as the cell layer was formed. For IL-4 and IL-13 addition, the values reached approximately 148% for IL-4 and 164% for IL-13 treatment. The statistical analysis, which was conducted by *t-test* for each condition comparing with the control, indicated that the changes in TEER values are not significant in all of pro-inflammatory cytokines treatment conditions (*P values* >0.05), comparing to the results of control cells.

Figure 5.1b shows the TEER measurements for Caco-2 cell culture during the 21 days exposure experiment. The results are expressed as percentage of baseline value (average TEER value at day 0 before cytokines addition and taken as 100%). Caco-2 control cells showed normal growth during the experiment (optical microscopy observation), and the TEER value rose gradually from 100% of the initial measurement to approximately 470% at the last day of experiment. In IL-4 and IL-13 conditions, TEER values were increased gradually from 100% to about 350% and 360%, respectively. T-test of significance confirmed that the changes in TEER values for both IL-4 and IL-13 interleukins are not significant in comparison to the control (*P values* >0.05). However, in TNF-α condition, it is clear that during the culture period TEER values are significantly lower than that of the control cells, increasing to only approximately 175% in the final measurement. Statistical analysis by t-test demonstrated that *P value* in this case is less than 0.00001, confirming statistically significant difference.

a)



b)

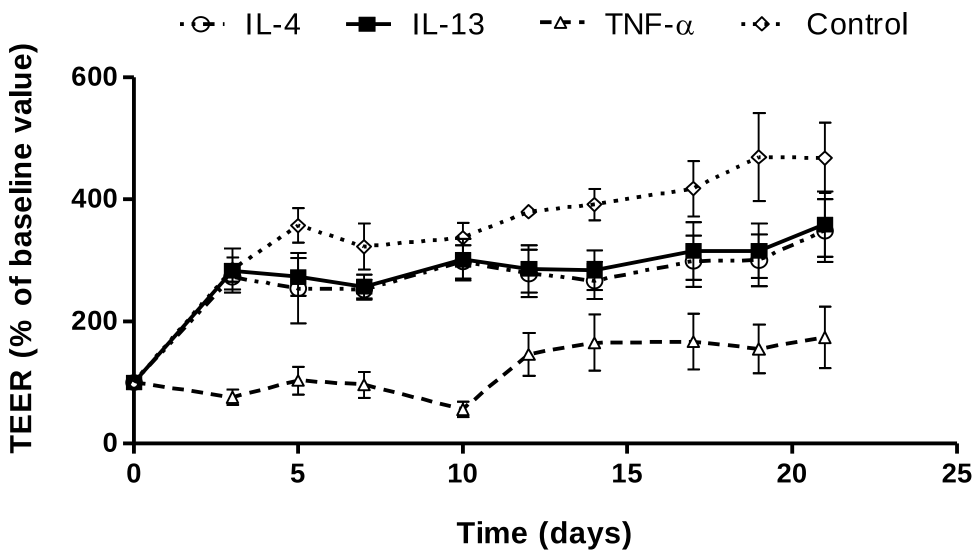


Figure 5.1 TEER profiles of a) Calu-3 and b) Caco-2 cells cultured on Transwell® filters and treated for 21 days with IL-4, IL-13 (5ng/ml) and TNF-α (25ng/ml).

*TEER is expressed as % change compared to baseline value (TEER at day 0 before cytokines addition). Background TEER due to the filter was subtracted from the reported TEER values. Data presented as the mean ± SD (n=4). Statistical analysis calculated by t-test (P value: * < 0.05, ** < 0.001, *** < 0.0001, **** < 0.00001 ns > 0.05).*

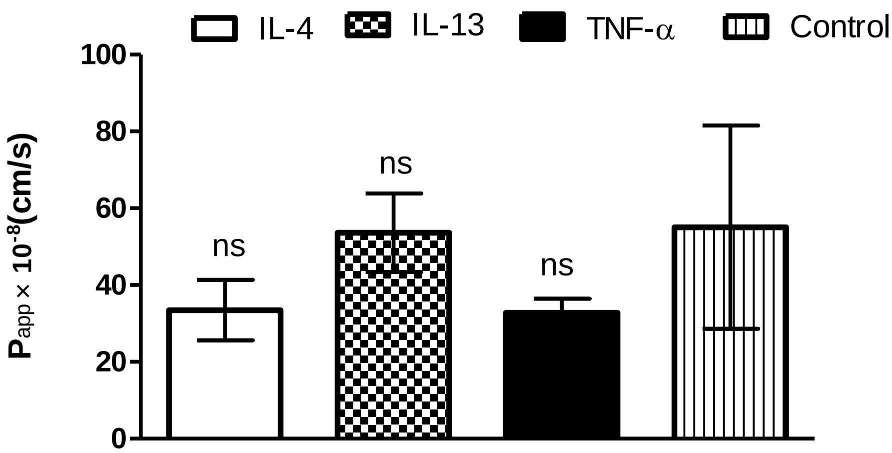
5.3.1.2 Effect on Cell Layers Permeability

In addition to TEER, changes in the permeability barrier of Calu-3 and Caco-2 cell layers following long-term cytokine exposure were evaluated by measuring the permeability of a macromolecular model compound, FD10. Figure 5.2 depicts FD10 permeability across polarised Calu-3 (grown at air-liquid interface) and Caco-2 cell layers, after a basolateral side treatment with the cytokines for 21 days. FD10 permeability is expressed as apparent permeability coefficient (P_{app}), which was calculated according to the equation described in section 2.2.4, following regular sampling of the basolateral solution (over 3 hours) and FD10 quantitation by fluorescence, as described in section 2.2.4.

Figure 5.2a shows the P_{app} values of Calu-3 cells treated with the cytokines. The apparent permeability of control cells was P_{app} ~ 55 ± 26 x 10^{-8} cm/s, while treated cells showed P_{app} ~ 33 ± 8 x 10^{-8} cm/s for IL-4, P_{app} ~ 53 ± 10 x 10^{-8} cm/s for IL-13 treated and P_{app} ~ 32 ± 4 x 10^{-8} cm/s for TNF-α exposure. All these values showed insignificant difference of long-term cytokines treatment on Calu-3 cells permeability (*P values* >0.05) compare to the control.

Figure 5.2b shows the effect of long-term cytokines treatment on the permeability of FD10 across Caco-2 monolayers. Control cells which were not treated with cytokines showed P_{app} of approximately 2 ± 1 x 10^{-8} cm/s, with IL-4 and IL-13 treated cells showing P_{app} around 7± 6 and 3 ± 1 x 10^{-8} cm/s, respectively. Statistical tests confirmed that both IL-4 and IL-13 interleukin's results were not significantly different from the control P_{app} (*p values* >0.05). In TNF-α situation, the P_{app} result demonstrated that FD10 permeability is approximately 17-times more than normal, untreated Caco-2 cells. P_{app} of FD10 was increased from around 2 ±1 x 10^{-8} in the control to around 38 ± 10 x 10^{-8} cm/s with TNF-α treatment, and t-test confirmed statistical difference (*p value* <0.00001).

a)



b)

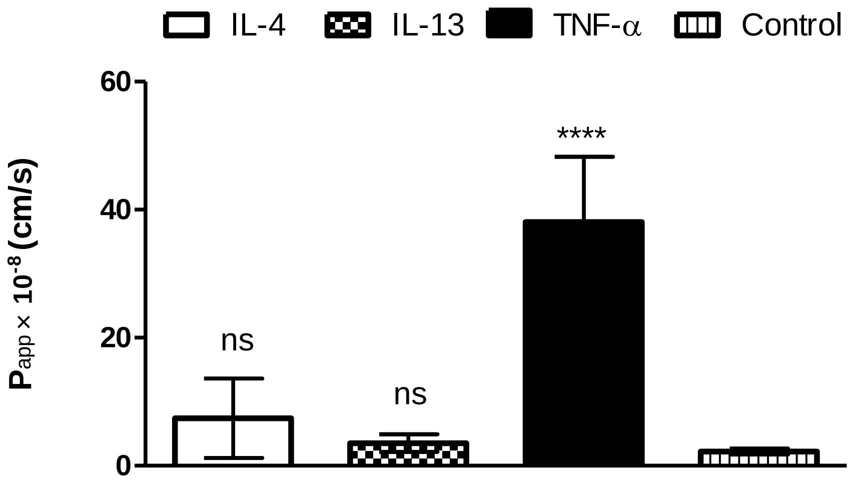


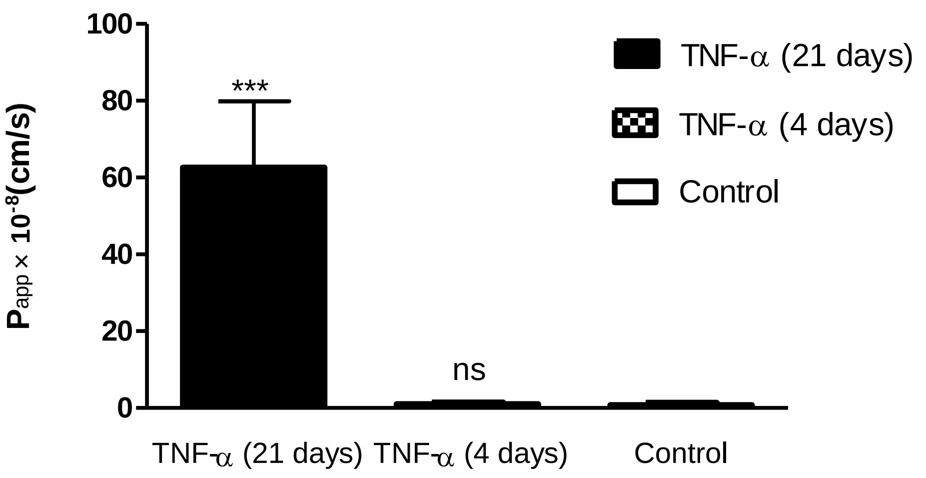
Figure 5.2 Effect of long-term cytokines treatment on FD10 permeability across a) Calu-3 and b) Caco-2 monolayers

*Cells were treated with IL-4, IL-13 (5ng/ml) and TNF-α (25ng/ml). FD10 permeability expressed as P_{app}, calculated using the equation of apparent permeability coefficient that described in section 2.2.4. Results presented as the mean ± SD (n=4). Statistical analysis calculated by t-test (P value: **** < 0.00001, ns > 0.05).*

5.3.1.3 Comparing Long-Term and Short-Term Treatment with TNF-α Effect on Permeability

To compare the difference between the effects of long and short-term application of cytokines on epithelial layers permeability, in a new experiment the FD10 transport across the cell layers in these experimental conditions was determined. Figure 5.3a shows a comparison between P_{app} of FD10 transport across Calu-3 layers (grown at air-liquid interface) in long-term (21 days) and short-term treatment (4 days). In 3 weeks of TNF-α treatment experiment, the result shows that this cytokine produced a significant effect on Calu-3 layer permeability with P_{app} around $62 \pm 17 \times 10^{-8}$ cm/s comparing to control of $0.8 \pm 0.5 \times 10^{-8}$ cm/s. However, short-term treatment did not provide a notable change in Calu-3 permeability showing P_{app} of ~ $1.1 \pm 0.4 \times 10^{-8}$ cm/s. Taken together, the permeability in long-term treatment was approximately 77.5-fold higher than the control cell layer, while short-term TNF-α exposure was only 1.25 times more than the control. Statistical analysis indicates that the result in long-term treatment are significantly different (*p value* < 0.001), and insignificantly increased in short-term treatment (*P values* >0.05), relative to the control.
Figure 5.3b shows a comparison between P_{app} of FD10 transport across Caco-2 monolayers in long-term treatment (21 days) and short-term treatment (4 days). Again the long-term treatment provided significant effect by showing P_{app} of $14.5 \pm 6.5 \times 10^{-8}$ cm/s in long-term treatment, comparing to control P_{app} of $1.0 \pm 0.2 \times 10^{-8}$ cm/s. This means that the increase in permeability was nearly 14 fold. With regard to short-term treatment, apparent permeability coefficient P_{app} was approximately $2.5 \pm 1.2 \times 10^{-8}$ cm/s, and by comparing this value with the P_{app} of control Caco-2 cells, the permeability is 2.5 times increased. T-test demonstrated that the P_{app} in long-term exposure is significantly increased (*p value* < 0.05), relative to the control, while P_{app} in short-term experiment showed no statistical difference to the control.

a)



b)

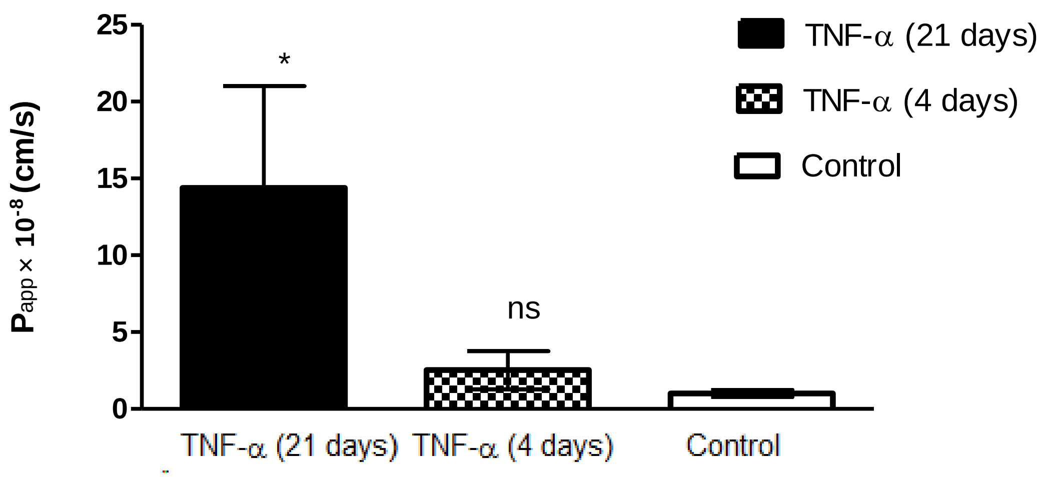


Figure 5.3 Comparison of the effect of long-term and short-term TNF-α (25ng/ml) treatment on FD10 permeability across a) Calu-3 and b) Caco-2 monolayers.

*FD10 permeability expressed as P_{app}, calculated using the equation of apparent permeability coefficient that described in section 2.2.4. Results presented as the mean ± SD (n=4). Statistical analysis calculated by t-test (P value: * < 0.05, ** < 0.001, *** < 0.0001, **** < 0.00001, ns > 0.05).*

5.4 Discussion

The present work aimed to assess a possible difference between the effects of long-term and short-term proinflammatory cytokines treatments on epithelial monolayers properties. Chapters 3 and 4 discussed the short-term effects in more details, and this chapter will focus on the long-term effects. Epithelial cells were shown to have the ability to release cytokines in inflammatory conditions [26]. It has been reported that in intestinal inflammatory diseases, proinflammatory cytokines generate important alteration of epithelial barrier structure and functionality, leading to an increase in the permeability of substances *via* paracellular route to intestinal lumen [4]. From the present data, a long term treatment of Caco-2 cells with IL-4 and IL-13 did not significantly affect transepithelial resistance. However, TNF-α showed an opposite result; the significantly decreased TEER values of Caco-2 layers during 21 days of experiment. These results are reflected in the FD10 transport across treated epithelium. The long-term exposure to pro-inflammatory cytokines did not provide notable effect on the P_{app} values for IL-4 and IL-13, but showed a dramatic effect in the TNF-α treatment with approximately 14.5- and 77.5-fold increase for Caco-2 and Calu-3 layers. The general trend of increased permeability following TNF- α treatment was seen in two separate experiments, although the absolute values are substantially different and potential future work would need to study more biological replicates. The future work would also need to consider a long term application of IL-4+IL-13 combination in CaCo-2 cells, as this has shown a significant effect in the short term experiment.

With regard to Caco-2 cells, our results for IL-4 and IL-13 are in agreement with a study which reported that no significant effect was observed with recombinant IL-4 and IL-13 treatment *in vitro* [26]. The possible explanation might be that the interleukins play a role in protecting the functionality and structure of the epithelium during long-term treatment. This observation also agrees with previous studies which demonstrated that IL-4 and IL-13 have anti-inflammatory properties in such cell lines, e.g. H-29 [26]. In contrast, another published work demonstrated that recombinant IL-4 and IL-13 contributed significantly to the damage of Caco-2 epithelial monolayers [27]. Potentially dual anti-inflammatory and pro-inflammatory action of IL-4 and IL-13 has been seen in several conditions [26], and this might be the most likely reason for different effect of IL-4 and IL-13 on epithelium in different cases. Taking this further, an intestinal study demonstrated that it could be a good strategy to use IL-4 and IL-13 to treat inflammatory intestinal conditions by inhibiting the effect of other pro-inflammatory cytokines [26]. Furthermore, both interleukins have the capacity to inhibit the production of pro-inflammatory cytokines in different inflammatory conditions [26].

With regard to intestinal cell line models, TNF-α was shown to produce a significant effect on epithelial barrier in HT29/B6 cell line, while in T_{84} cell model TNF-α it did not produce notable effects [4]. Another recent study documented that TNF-α can stimulate the tight junction modification and produce chronic damage of *in vitro* epithelial layer models [28]. The effect of TNF-α on intestinal cells was not ascribed to apoptosis action [4], but the TNF-α capacity to produce significant alteration in Caco-2 epithelial barrier characteristics [29], which consequently increased the permeability. The dose of cytokines that were applied on epithelial cells may play a role in the intensity of its effect [26]. This has not been assessed in the present study, but the future potential work would need to consider such experiments. Moreover, the prolonged contact time with cytokine would increase the probability of producing significant effect, as shown in the present study in the experiment that compare the influences of short-term and long-term TNF-α.

A study demonstrated that epithelial monolayers could release cytokines in inflammatory condition [30]. In asthma, proinflammatory cytokines that are released by epithelium can produce potent effects on remodelling of tight junction structure [31]. Inflammation reaction such as asthma could cause irreversible destruction in epithelial tissue [30]. In case of AIC Calu-3 treatment, different results were obtained from treating the cells with IL-4, IL-13 and TNF-a for 21 days. Our TEER and FD10 permeability data demonstrated that short exposure of the formed airway epithelial layer to the pro-inflammatory cytokines used in this study did not cause significant influence. Long-term / chronic treatment with TNF-a however provided a significant effect on Calu-3 permeability in one of the experiments conducted, and no significant effect in the other. The effect seen could be resulting from a prolonged interaction time between the recombinant cytokine and airway epithelial monolayers, however the experiment would need to be repeated to confirm this. In chapter 3 and 4, we verified the effects of tested cytokines following short-term exposure (not more than 4 days), as reported in a few studies discussed. There is however no available data from literatures on the influence of chronic treatment on intestinal and respiratory cell line models.

In asthmatic tissue, IL-4 and IL-13 might produce significant structural modification in epithelium [30]. IL-4 and IL-13 play important function on impairment of Calu-3 epithelial cells and prolong residence time could induce changes in the expression of epithelial membrane proteins, such as occluding expression [27]. Concerning the effect of IL-13 on Calu-3, it was shown to downregulate some tight junction proteins and increase an influx of molecules across the epithelial layer [29]. Similar observation was noticed in asthma individuals [27]. Other possible mechanism for the influence of IL-4 and IL-13 on airway epithelium is a decrease of cell migration, which could delay wound healing process [27]. In our study however, TEER and FD10 permeability data show that long-term treatment with tested of IL-4 and IL-13 interleukins did not have negative effects but may potentially generate protective effect on epithelium overtime. Our results are consistent with other observations about the protective properties of these cytokines [26]. Further investigations, particularly on epithelial membrane gene expression, would be important to prove this hypothesis (see Chapter 6).

Tumour necrosis factor has been shown to have a significant harmful effect on several cells [32]. A number of investigations concluded that TNF-α plays an essential function in asthma disorder [22]. TNF-α play a key role in induce the asthma inflammation [33]. Elevated in TNF-α expression was observed in analysis from asthmatic individual [17][20].

5.5 Conclusions

Using Caco-2 and Calu-3 cells the present study investigated the effect chronic exposure with different cytokines on epithelial cells properties. We demonstrated that long-term treatment with proinflammatory cytokine TNF-α has an important effect on the cell layers. TNF-α has exhibited significant effect on TEER and FD10 permeability. However, IL-4 and IL-13 did not produce significant effects on the same cell lines. In Calu-3, no significant effect was observed with these cytokines in TEER, as well as in transport of FD10. This observation might be due to suggested anti-inflammatory properties of IL-4 and IL-13.. It should be noted that no much work was published about the effect of these cytokines on Calu-3 and Caco-2 epithelial monolayers, particularly during prolonged incubation [26]. Furthermore, the data suggest that it may be useful to start further work on gene expression analysis to examine the regulation properties of proinflammatory cytokines on epithelial cells protein components expression [30].

5.6 References

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Chapter 6

Effect of Cytokines on the Expression of Human Intestinal and Airway Epithelial Barrier Genes

6.1 Introduction

Inflammation is an immunological reaction stimulated usually by endogenous and exogenous mediators via different mechanisms [1]. Several components of epithelium have been shown to be involved in the inflammation process mechanism in a wide range of inflammatory disorders [2]. Shaoyong *et al*. stated that a number of epithelial membrane proteins could be significant constituent of inflammation progression in human [3].

Epidemiological studies reported that modification in the expression of epithelial barrier genes is one of the important factors that might play a significant function in such intestinal inflammatory disorders [4][5][6]. In addition, in pulmonary inflammatory conditions, there is strong evidence that the development of asthma, for instance, is based mainly on changes in the expression of such airway barrier genes, which might drive medical research in future into new insight of therapy [7][8][9][10]. Most patients suffering from inflammatory diseases are dissatisfied with current anti-inflammatory treatment, while good understanding of the contribution of genes up- or down-regulation in the inflammation process could lead to discover a novel therapeutic strategy for clinical applications [6][11][12]. In addition, understanding of the expression features of epithelium in inflammation reaction will help inventors to create well-characterised *in vitro* models of inflamed cells, and this will support researchers to examine the efficacy of new anti-inflammatory agents.

Recently, numerous investigations have demonstrated that the remodelling of epithelial tight junction structure is considered a key feature of the pathophysiology of inflammatory disease, and the epithelial membrane proteins might play a central function in this alteration [13][14][15][16]. The literature reports different epithelial membrane genes as shown in Table 6.1 that may provide important functions in formation, regulation and transport processes across epithelial membranes. It can be hypothesised that these genes play vital roles in the modulation of tight junction complex structure and function in inflamed epithelial tissue.

Gene Symbol	Gene Name	Function	Reference
TJP1	tight junction protein 1 (zona occludens 1)	Tight junction formation and regulation	[17]
TJP2	tight junction protein 2 (zona occludens 2)	Tight junction formation and regulation	[18][19]
TJP3	tight junction protein 3 (zona occludens 3)	Tight junction formation and regulation	[20][21]
CDH1	cadherin 1, type 1, E-cadherin (epithelial)	Regulating cell-cell adhesions (adherence junctions protein)	[22]
CAV1	caveolin 1, caveolae protein	Transport via endocytosis pathway	[23]
LAMP1	lysosomal-associated membrane protein 1	Late endosome and lysosomes	[24][25]
CLTC	clathrin, heavy chain (Hc)	Regulation endocytosis pathway	[26]
CLDN1	claudin 1	Formation of tight junction	[27]
CLDN4	claudin 4	Formation of tight junction	[28]
CLDN5	claudin 5	Formation of tight junction	[29]
FCGRT	Fc fragment of IgG, receptor, transporter, alpha	Transfer of IgG from mother to fetus	[30]
CUBN	cubilin (intrinsic factor-cobalamin receptor)	Endocytic receptor	[31]
FOLR1	folate receptor 1 (adult)	Membrane-bound protein	[32]
EEA1	early endosome antigen 1	Endosome fusion	[33]
RAB4A	RAB4A, member RAS oncogene family	Cell adhesion and early endosome	[34][35]
RAB5A	RAB5A, member RAS oncogene family	Fusion of plasma membranes and early endosome	[36][37]
RAB7A	RAB7A, member RAS oncogene family	Late endocytic transport	[38]
RAB9A	RAB9A, member RAS oncogene family	Late endocytic transport	[39]

RAB11A	RAB11A, member RAS oncogene family	Regulates endocytic recycling	[40]
M6PR	mannose-6-phosphate receptor (cation dependent)	Late endocytic transport	[41]
FPR1	formyl peptide receptor 1	Activation of neutrophils	[42]
OCLN	occludin	Formation and regulation of the tight junction	[43]

Table 6.1 Several genes whose expression features could play key roles in remodelling of the epithelium in inflammatory conditions.

From the table above, the candidate genes would be analysed by using gene expression microarray data from the Gene Expression Omnibus (GEO) database to distinguish the difference of gene expression in normal and inflammatory condition and to select the genes of interest that are suitable to be investigated in this chapter.

Gene Expression Omnibus (GEO) is a public microarray databank created in 2000 by the National Centre for Biotechnology Information (NCBI) to archive experimental information of mRNA, protein molecules and genomic DNA, which are used routinely in biomedical and molecular biology research [44][45][46][47]. Nowadays, numerous researchers mine the GEO database to identify candidate genes, to confirm their results and to design further studies [48].

In 2006, GEO contained more than 20.000 studies, about 33 billion individual measurements and about half a million samples [49]. GEO database contains three types of data which are series, platform and sample [50]. Platform is the type of array used in the experiment and identified by (GPL) prefix [51]. Sample is the collection of a summary of the materials and general methodology used in the experiment and known by (GSM) prefix [52]. Finally, series is a collection of samples which composed part of a study and is known by (GSE) prefix

[49]. Also, there is a further tool called Dataset which is known by (GDS) prefix, and contains complete summary of final values [53]. GEO database can be accessed freely on (GEO; http://www.ncbi.nlm.nih.gov/geo).

In an attempt to investigate the contribution of specific genes involved in epithelial transport under conditions of increased inflammatory mediators in epithelial tissue, Caco-2 and Calu-3 cell lines were used. Caco-2 monolayers are a well characterised cell line that represents an *in vitro* model of intestinal mucosa and is used widely among varieties of pharmaceutical research [55]. The Calu-3 cell line, originally derived from bronchial tissue, is used commonly as a model of the *in vivo* situation of airway epithelium in normal state [56][57].

The expression patterns of selected genes were examined in this chapter in three different conditions. First, epithelial cells treated with a proinflammatory cytokine (TNF-α) for 21 days, mimicking a chronic inflammatory condition. Second condition, 4 days of treatment was used in Caco-2 and Calu-3 monolayers, mimicking an acute inflammatory condition. Finally, cells were cultured without cytokine treatment. We hypothesized that the gene expression of intestinal and airway epithelium would be similar to that of Caco-2 and Calu-3, respectively. It should be noted that proinflammatory cytokines play a central role in stimulating the inflammation process as host defence mechanism [58][59], as mentioned in detail in previous chapters.

In general, it is still unclear how these genes contribute in the alteration of epithelial barrier structure and function in inflammatory disorders. Hence, this chapter seeks to define the correlation between inflammatory state and gene expression using *in vitro* models of epithelial membrane treated with proinflammatory mediators. Good understanding of this correlation could help researchers in academia or the pharmaceutical industry to employ these systems as models of inflamed airway and intestinal epithelium.

6.2 Methods

6.2.1 Cell Culture and Cytokine Treatment

Caco-2 Cells were originated from human colorectal adenocarcinoma and purchased from the European Collection of Cell Cultures (ECACC number; 86010202). Caco-2 cells were used in this work with passage number (69), and cultured using Dulbecco's Modified Eagles Medium (DMEM). Calu-3 cells were originated from human bronchial adenocarcinoma and obtained from the American Type Culture Collection (ATCC number; HTB-55). Calu-3 cells were used with passage number (36) and cultured using Eagle`s Minimum Essential Medium (EMEM). Both DMEM and EMEM were purchased from Sigma-Aldrich (UK). Caco-2 and Calu-3 cells were seeded with 100.000 cells/well seeding density in 12 mm diameter and 0.4 µm pore size polystyrene permeable inserts (Transwell®) were supplied by Corning Life Sciences (USA). Cells were incubated at 37˚C in a humidified incubator with 5% CO2.

Recombinant human proinflammatory cytokine TNF-α was purchased from R&D Systems (UK) and mixed with master medium with (25ng/ml) concentration. Three conditions were created for each cell line: cells with TNF-α for21 days, cells with TNF-α for 4 days and control (cells without TNF-α treatment). Following treatment with TNF-α, Caco-2 and Calu-3 cells were lysed for gene expression analysis. More information about this study was explained minutely in Chapter 2.2.6.

6.2.2 Primer Design for PCR

Primer design for the RT–PCR experiment was made by Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers were designed across an intron to discriminate cDNA from genomic DNA contaminants, and taking into account the existence of known splice variants. The specificity was checked using Primer- BLAST. Primers (desalted) were purchased from Sigma-Aldrich and dissolved in water as 100 µM stock solutions. All primers were analysed for optimal annealing temperature using a gradient cycler. In these experiments, the human housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as control. The primers used in this study are as follows:

Gene	Forward primer	Reverse primer	Product size
TJP2 v1	CAGGCATGGAAGAGCTGATA	CCGGGAGCACATCAGAAAT	150 bp
TJP2 v2	GGAGGATGTGCTTCATTCGT	CGCATGGTCTTGGTCCAG	352 bp
TJP3 v1	GGGACCTGCCTCCCTGTTGC	GGTCGCCTGTCTGTAGCCTGC	342 bp
TJP3 v2	GGGTGGGGGCCGATTGACTG	GTCGCCTGTCTGTAGCCTGCC	264 bp
LAMP1	CACGCTGTGAACAAGACAGG	TGTTGGGGTTGATGTTGAGA	219 bp
RAB4A	TGATATCACCAGCCGAGAAA	GAGCAAATCTGGAGGCTTCT	158 bp
RAB5A	CTCGGCTTGCTGCGGTCTCA	TGCCAACAGCGGACTCTCCCA	211 bp

Table 6.2 List of forward and reverse primers used to examine the expression features of candidate genes.

6.2.3 PCR Analysis

Total mRNA was isolated and reverse transcribed into cDNA using the OneStep cDNA synthesis kit (Miltenyi Biotec) following the manufacturer's protocol. Each PCR reaction contained (10µl) of Jump Start Taq DNA polymerase mix (Sigma-Aldrich), (2µl) of primers, (1µl) of cDNA (for specific condition) and (7µl) free DNA and RNA water. 20 µl of PCR samples were mixed in PCR tubes. A 96-Well PCR Thermal Cyclers (Thermocyclers) was used with 58˚C annealing temperature and 35 cycles of heating and cooling. The 58˚C was used as a suitable annealing temperature for all examined genes.

The amplified products were confirmed by their expected sizes using 2% agarose gel electrophoresis run in 0.5x Tris-Borate-EDTA (TBE) buffer stained with 5 µg/ml ethidium bromide and imaged using SynGene Genius equipment and GeneSNAP imaging software.

6.3 Results

6.3.1 Expression Analysis of Candidate Genes

The list of candidate genes in Table 6.1 whose expression features could play a significant role in transport processes across the epithelial membrane were analysed by normalising the expression microarray data of each gene against appropriate housekeeping genes [54] in normal and inflammation state (asthma) and the results are shown in Table 6.3.

genes	Study 1 (sample NO. 12)	Study 2 (sample NO. 16)	Study 3 (sample NO.26)	Study 4 (sample NO.30)
FPR1	ns	ns	ns	↑*
CDH1	ns	ns	ns	ns
TJP3	ns	ns	↓*	↓*
TJP1	ns	ns	↓*	↓*
TJP2	ns	ns	↓**	ns
CAV1	ns	↑**	↓*	ns
CLTC	ns	ns	↓*	ns
VCFS	ns	↑*	ns	↓*
CLDN4	ns	ns	↓*	↓**
FCGRT	ns	↓**	ns	↑**
CUBN	ns	↑*	ns	ns
FOLR1	ns	ns	↓**	↑**
EEA1	ns	↑***	↓*	↑**
RAB4A	ns	↑*	↑*	↑***
RAB5A	ns	ns	ns	↓**
RAB9A	ns	ns	ns	ns
RAB11A	ns	ns	↑**	↑**
LAMP1	↑*	↓*	↓***	↑**
M6PR	ns	ns	ns	↑**
OCLN	ns	ns	ns	↓**
CLDN1	ns	↑*	ns	↑*
RAB7A	ns	ns	↓*	↓**

*Table 6.3 Summary of gene expression data analysis performed on the lung tissue of asthmatic and non-asthmatic individuals for a list of candidate genes and examined by GEO database (ns: not significant, ↓: downregulated, ↑: upregulated). Statistical analysis was calculated by t-test, P value (ns > 0.05, * < 0.05, ** < 0.001, *** < 0.0001).*

Table 6.3 shows the result summary of analysed expression data of the candidate gene microarray in four different studies with different sample numbers. From the table, five different genes were selected to be further investigated in this chapter. These genes are TJP2, TJP3, LAMP1, RAB4A, and RAB5A. These genes show interesting expression data from primary data analysis and it would therefore be useful to confirm their enrolment in the inflammation reaction in more depth. TJP2 and TJP3 are main components of paracellular pathway of transport in epithelial membrane, while the rest of the genes are involved mainly in the endocytosis pathway. Studies stated that TJP2 and TJP3 are corner-stones in the formation of tight junction complex in epithelium [18][20]. In addition, LAMP1 and RAB5A contribute in late and early endosome process, respectively [24][36]. Furthermore, RAB4A is reported to play a role in cell-cell adhesion in epithelial tissue and is also involved in endocytosis pathway [34][35]. The figures below reported that these genes show significant difference in expression level in inflammatory condition (asthma) compared with healthy control confirmed by t-test of significance.

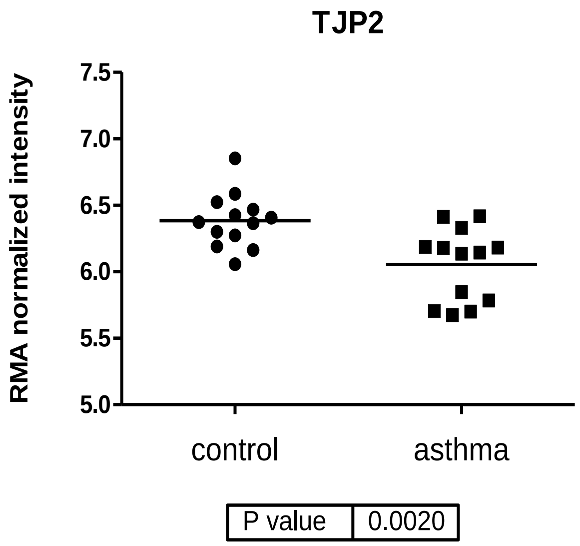
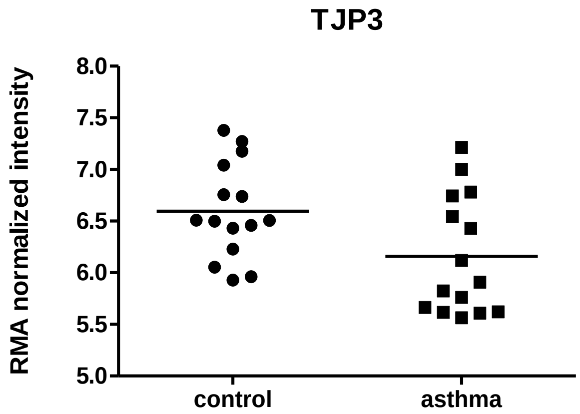
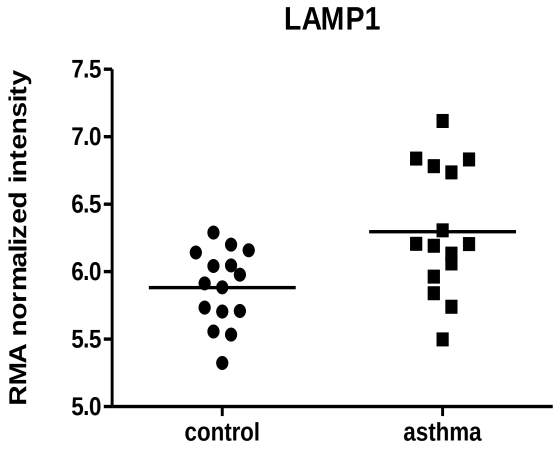


*Figure 6.1 Graph shows significant difference between the expression of normal control (healthy individuals) and diseased condition (asthmatic patients) in TJP2 gene. Statistical analysis made by t-test of significance. P value (ns > 0.05, * < 0.05, ** < 0.001, *** < 0.0001)*



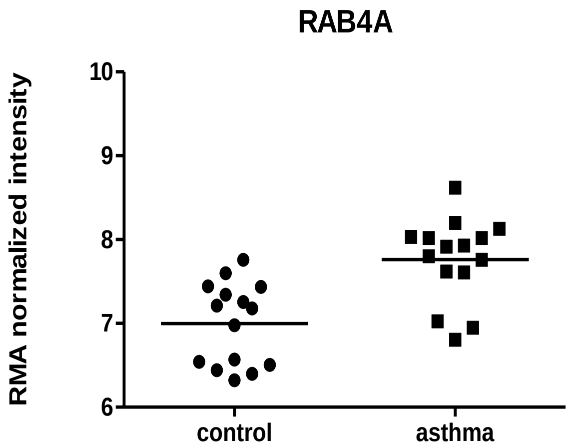
P value	0.0295

Figure 6.2 Graph shows significant difference between the expression of normal control (healthy individuals) and diseased condition (asthmatic patients) in TJP3 gene. Statistical analysis made by t-test of significance.



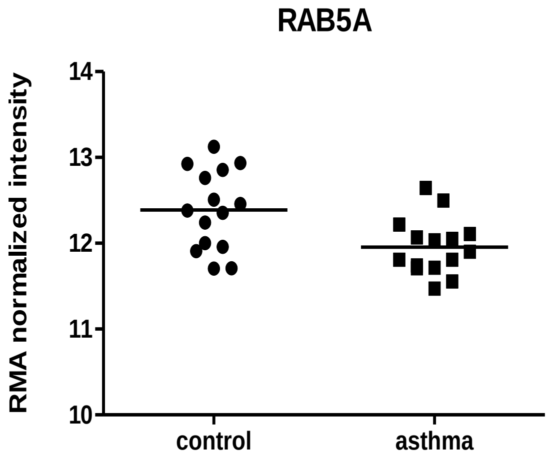
P value	0.0063

Figure 6.3 Graph shows significant difference between the expression of normal control (healthy individuals) and diseased condition (asthmatic patients) in LAMP1 gene. Statistical analysis made by t-test of



P value	0.0002

*Figure 6.4 Graph shows significant difference between the expression of normal control (healthy individuals) and diseased condition (asthmatic patients) in RAB4A gene. Statistical analysis made by t-test of significance. P value (ns > 0.05, * < 0.05, ** < 0.001, *** < 0.0001)*



P value	0.0063

*Figure 6.5 Graph shows significant difference between the expression of normal control (healthy individuals) and diseased condition (asthmatic patients) in RAB5A gene. Statistical analysis made by t-test of significance. P value (ns > 0.05, * < 0.05, ** < 0.001, *** < 0.0001)*

These results led us to investigate the expression levels of these genes in inflammation using an *in vitro* model of cells exposed for prolonged period of time to the inflammatory mediators.

6.3.2 Effect of TNF-α on the Expression of Caco-2 and Calu-3 Barrier Genes

Figure 6.6 shows the time dependent influence of recombinant human proinflammatory cytokine TNF-α (25ng/ml) on mRNA expression features of protein components of Caco-2 and Calu-3 epithelial cell layers. The isolated mRNA was reverse transcribed into cDNA and examined by PCR analysis technique.

In case of the first splice variant of TJP2 gene, Caco-2 cell line did not express this gene showing by comparison of Caco-2 expression with GAPDH control bands. However, TJP2 v1 shows interesting expression results on Calu-3 cells. TNF-α was observed to cause down regulation of this gene. The expression bands show TJP2 v1 expressed constitutively in Calu-3 control, while Calu-3 cells treated with TNF-α show knockdown of TJP2 v1 and this effect would increase with exposure time. The time course of the TNF-α induced decrease in TJP2 v1 expression correlated with the time of treatment showing stronger down regulation effect on TJP2 v1 in 21 days than in 4 days of TNF-α treatment. With respect to the expression features of the second splice variant of TJP2, Caco-2 and Calu-3 cell lines did not show changes in gene expression with TNF-α treatment. By comparing GAPDH bands with TJP2 v2 bands, no significant effect was observed in mRNA expression. In addition, TNF-α did not influence the TJP2 v2 expression correlated with increased time of treatment.

In case of the first splice variant of TJP3 gene, no meaningful effect of TNF-α time-dependent treatment was detected. This result confirmed that TNF-α does not provide significant effect on mRNA expression of variant 1 of TJP3 showing by comparing the expression bands on Caco-2 and Calu-3 conditions with GAPDH. With regards to the second splice variant of TJP3, prolonged TNF-α treatment appeared to cause up regulation in both Caco-2 and Calu-3 cell lines. By comparing the control bands in both cells with the bands of treated cells, increased expression of TJP3 v2 was observed with TNF-α treated cells. The duration of the TNF-α treatment did not appear to affect TJP3 v2 mRNA expression as suggested by the similar levels with both incubation times (4 and 21 days).

With regard to LAMP1, RAB4A and RAB5A genes expression, Caco-2 and Calu-3 cell lines constitutively expressed the mRNA for these genes in the presence or absence of the proinflammatory cytokine TNF-α. These results suggest that TNF-α does not have a significant effect on the expression of these genes on epithelial barriers of intestinal and pulmonary tissues showing by comparing the obtained bands with the expression of control mRNA (GAPDH).

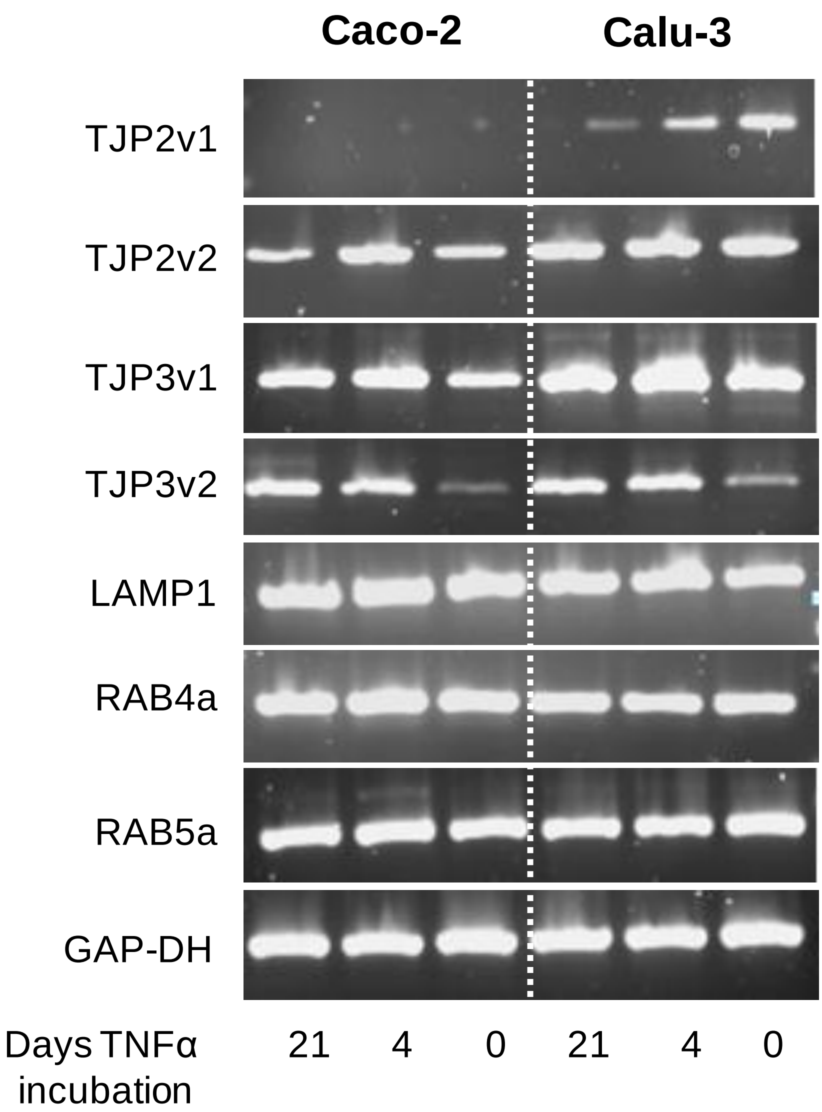


Figure 6.6 Effect of time-dependent proinflammatory cytokine TNF-α (25ng/ml) treatment on mRNA expression of Caco-2 and Calu-3 epithelial membrane genes. The genes examined in this study were TJP2v1, TJP2v2, TJP3v1, TJP3v2, LAMP1, RAB4A, and RAB5A. Human GAPDH was used as a housekeeping gene (control).

6.4 Discussion

In the past, numerous laboratories have attempted to design well-characterised *in vitro* models of inflamed epithelium in order to understand the basic mechanisms of such inflammatory reactions, to study the effect of diseased tissue on drug delivery properties and to examine anti-inflammatory action of such new therapeutics. At present, there are no available methods accepted to be a good *in vitro* model for inflammation. Caco-2 and Calu-3 cell lines are used widely in research as an *in vitro* model of intestinal and airway epithelium, respectively. We hypothesised that treating these cell lines with proinflammatory cytokines under specific conditions would establish a good *in vitro* model of inflamed epithelium.

In vivo, such TNF-α may be released by local resident mast cells in close proximity of the epithelium, which may also be increased during chronic inflammation.

The results in previous chapters 3, 4, and 5, indicate that the treatment of Caco-2 and Calu-3 with proinflammatory cytokines could result (although experiments would need to be repeated) in significant effect on TEER profiles of cells and on tight junction formation, particularly with TNF-α.

In this chapter, the work aimed to investigate the expression features of such epithelial membrane genes (TJP2, TJP3, LAMP1, RAB4A, and RAB5A) which could play an essential function in regulation of mucosal barriers using PCR. Results will provide a primary overview of gene expression features and would allow drawing of an initial assumption about the capacity of these cells to be appropriate models of inflammation. These genes are expressed widely in cultured cells, and TJP2 and TJP3 are known to be components of tight junctions and are used as indicators of tight junction formation and epithelial membrane integrity, whilst the rest of the genes are usually involved in endocytosis pathway. In this work, samples were obtained for RT-PCR at day 21 and cells were examined at the same time of culture and taken from the same batch of cells to decrease the variation in experiments.

Tight junction proteins TJP2 and TJP3 (a.k.a. ZO-2 and ZO-3) are important components in regulation and formation of tight junction and control the transport across epithelial barrier via paracellular pathway [60][61]. Under normal (i.e. non-inflammatory) conditions, TJP2 and TJP3 are expressed normally in both Caco-2 and Calu-3 cells [62][63] and help to prevent harmful substances from entering the tissues from the host lumen of intestine and lung, while in inflammatory conditions, tight junctions might be characterised by increased permeability via the paracellular route [64].

From our results, we found that TJP2 showed time-dependent down regulation in Calu-3 cells, where TNF-α stimulated the up regulation of TJP3 in both Caco-2 and Calu-3 cells. These results suggest that tight junction complex may be an attractive target for anti-inflammatory mediators in the *in vivo* system. In addition, TJP2 might be involved in the regulation of epithelial barriers in the *in vitro* model of airway, while TJP3 could be involved in the intestinal model as well. The significance of the different splice variants of TJP2 and TJP3, and of their differential regulation by TNF-α, is currently unclear.

In inflammatory disorders, one important issue is the increased permeability of noxious elements across epithelial layer due to disruption of the tight junction barrier [55]. Numerous studies have reported that inflammatory diseases occur perhaps due to disruption of the epithelial membrane and changing the expression of tight junction proteins might play an important role in this defect [62]. We can suggest that tight junctions were involved importantly in inflammation reaction on epithelial membranes.

Time-course is indeed responsible for the TNF-α facilitated stimulation of TJP2 v1 expression in Calu-3 monolayers. We observed in chapter 5 that TNF-α increase permeability of epithelial membrane with increase in the time of treatment. Taken together, it could be established that TNF-α plays a role in dysfunction of tight junction, possibly by down regulation TJP2; this effect would be increased with prolonged exposure to TNF-α. However, the differences in expression of tight junction proteins variants remain unclear. In the literature, nothing is mentioned about the expression of different variants of tight junction proteins.

On the other hand, several studies demonstrated that LAMP1, RAB4A and RAB5A contribute in transport elements across epithelium via endocytosis pathway [25][34][36]. Study reported that LAMP1 plays multiple functions in transport across the cell membrane via endocytosis [65]. RAB4A and RAB5A are important members of the RAB family and they have a role in regulation of early and recycling endosomes process, respectively and also endocytosis pathway of transport in a wide range of cells [35][66][67][68]. LAMP1, RAB4A, and RAB5A did not show alteration in expression during treatment of the cells with proinflammatory cytokines. By combining the previous results of tight junction proteins with these results, it can be suggested that the increased permeability observed during inflammation could be mediated due to disruption of tight junction complex resulting in increased transport across epithelial membranes through paracellular pathway rather than through the endocytosis route. To the best of our knowledge, we have not been able to find any information regarding expression features of these genes in inflammatory conditions in the literature.

6.5 Conclusion

The data in this chapter show that treatment of the cells with cytokines did not produce a significant effect on the expression array of the protein components of endocytosis membrane. It would be concluded that tight junctions might be involved in the epithelial barriers modifications more than endocytosis pathway during inflammation reaction. *In vivo* or *ex vivo* (e.g. using biopsies from patients) studies should be established to confirm these results, to find out if pre-treated cell lines mimic the *in vivo* situation or not. Furthermore, additional investigations should be focused in the direction of critical problems addressing the fundamental character of inflammation, the functional importance of intestinal and airway remodelling, and the role of gene expression in disease states.

6.6 References

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Chapter 7

Summary and Future Work

7.1 Overall Summary

Mucosal barriers have a protective role by acting as a permeability barrier limiting the movement of undesirable materials from the external environment into the systemic circulation. However, it has been realized the barrier properties of mucosal epithelium can be significantly altered in inflammation conditions, in terms of its function and structure. Alterations in the tight junction complex have been reported to occur in inflammatory disorders affecting the epithelium. As a result, the mucosal barriers in inflammatory conditions have increased the paracellular permeability. The changes in epithelium, including the tight junctions, have been attributed to up-regulation of proinflammatory cytokines.

To investigate the effect of pro-inflammatory cytokines on permeability function of mucosal membrane *in vitro,* we need a cell line model of the tissues of interest. The cell line models provide several benefits in comparing with primary cells and animal models as they are cost-effect, easier to maintain and show higher in reproducibility. In this work, Calu-3 and Caco-2 cell lines were used to represent the airway and intestinal epithelium, respectively. Both these cell lines are capable of producing polarised layers when cultured appropriately. Another benefit of using Calu-3 cells is their ability to produce mucus when cultured adequately. The

Caco-2 cell line has a capacity to mimic the *in vivo* situation of intestinal epithelium more closely than other cell lines. Chapter 1 in this thesis discussed these cell lines in more details.

The effect of proinflammatory cytokines on the epithelial barrier of the cell monolayers (Calu-3 and Caco-2) was investigated by treating the cells with cytokines and assessing the barrier function. IL-4, IL-13, and TNF-α were tested in this work and the barrier function was assessed by measuring TEER, permeability and structural changes in the tight junctions.

Chapter 3 assessed the effect of the tested cytokines on the barrier characteristic of Calu-3 cell layers. The data demonstrated that TNF-α is the most effective cytokine in altering the barrier function of Calu-3 cell layers, as shown by a significant decrease in TEER and increase in FD10 permeability. On the other hand, IL-4 and IL-13 failed to significantly influence the TEER and permeability. Applying these cytokines in combination produced a synergetic effect, when TNF-α was present in the combination. The tested cytokines seemed to influence the distribution of a tight junction protein, ZO-1, suggesting that alteration in epithelial barrier results from a tight junction effect.

The influence of cytokines on the intestinal epithelium, using Caco-2 cells as a model, was assessed in chapter 4. The data were less clear in this instance, with some evidence that TNF-α and IL-4 produce some effects on the barrier when applied alone. When applied in combination all the tested cytokines markedly decreased cell layer TEER, though they failed to significantly increase FD10 permeability, except with IL-4/IL-13 combination. The data from Chapters 3 and 4 suggested that proinflammatory cytokines could increase the membrane permeability of airway and intestinal epithelium via the remodelling action on mucosal barriers.

While both chapter 3 and 4 aimed to establish the effect of short-term exposure of the cells with proinflammatory cytokines on the epithelial barrier, in chapter 5 the work explored the influence of long-term treatment of the cells with the same cytokines. Long-term treatment lasted for the whole duration of cell growth on filter supports. TNF-α long-term treatment showed significant effect on Caco-2 cells, but no significant effect was observed with IL-4 or IL-13.

Data shown in Chapter 6 aimed to examine if the expression of epithelial cell 'markers' of interest if affected during inflammation reaction, mimicked by the TNF-α application. In this experiment, Calu-3 and Caco-2 cells were incubated with TNF-α for short and long time period (4 and 21 days, respectively). The PCR results demonstrated that down-regulation of TJP2 and up-regulation of TJP3 might play important role in paracellular tight junction remodelling in inflammatory disorders. Conversely, LAMP1, RAB4A, and RAB5A did not show difference in expression during the experiment, suggesting no involvement in epithelial modification.

In conclusion, this work confirmed the previous reports that proinflammatory cytokines, the expression of which is up-regulated in inflammatory conditions affecting the epithelium (e.g. asthma and Crohn's disease), alter the epithelial barrier. This alteration is likely to result from an effect on the tight junctions, as shown by changes in the appearance and the level of expression of these proteins following epithelial cell treatment with the cytokines. The defective mucosal barrier in inflammatory conditions such as asthma or Crohn's disease can potentially be exploited for mucosal delivery of biotherapeutics.

7.2 Future Work

Whilst this work showed the epithelial barrier-disruptive effects of three tested cytokines, it would be interesting to identify precisely the conditions of the epithelium in different inflammatory diseases. Specifically, the levels of other pro-inflammatory (as well as anti-inflammatory) cytokines are likely to be altered (upregulated or downregulated) under inflammatory conditions. Establishing the effect of these precise conditions on the barrier property of the epithelium would give a more accurate indication of how the epithelium is affected in inflammatory diseases of the epithelium.

This work has also implications in research related to establishing suitable models of inflamed epithelial tissue to study the disease itself, as well as drug delivery in these conditions. This is important as the use of standard models (e.g. cell line based models or excised tissue) to predict drug absorption in inflammatory epithelial conditions is not appropriate. Models based on culture of epithelial cell lines with proinflammatory cytokines may provide appropriate models, though these models need to be validated extensively in terms of their similarity (e.g. protein expression and permeability) to the inflamed mucosal tissue *in vivo*.

Applying proinflammatory cytokines to the current *in vitro* models of epithelium, either lung or intestinal, under specific conditions could potentially create an *in vitro* model of inflamed epithelial tissue which could find its application in academia or pharmaceutical industry.

Chapter 8

Appendices

8.1 The Effect of TNF-α on Calu-3 Cell Layers at Concentrations of (10, 25, and 50ng/ml)

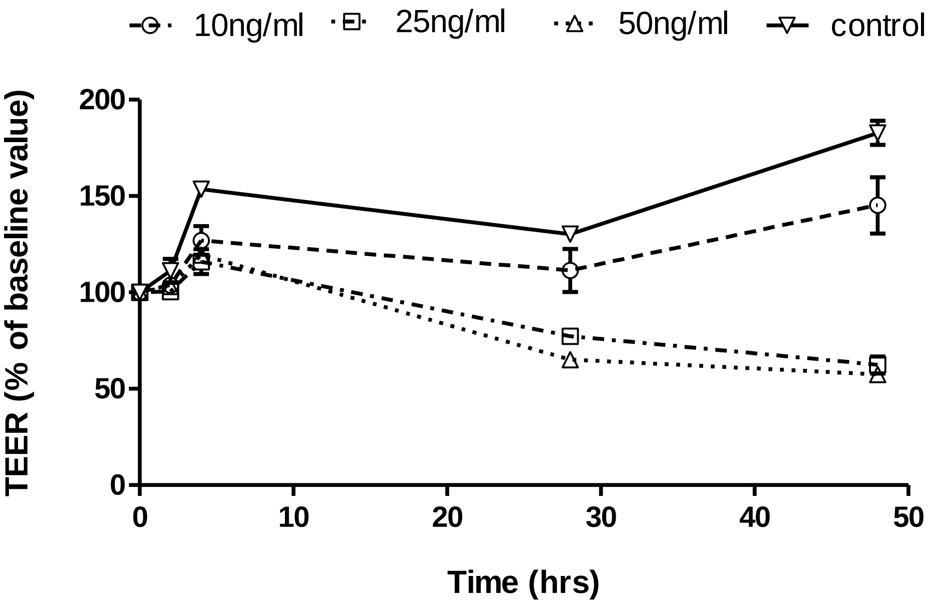

Figure 8.1 Effect of 10, 25, and 50ng/ml of TNF-α on TEER of Calu-3 layer.

8.2 Affymetrix Array of Candidate Genes from (GeneCards)

Formyl peptide receptor 1 (FPR1)

Affymetrix probe-set	Array
36919_r_at	U95-A
205119_s_at	U133-A
205118_at	U133-A
205119_s_at2	U133Plus2
205118_at2	U133Plus2

Tight junction protein 1 (TJP1)

Affymetrix probe-set	Array
32532_at	U95-A
78685_at	U95-D
214168_s_at	U133-A
202011_at	U133-A
214168_s_at2	U133Plus2
202011_at2	U133Plus2

Tight junction protein 2 (TJP2)

Affymetrix probe-set	Array
36655_at	U95-A
54505_at	U95-B
50087_at	U95-B
202085_at	U133-A
232017_at	U133-B
202085_at2	U133Plus2
232017_at2	U133Plus2

Tight junction protein 3 (TJP3)

Affymetrix probe-set	Array
35148_at	U133-A
35148_at	U133-A
213412_at	U133-A
35148_at2	U133Plus2
213412_at2	U133Plus2

Caveolin 1 (CAV1)

Affymetrix probe-set	Array
36119_at	U95-A
212097_at	U133-A
203065_s_at	U133-A
212097_at2	U133Plus2
203065_s_at2	U133Plus2

Clathrin (CLTC)

Affymetrix probe-set	Array
91168_at	U95-E
84461_at	U95-E
41159_at	U95-A
200614_at	U133-A
200614_at2	U133Plus2

Claudin 5 (VCFS)

Affymetrix probe-set	Array
38995_at	U95-A
204482_at	U133-A
204482_at2	U133Plus2

Claudin 4 (CLDN4)

Affymetrix probe-set	Array
35276_at	U95-A
87864_i_at	U95-E
201428_at	U133-A
1569421_at2	U133Plus2
201428_at2	U133Plus2

Cubilin (CUBN)

Affymetrix probe-set	Array
35416_at	U95-A
35417_at	U95-A
206775_at	U133-A
206775_at2	U133Plus2

Early endosome antigen 1 (EEA1)

Affymetrix probe-set	Array
39627_at	U95-A
50373_at	U95-B
204840_s_at	U133-A
225885_at	U133-B
204841_s_at	U133-A
204840_s_at2	U133Plus2
225885_at2	U133Plus2
204841_s_at2	U133Plus2

Lysosomal-associated membrane protein 1 (LAMP1)

Affymetrix probe-set	Array
39758_f_at	U95-A
64344_at	U95-C
201551_s_at	U133-A
201553_s_at	U133-A
201552_at	U133-A
213728_at	U133-A
201551_s_at2	U133Plus2
201553_s_at2	U133Plus2
201552_at2	U133Plus2
213728_at2	U133Plus2

Cadherin 1 (CDH1)

Affymetrix probe-set	Array
2082_s_at	U95-A
977_s_at	U95-A
201130_s_at	U133-A
201131_s_at	U133-A
201130_s_at2	U133Plus2
201131_s_at2	U133Plus2

Tight junction protein occludin (OCLN)

Affymetrix probe-set	Array
82255_at2	U95-E
43530_at	U95-B
209925_at	U133-A
231022_at2	U133-B
227492_at	U133-B
209925_at2	U133Plus2
231022_at2	U133Plus2
227492_at2	U133Plus2

Claudin1 (CLDN1)

Affymetrix probe-set	Array
46260_at	U95-B
77660_at	U95-E
222549_at	U133-B
218182_s_at	U133-A
222549_at2	U133Plus2
218182_s_at2	U133Plus2

Fc fragment of IgG receptor transporter alpha1 (FCGRT)

Affymetrix probe-set	Array
52415_s_at	U95-B
31431_at	U95-A
54971_at	U95-C
31432_g_at	U95-A
218831_s_at	U133-A
218831_s_at2	U133Plus2

Folate receptor 1 (FOLR1)

Affymetrix probe-set	Array
534_s_at	U95-A
821_s_at	U95-A
39311_at	U95-A
204437_s_at	U133-A
204437_s_at2	U133Plus2

Member RAS oncogene family (RAB4A)

Affymetrix probe-set	Array
621_at	U95-A
39244_at	U95-A
203581_at	U133-A
203582_s_at	U133-A
206272_at	U133-A
203581_at2	U133Plus2
203582_s_at2	U133Plus2
206272_at2	U133Plus2

Member RAS oncogene family (RAB5A)

Affymetrix probe-set	Array
600_at	U95-A
36110_at	U95-A
206113_s_at	U133-A
209089_at	U133-A
240990_at	U133-B
206113_s_at2	U133Plus2
209089_at2	U133Plus2
240990_at2	U133Plus2

Member RAS oncogene family (RAB7A)

Affymetrix probe-set	Array
45003_r_at	U95-B
211960_s_at	U133-A
211961_s_at	U133-A
211960_s_at2	U133Plus2
211961_s_at2	U133Plus2
1570061_at2	U133Plus2

Member RAS oncogene family (RAB9A)

Affymetrix probe-set	Array
50332_r_at	U95-B
39628_at	U95-A
50329_s_at	U95-B
221808_at	U133-A
221808_at2	U133Plus2

Member RAS oncogene family (RAB11A)

Affymetrix probe-set	Array
36660_at	U95-A
61624_at	U95-C
200863_s_at	U133-A
200864_s_at	U133-A
234998_at	U133-B
200863_s_at2	U133Plus2
200864_s_at2	U133Plus2
234998_at2	U133Plus2

Mannose-6-phosphate receptor (M6PR)

Affymetrix probe-set	Array
32547_at	U95-A
200901_s_at	U133-A
200900_s_at	U133-A
200901_s_at2	U133Plus2
200900_s_at2	U133Plus2

8.3 Normalizing Gene expression Data from (GEO)

8.3.1 Study 1

Series: GSE470, Platform: GPL8300, Sample number: 12 (6 healthy, 6 asthmatic).

			RPL27		FPR1		CDH1		CDH1		TJP1		TJP2		CAV1	
			39830_at		36919_r_at		2082_s_at		977_s_at		32532_at		36655_at		36119_at	
GSM3909	asthma		17201.5		62.79898	62.79897	924.401123	924.401	9894.5615	9894.56	137.7786	137.7786	563.7983	563.7982	4163.757	4163.757
GSM3910	asthma		27250.78		56.59931	35.72717	667.302246	421.221	11800.146	7448.602	119.6376	75.5188	655.6877	413.8896	6501.623	4104.018
GSM3911	asthma		9402.37		124.6697	228.0815	622.21405	1138.332	7530.3042	13776.58	114.5945	209.649	1019.407	1864.99	4864.226	8899.03
GSM3912	asthma		10717.07		71.87707	115.3667	877.76355	1408.859	9747.9785	15646.05	71.50285	114.7661	752.3993	1207.643	6119.465	9822.083
GSM3913	asthma		15995.54		13.34972	14.3562	974.080261	1047.52	8070.875	8679.367	170.8493	183.7302	1333.343	1433.868	7509.447	8075.611
GSM3914	asthma		16901.96		71.04765	72.30677	855.324951	870.4832	9200.3545	9363.406	91.1652	92.78085	532.4933	541.9303	5515.01	5612.748
GSM3916	normal		32125.71		235.7453	126.2282	314.828522	168.5728	6228.019	3334.752	252.1188	134.9953	997.0525	533.8651	4520.05	2420.231
GSM3917	normal		25124.97		21.63008	14.80877	660.907654	452.4822	9655.9365	6610.817	184.8999	126.5894	985.3081	674.5789	5633.734	3857.066
GSM3918	normal		14018.19		55.07094	67.57668	662.337708	812.7441	6794.1763	8337.026	97.78664	119.9924	1701.188	2087.501	5064.507	6214.577
GSM3919	normal		15505.32		137.2409	152.2542	1468.88501	1629.571	8900.4053	9874.051	89.8802	99.7125	1112.11	1233.768	7082.981	7857.812
GSM3915	normal		13638.43		105.3802	132.911	918.296509	1158.204	9416.9551	11877.16	97.58666	123.0814	796.7094	1004.852	8054.281	10158.48
GSM3920	normal		27269.54		103.5159	65.29738	550.481201	347.241	8469.4863	5342.513	26.05685	16.43654	629.6876	397.2041	7063.41	4455.567

CLTC		VCFS		CLDN4		FCGRT		CUBN		FOLR1		EEA1		RAB4A	
41159_at		38995_at		35276_at		31431_at		35417_at		534_s_at		39627_at		621_at	
215.5891	215.5891	85.09485	85.09484	10112.91	10112.91	1381.802	1381.802	70.18961	70.1896	3794.796	3794.796	4.929906	4.929905	290.1913	290.1913
225.0362	142.0496	132.8929	83.88597	8351.86	5271.942	1411.874	891.2166	71.11766	44.89158	4701.909	2967.985	9.416497	5.943973	301.3016	190.1905
256.5053	469.2727	96.14056	175.8878	11482.31	21006.72	1677.177	3068.372	72.1087	131.9218	5084.599	9302.2	31.29861	57.26035	359.7787	658.2099
241.627	387.8249	107.5829	172.6765	8262.371	13261.57	1721.475	2763.063	67.246	107.9336	5709.888	9164.688	7.764824	12.46298	530.9393	852.1871
947.8531	1019.315	128.7689	138.4773	5770.931	6206.022	945.7314	1017.033	94.63753	101.7726	3641.519	3916.066	38.40062	41.29578	667.4117	717.7303
194.9348	198.3895	101.1834	102.9766	7328.156	7458.027	1515.708	1542.57	48.60173	49.46306	4936.184	5023.664	14.40051	14.65572	476.5924	485.0387
221.2935	118.4901	89.69572	48.02698	11107.81	5947.604	1100.69	589.3571	99.89535	53.4883	5056.399	2707.415	11.85997	6.350343	57.18189	30.61767
194.5467	133.194	150.4706	103.0178	15483.86	10600.83	985.0404	674.3957	62.69239	42.92156	4803.866	3288.907	6.652773	4.554738	325.4477	222.8137
447.7437	549.4193	102.6669	125.981	10165.44	12473.85	1405.962	1725.234	210.5631	258.3786	6130.956	7523.199	2.78129	3.412877	331.8394	407.1949
334.3925	370.9728	117.5182	130.3739	10724.67	11897.87	1689.137	1873.917	50.04365	55.5181	4945.511	5486.517	15.7957	17.52364	416.6909	462.2742
339.209	427.8282	85.5839	107.9429	7074.633	8922.899	1644.71	2074.395	119.748	151.0324	3919.528	4943.515	1.77953	2.244437	454.9957	573.8644
194.4593	122.6641	157.5889	99.40637	10996.95	6936.824	1633.256	1030.251	117.7116	74.25193	4998.483	3153.02	7.334524	4.626584	374.6732	236.3422

RAB5A		RAB9A		RAB11A		LAMP1		M6PR		FCGRT		CUBN		FOLR1		FOLR1	
600_at		39628_at		36660_at		at		32547_at		31432_g_at		35416_at		821_s_at		39311_at	
88.92399	88.92398	1549.282	1549.282	1410.557	1410.557	14099.79	14099.79	2254.636	2254.635	2046.744	2046.744	21.60967	21.60966	488.4326	488.4325	442.1562	442.1561
114.4115	72.21991	2038.204	1286.575	1089.31	687.6049	23439.54	14795.74	1720.208	1085.846	1910.849	1206.184	16.7766	10.58989	414.2038	261.4577	558.1496	352.3206
62.52706	114.3924	1610.721	2946.791	921.8052	1686.429	8887.098	16258.82	2448.36	4479.239	2139.846	3914.818	26.76158	48.95992	613.9946	1123.294	670.5855	1226.826
109.5007	175.7548	1521.036	2441.347	1174.151	1884.578	10556.7	16944.1	2110.35	3387.23	2236.485	3589.684	25.78625	41.38837	571.7582	917.7038	707.2238	1135.134
388.6642	417.9669	2448.818	2633.444	2062.469	2217.966	11239.84	12087.26	2388.391	2568.46	1683.474	1810.397	24.05052	25.86378	352.7494	379.3445	409.1276	439.9732
102.4228	104.2379	1548.599	1576.043	911.1465	927.2941	13681.3	13923.76	1926.218	1960.355	2075.319	2112.098	14.47199	14.72847	578.3781	588.6282	713.2881	725.9292
7.954841	4.259367	1051.085	562.7966	862.9199	462.0448	17657.95	9454.83	2075.738	1111.44	1457.368	780.3379	10.98216	5.880322	884.2571	473.4696	813.019	435.3257
109.0722	74.67492	1581.759	1082.932	746.8663	511.3327	20842.46	14269.53	1634.592	1119.103	1479.231	1012.737	28.53967	19.53933	442.6971	303.0871	560.3146	383.6124
127.1666	156.0441	1267.778	1555.671	1390.576	1706.354	9708.167	11912.74	2216.991	2720.434	1847.621	2267.187	20.18874	24.77328	673.8727	826.8985	652.8766	801.1345
268.6753	298.0666	1529.165	1696.445	1281.624	1421.825	11351.01	12592.74	1442.402	1600.191	1968.007	2183.294	24.06997	26.70307	677.2744	751.3638	603.1606	669.1424
119.4805	150.6951	1629.084	2054.687	1416.472	1786.528	10131.7	12778.64	1663.523	2098.123	2467.1	3111.636	13.63587	17.19828	353.2547	445.5434	710.7446	896.4284
41.47525	26.1624	1045.364	659.4106	741.4476	467.7018	20982.59	13235.72	1204.571	759.8379	2051.769	1294.246	45.0807	28.4367	425.7005	268.53	611.8331	385.9415

M6PR		FCGRT		CUBN		FOLR1		FOLR1		RAB4A		RAB5A			
32547_at		31432_g_at		35416_at		821_s_at		39311_at		39244_at		36110_at			
2254.636	2254.635	2046.744	2046.744	21.60967	21.60966	488.4326	488.4325	442.1562	442.1561	232.7628	232.7628	901.9968	901.9967	GSM3909
1720.208	1085.846	1910.849	1206.184	16.7766	10.58989	414.2038	261.4577	558.1496	352.3206	358.6654	226.4002	887.3479	560.1203	GSM3910
2448.36	4479.239	2139.846	3914.818	26.76158	48.95992	613.9946	1123.294	670.5855	1226.826	282.066	516.0356	901.3221	1648.956	GSM3911
2110.35	3387.23	2236.485	3589.684	25.78625	41.38837	571.7582	917.7038	707.2238	1135.134	413.8976	664.3287	752.9396	1208.51	GSM3912
2388.391	2568.46	1683.474	1810.397	24.05052	25.86378	352.7494	379.3445	409.1276	439.9732	463.7838	498.7502	2379.056	2558.421	GSM3913
1926.218	1960.355	2075.319	2112.098	14.47199	14.72847	578.3781	588.6282	713.2881	725.9292	319.9934	325.6644	740.9291	754.0601	GSM3914
2075.738	1111.44	1457.368	780.3379	10.98216	5.880322	884.2571	473.4696	813.019	435.3257	343.4234	183.8838	858.3215	459.5826	GSM3916
1634.592	1119.103	1479.231	1012.737	28.53967	19.53933	442.6971	303.0871	560.3146	383.6124	282.7172	193.5588	1126.977	771.5708	GSM3917
2216.991	2720.434	1847.621	2267.187	20.18874	24.77328	673.8727	826.8985	652.8766	801.1345	205.953	252.7216	1112.522	1365.158	GSM3918
1442.402	1600.191	1968.007	2183.294	24.06997	26.70307	677.2744	751.3638	603.1606	669.1424	302.6194	335.7239	1513.317	1678.864	GSM3919
1663.523	2098.123	2467.1	3111.636	13.63587	17.19828	353.2547	445.5434	710.7446	896.4284	411.799	519.3826	1054.093	1329.477	GSM3915
1204.571	759.8379	2051.769	1294.246	45.0807	28.4367	425.7005	268.53	611.8331	385.9415	263.608	166.2827	925.0057	583.4894	GSM3920

8.3.2 Study 2

Series: GSE18965, Platform: GPL96, Sample number: 16 (8 healthy, 8 asthmatic).

patient code		SDHA		FPR1		CDH1		TJP3		TJP1		TJP2		CAV1	
		201093_x_at		205119_s_at		201130_s_at		35148_at		214168_s_at		202085_at		212097_at	
GSM469508	healthy	5.89696		6.70533	6.70533	2.36889	2.36889	5.3928	5.3928	2.57169	2.57169	6.51257	6.51257	2.48482	2.48482
GSM469513	healthy	5.83458		3.3795	3.41563	2.41269	2.43849	6.31841	6.38596	2.61884	2.64684	5.59573	5.65556	2.52593	2.55294
GSM469519	healthy	6.02012		7.65792	7.50125	2.94048	2.88032	5.07659	4.97273	2.59207	2.53904	5.79628	5.6777	2.477	2.42632
GSM469515	healthy	6.20903		5.52318	5.24558	2.47145	2.34724	5.54559	5.26686	2.48933	2.36422	6.34292	6.02412	2.51498	2.38858
GSM469516	healthy	6.10971		6.2498	6.03217	2.78068	2.68385	6.38009	6.15792	2.50361	2.41643	6.81338	6.57613	2.4948	2.40793
GSM469517	healthy	5.88002		8.11873	8.14211	2.66237	2.67004	4.13732	4.14924	3.14006	3.1491	6.16599	6.18375	2.47369	2.48081
GSM469521	healthy	6.1404		2.9662	2.8486	3.99945	3.84089	6.56998	6.30951	2.5076	2.40819	6.89364	6.62034	2.50476	2.40546
GSM469522	asthma	5.66781		4.75718	4.94951	2.40913	2.50653	6.05057	6.29519	2.58173	2.6861	5.94694	6.18737	2.56461	2.6683
GSM469523	asthma	5.86247		3.27882	3.29811	2.5599	2.57496	5.24072	5.27154	2.68688	2.70269	5.64128	5.67447	2.60587	2.6212
GSM469509	asthma	6.01892		8.34375	8.17468	2.5566	2.50479	6.75199	6.61517	2.73226	2.67689	4.47593	4.38523	2.83845	2.78094
GSM469510	asthma	5.19006		6.86164	7.79621	2.51315	2.85544	5.24481	5.95916	2.75483	3.13004	6.16497	7.00465	2.75075	3.1254
GSM469511	asthma	5.93214		3.33047	3.31072	2.56864	2.55341	5.9688	5.9334	2.70397	2.68793	6.27327	6.23607	2.80301	2.78639
GSM469512	asthma	6.10134		4.4549	4.30567	2.4581	2.37576	5.25785	5.08172	2.65914	2.57007	5.69809	5.50722	2.64434	2.55576
GSM469514	asthma	5.93001		3.94539	3.9234	2.70129	2.68624	5.6948	5.66306	2.91084	2.89462	5.7342	5.70225	2.69149	2.67649
GSM469518	asthma	5.79182		4.72681	4.81261	2.5412	2.58733	5.68144	5.78457	2.59098	2.63801	5.93589	6.04364	2.55457	2.60094
GSM469520	asthma	5.91422		3.08053	3.07154	2.55907	2.5516	4.92255	4.90819	2.63806	2.63036	5.94527	5.92792	2.53523	2.52783

CLTC		VCFS		CLDN4		FCGRT		CUBN		FOLR1		EEA1		RAB4A		RAB5A	
200614_at		204482_at		201428_at		218831_s_at		206775_at		204437_s_at		204840_s_at		203581_at		206113_s_at	
9.74963	9.74963	3.20166	3.20166	7.18012	7.18012	7.2821	7.2821	3.16137	3.16137	4.92861	4.92861	3.47774	3.47774	5.64911	5.64911	3.97409	3.97409
8.65781	8.75037	3.20099	3.23521	7.87628	7.96048	5.95633	6.02001	3.21784	3.25224	7.83238	7.91612	3.89535	3.93699	5.45421	5.51252	3.47246	3.50958
9.21218	9.02371	3.19803	3.1326	6.60727	6.47209	6.95805	6.81569	3.17639	3.11141	5.42066	5.30976	3.27805	3.21098	5.79115	5.67267	3.93748	3.85692
9.18705	8.7253	3.23195	3.06951	7.85454	7.45977	6.20731	5.89533	3.54948	3.37108	6.76664	6.42654	3.68084	3.49584	5.93251	5.63434	3.41259	3.24107
9.01735	8.70336	3.06136	2.95476	8.20133	7.91575	6.32748	6.10715	3.17012	3.05973	6.47271	6.24732	3.27777	3.16364	6.26367	6.04556	3.59009	3.46508
9.02472	9.05072	2.7044	2.71219	6.64914	6.66829	6.57328	6.59222	3.15062	3.15969	4.90625	4.92038	3.22334	3.23263	5.12667	5.14144	4.62169	4.635
9.45897	9.08397	3.20035	3.07347	7.76691	7.45899	6.53796	6.27876	3.18028	3.05419	8.35138	8.02029	3.34974	3.21693	5.60441	5.38222	3.5941	3.45161
8.8215	9.17815	3.20208	3.33154	7.32168	7.61769	5.7327	5.96447	3.22138	3.35162	5.3316	5.54715	4.36787	4.54446	5.51332	5.73622	3.33153	3.46623
8.76104	8.81257	3.35719	3.37694	8.3233	8.37226	5.53439	5.56695	3.37115	3.39098	5.46693	5.49909	4.17641	4.20097	5.88836	5.923	3.37014	3.38996
8.84117	8.66202	3.23263	3.16713	7.845	7.68604	5.8333	5.7151	3.36575	3.29755	4.86601	4.7674	5.60644	5.49284	6.10904	5.98525	3.64377	3.56994
8.87811	10.0873	3.1866	3.62063	6.31263	7.17242	4.5613	5.18255	3.26538	3.71013	3.22732	3.66689	5.36676	6.09772	5.18297	5.8889	3.72266	4.22969
9.31421	9.25897	3.3598	3.33988	7.13014	7.08785	5.7212	5.68726	3.295	3.27546	6.57077	6.5318	5.26187	5.23066	5.67892	5.64524	3.51467	3.49382
8.96344	8.66318	3.38809	3.2746	6.91561	6.68395	5.58009	5.39317	3.51691	3.3991	5.07043	4.90058	5.07614	4.9061	5.7599	5.56696	3.49578	3.37868
8.9568	8.90688	3.21126	3.19336	6.77531	6.73755	5.17509	5.14624	3.26492	3.24672	5.5901	5.55894	4.78073	4.75409	5.78097	5.74875	3.61313	3.59299
8.82218	8.98232	3.06844	3.12414	6.97086	7.09739	5.90411	6.01128	3.17288	3.23047	6.82293	6.94678	3.83	3.89952	5.89233	5.99929	3.61849	3.68417
9.28627	9.25917	3.20931	3.19994	7.58981	7.56766	6.3792	6.36058	3.24108	3.23162	7.18074	7.15979	3.86379	3.85252	6.32941	6.31094	3.7657	3.75471

RAB9A		RAB11A		LAMP1		M6PR		OCLN		CLDN1		RAB7A		patient code
221808_at		200863_s_at		201551_s_at		200901_s_at		209925_at		218182_s_at		211960_s_at		
5.92251	5.92251	8.02636	8.02636	5.32208	5.32208	7.59472	7.59472	3.30745	3.30745	3.17452	3.17452	4.64551	4.64551	GSM469508
4.64244	4.69207	7.57732	7.65833	2.33681	2.36179	6.29179	6.35906	3.27555	3.31057	3.33247	3.3681	3.68107	3.72043	GSM469513
5.86171	5.74178	8.31236	8.1423	4.50955	4.41729	6.44213	6.31033	3.34573	3.27728	3.00944	2.94787	4.57299	4.47943	GSM469519
5.55081	5.27182	8.04234	7.63812	3.88728	3.69191	5.99686	5.69545	3.58656	3.4063	3.29251	3.12703	3.56637	3.38712	GSM469515
6.05805	5.8471	8.96736	8.6551	3.92374	3.78711	6.04054	5.8302	3.94545	3.80807	3.28791	3.17342	4.33376	4.18285	GSM469516
6.42284	6.44134	8.47598	8.50039	4.23865	4.25086	6.79788	6.81746	3.3973	3.40709	3.23926	3.24859	4.58709	4.6003	GSM469517
5.70235	5.47628	8.55929	8.21995	4.25757	4.08878	6.06519	5.82473	3.98044	3.82263	3.27095	3.14127	3.89988	3.74527	GSM469521
5.13959	5.34738	8.08705	8.41401	2.84815	2.9633	4.8067	5.00104	3.18354	3.31224	3.22631	3.35675	3.65303	3.80073	GSM469522
5.21901	5.24971	7.89994	7.94641	3.25661	3.27576	5.73187	5.76558	3.3309	3.35049	3.40528	3.42531	3.82808	3.85059	GSM469523
5.35017	5.24176	8.3389	8.16992	2.78685	2.73038	6.29631	6.16873	3.38597	3.31736	3.57532	3.50288	4.47314	4.3825	GSM469509
5.36627	6.09716	8.76978	9.96424	2.48759	2.82641	6.45681	7.33624	3.87037	4.39752	3.4583	3.92932	3.96416	4.50408	GSM469510
5.57263	5.53958	8.01292	7.96539	2.51063	2.49574	5.20223	5.17137	3.57937	3.55814	3.33164	3.31188	3.63303	3.61148	GSM469511
5.56432	5.37793	8.4965	8.21189	2.4718	2.38899	5.43621	5.25411	3.65465	3.53223	3.35909	3.24657	3.7032	3.57915	GSM469512
5.80033	5.768	8.72675	8.67812	2.33296	2.31996	4.81174	4.78492	3.66145	3.64104	3.66033	3.63993	3.66828	3.64783	GSM469514
5.51364	5.61373	8.23059	8.37999	3.10123	3.15753	5.63421	5.73649	3.59903	3.66436	3.20191	3.26003	3.93017	4.00151	GSM469518
5.87166	5.85453	8.55025	8.5253	4.50313	4.48999	6.06835	6.05064	3.67799	3.66725	3.34559	3.33583	3.62675	3.61616	GSM469520

8.3.3 Study 3

Series: GSE4302, Platform: GPL570, Sample number: 26 (13 healthy, 13 asthmatic).

		RPL27		FPR1		CDH1		TJP3		TJP1		TJP2		CAV1	
		200025_s_at		205119_s_at		201130_s_at		35148_at		214168_s_at		202085_at		212097_at	
GSM98210	asthma	12.49463		6.83161	6.83161	5.298821	5.29882	11.37572	11.3757	5.935904	5.9359	9.320962	9.32096	4.707092	4.70709
GSM98208	asthma	12.57797		6.296167	6.25445	5.203255	5.16878	11.39271	11.3172	6.055079	6.01496	9.847209	9.78196	5.180606	5.14628
GSM98141	asthma	12.53602		6.940215	6.9173	5.063536	5.04682	11.50874	11.4707	6.40681	6.38566	9.669104	9.63718	5.157019	5.13999
GSM98144	asthma	12.51973		9.679726	9.66032	5.20244	5.19201	11.49937	11.4763	6.256196	6.24365	9.852286	9.83253	5.050436	5.04031
GSM98145	asthma	12.46433		6.52939	6.54526	5.17192	5.18449	10.62314	10.649	6.034719	6.04939	9.667788	9.69129	4.770873	4.78247
GSM98148	asthma	12.629		6.02825	5.96411	5.4104	5.35283	11.3974	11.2761	6.17537	6.10967	10.033	9.92627	4.83395	4.78252
GSM98149	asthma	12.565		7.6224	7.57968	5.32576	5.29592	10.0615	10.0051	6.20461	6.16984	9.8708	9.81549	4.69843	4.6721
GSM98152	asthma	12.5454		6.45724	6.43109	5.16724	5.14631	11.3362	11.2903	6.09643	6.07174	9.47106	9.4327	4.86022	4.84053
GSM98153	asthma	12.6881		6.42478	6.32682	5.07144	4.99412	11.1026	10.9333	6.52653	6.42701	10.0802	9.92646	5.49292	5.40917
GSM98155	asthma	12.6665		6.7722	6.68032	5.19752	5.127	11.4995	11.3434	6.19147	6.10747	9.86385	9.73002	5.47135	5.39712
GSM98158	asthma	12.7625		6.06208	5.93483	4.93181	4.82829	11.1276	10.894	5.98709	5.86142	9.62133	9.41938	5.89684	5.77306
GSM98159	asthma	12.2819		6.49374	6.6062	5.43183	5.5259	10.6584	10.8429	6.1111	6.21692	9.78072	9.95009	4.53122	4.60969
GSM98161	asthma	12.1331		6.43776	6.62957	5.16232	5.31613	10.7587	11.0793	6.16908	6.35289	10.0904	10.391	4.46669	4.59978

CLTC		VCFS		CLDN4		FCGRT		CUBN		FOLR1		EEA1		RAB4A		RAB5A	
200614_at		204482_at		1569421_at		218831_s_at		206775_at		204437_s_at		204840_s_at		203581_at		206113_s_at	
11.31567	11.3157	5.852132	5.85213	6.316325	6.31633	8.919625	8.91963	5.394624	5.39462	8.994543	8.99454	7.267729	7.26773	10.79304	10.793	5.776677	5.77668
11.47094	11.3949	5.672726	5.63514	6.438212	6.39555	8.639134	8.58189	5.486231	5.44988	9.596956	9.53336	7.283684	7.23542	10.57397	10.5039	5.890678	5.85164
11.49207	11.4541	5.575212	5.5568	6.352084	6.33111	9.094909	9.06488	5.427845	5.40992	9.242893	9.21238	7.16381	7.14016	10.75592	10.7204	5.982931	5.96318
11.62431	11.601	5.591952	5.58074	6.435859	6.42295	9.295758	9.27712	5.347101	5.33638	9.143316	9.12498	7.442128	7.4272	10.59387	10.5726	5.939136	5.92723
11.64493	11.6732	5.5844	5.59797	6.292601	6.30789	9.087655	9.10974	5.501166	5.51454	8.625259	8.64622	6.643081	6.65923	10.98334	11.01	5.758452	5.77245
11.4949	11.3726	5.801	5.73928	6.47739	6.40847	8.79668	8.70308	5.32687	5.27019	8.58429	8.49295	6.80845	6.73601	10.916	10.7999	5.85254	5.79027
11.6931	11.6276	5.53675	5.50572	6.31187	6.2765	9.05784	9.00709	5.21078	5.18158	8.24094	8.19476	7.41344	7.3719	11.0746	11.0125	5.70158	5.66963
11.5413	11.4946	5.72492	5.70174	6.57234	6.54573	9.00365	8.96719	5.49844	5.47617	8.73237	8.69701	6.85411	6.82635	10.9401	10.8958	5.73117	5.70796
11.5761	11.3996	5.76617	5.67825	6.42573	6.32775	9.06939	8.9311	5.23509	5.15527	9.09663	8.95793	6.93528	6.82954	11.1317	10.9619	5.82113	5.73238
11.506	11.3498	5.7966	5.71796	6.31352	6.22786	9.10459	8.98106	5.33355	5.26119	9.23107	9.10583	6.89237	6.79886	10.7329	10.5873	5.55836	5.48295
11.3106	11.0732	5.42816	5.31422	6.43155	6.29655	9.07632	8.88581	5.78914	5.66763	7.30665	7.15329	6.97077	6.82446	10.7239	10.4988	5.56544	5.44862
11.5897	11.7904	5.6686	5.76676	6.50015	6.61271	8.45011	8.59644	5.50154	5.59681	9.5348	9.69991	7.48207	7.61163	10.7404	10.9264	5.89314	5.99519
11.7723	12.123	5.67558	5.84468	6.23397	6.41971	8.47487	8.72739	5.41275	5.57403	9.25669	9.5325	7.86089	8.09511	10.8537	11.1771	6.07867	6.25978

RAB9A		RAB11A		LAMP1		M6PR		OCLN		CLDN1		RAB7A		FPR1		TJP1	
221808_at		200863_s_at		201551_s_at		200901_s_at		209925_at		222549_at		211960_s_at		205118_at		202011_at	
8.221521	8.22152	11.29801	11.298	5.30107	5.30107	9.456651	9.45665	6.828783	6.82878	8.590227	8.59023	9.033182	9.03318	4.251014	4.25101	11.21476	11.2148
8.006817	7.95376	10.96207	10.8894	5.03393	5.00057	9.177988	9.11717	6.774981	6.73009	8.663756	8.60635	8.87395	8.81515	4.304376	4.27585	11.52851	11.4521
8.153467	8.12655	11.24135	11.2042	5.453409	5.4354	9.262841	9.23226	6.885933	6.8632	8.726713	8.6979	9.322381	9.2916	4.177473	4.16368	11.23765	11.2005
8.862685	8.84491	11.6332	11.6099	5.657883	5.64654	9.506516	9.48745	6.902005	6.88816	8.552468	8.53532	9.521378	9.50229	4.140408	4.13211	11.35753	11.3348
8.882879	8.90447	11.52944	11.5575	5.515419	5.52882	10.30256	10.3276	7.068307	7.08549	9.400009	9.42286	9.650456	9.67391	4.099663	4.10963	11.44196	11.4698
8.33158	8.24293	11.4949	11.3726	5.71063	5.64987	9.20576	9.1078	6.82661	6.75397	8.86868	8.77431	9.26556	9.16697	4.21088	4.16608	11.407	11.2857
8.96217	8.91195	11.226	11.1631	5.48449	5.45375	10.5194	10.4604	7.26758	7.22686	9.95185	9.89608	9.88938	9.83396	3.93218	3.91014	11.5312	11.4666
8.46066	8.42639	11.3258	11.28	5.17824	5.15727	9.32072	9.28297	6.97783	6.94957	7.79751	7.76593	9.1267	9.08974	4.22991	4.21278	11.0725	11.0277
9.04834	8.91038	11.7902	11.6104	5.46395	5.38064	9.32856	9.18633	7.08083	6.97287	8.98122	8.84428	9.601	9.45461	4.13749	4.0744	11.61	11.433
8.08874	7.97899	11.2904	11.1372	5.40132	5.32804	9.0997	8.97624	6.7739	6.68199	8.6148	8.49792	9.44072	9.31263	4.15084	4.09452	11.4483	11.293
8.29543	8.12131	11.5297	11.2877	5.28084	5.17	9.16977	8.9773	6.63012	6.49095	9.36942	9.17276	9.10763	8.91646	4.22405	4.13538	11.379	11.1401
8.55368	8.70181	10.3272	10.5061	5.42244	5.51634	10.1552	10.331	6.90215	7.02167	8.95752	9.11264	9.2104	9.36989	4.20306	4.27584	10.707	10.8924
9.04431	9.31379	10.7394	11.0594	5.64267	5.8108	10.4155	10.7258	7.44837	7.67029	9.3775	9.65691	9.50324	9.78639	4.11822	4.24092	11.3876	11.7269

CDH1		TJP2		TJP3		CAV1		OCLN		OCLN		CLDN1		CLDN4		EEA1	
201131_s_at		232017_at		213412_at		203065_s_at		227492_at		231022_at		218182_s_at		201428_at		204841_s_at	
11.40549	11.4055	5.783413	5.78341	9.722132	9.72213	4.721342	4.72134	10.07896	10.079	7.299481	7.29948	5.572475	5.57248	9.632591	9.63259	5.858612	5.85861
11.16515	11.0912	6.372671	6.33044	9.358309	9.2963	5.037321	5.00394	10.07976	10.013	7.714076	7.66296	5.031597	4.99826	9.101593	9.04128	6.543416	6.50006
11.20325	11.1663	6.206776	6.18628	9.556376	9.52483	4.794368	4.77854	9.622515	9.59075	7.39575	7.37133	4.905668	4.88947	9.185964	9.15564	6.557732	6.53608
11.08645	11.0642	5.685996	5.67459	9.523834	9.50474	4.702407	4.69298	9.784801	9.76518	7.471625	7.45664	4.771923	4.76235	9.313926	9.29525	5.728466	5.71698
11.61766	11.6459	6.121152	6.13603	9.146491	9.16872	4.568968	4.58007	9.965638	9.98986	7.346991	7.36485	5.204279	5.21693	9.769262	9.79301	5.890222	5.90454
11.3717	11.2507	5.91077	5.84788	9.68533	9.58228	4.60397	4.55499	10.1048	9.99729	7.6994	7.61748	5.16329	5.10835	9.62639	9.52396	5.66829	5.60798
11.9311	11.8642	5.73408	5.70195	8.82657	8.77711	4.7793	4.75252	10.2378	10.1805	7.32419	7.28315	4.89407	4.86665	9.62369	9.56976	6.19909	6.16435
11.4646	11.4182	6.17071	6.14572	9.41245	9.37433	4.94093	4.92092	9.54169	9.50305	7.3284	7.29872	5.04464	5.0242	9.33461	9.2968	6.49864	6.47232
11.3556	11.1825	5.79453	5.70618	9.28954	9.1479	4.66002	4.58896	9.88918	9.7384	7.40215	7.28928	5.14315	5.06473	9.42446	9.28076	5.70361	5.61665
11.1137	10.963	6.2643	6.17931	9.60847	9.47811	5.04347	4.97504	9.79937	9.66642	7.4147	7.3141	5.20649	5.13585	9.2579	9.1323	6.25793	6.17302
11.3346	11.0967	6.3139	6.18137	9.28595	9.09104	5.31423	5.20269	9.55993	9.35927	7.27878	7.126	5.25984	5.14944	8.97878	8.79032	6.80085	6.6581
11.6139	11.8151	6.30895	6.4182	9.23814	9.39812	4.64549	4.72594	9.26616	9.42662	7.17533	7.29959	4.97447	5.06061	10.0267	10.2003	6.01686	6.12105
12.0289	12.3873	6.22765	6.41321	9.33905	9.61731	4.69901	4.83902	10.4235	10.7341	7.70591	7.93551	5.44186	5.60401	9.9759	10.2731	5.72271	5.89322

RAB4A		RAB4A		RAB5A		RAB5A		RAB7A		RAB7A		RAB11A		RAB11A		LAMP1	
206272_at		203582_s_at		240990_at		209089_at		1570061_at		211961_s_at		234998_at		200864_s_at		201553_s_at	
9.446135	9.44613	6.005973	6.00597	5.850639	5.85064	10.61149	10.6115	5.201373	5.20137	10.99294	10.9929	9.097529	9.09753	9.425798	9.4258	12.74539	12.7454
9.747499	9.68291	5.430214	5.39423	5.872524	5.83361	10.68971	10.6189	5.309934	5.27475	11.09117	11.0177	8.824088	8.76562	8.455332	8.3993	12.99282	12.9067
9.737688	9.70554	5.29907	5.28158	5.706305	5.68747	10.78309	10.7475	5.316454	5.2989	11.41369	11.376	8.954215	8.92465	8.524943	8.4968	13.25339	13.2096
9.832425	9.81271	5.565311	5.55415	5.686988	5.67558	10.95415	10.9322	5.223205	5.21273	11.30981	11.2871	8.881155	8.86335	9.361292	9.34252	13.02044	12.9943
9.953277	9.97747	6.154006	6.16896	5.636612	5.65031	11.0559	11.0828	4.977266	4.98936	10.88789	10.9143	9.035235	9.0572	9.730219	9.75387	12.79368	12.8248
9.84331	9.73857	6.22502	6.15878	5.77283	5.71141	10.7787	10.664	5.12973	5.07515	11.3126	11.1923	9.33277	9.23347	9.26987	9.17124	13.0276	12.889
10.1729	10.1159	6.66024	6.62291	5.44475	5.41424	11.3468	11.2832	5.00987	4.9818	11.1377	11.0753	9.27561	9.22363	10.4025	10.3442	13.0292	12.9562
9.77447	9.73488	5.67087	5.6479	5.48428	5.46206	10.5532	10.5105	5.34874	5.32708	11.2368	11.1913	9.04706	9.01042	8.94736	8.91113	12.9172	12.8649
10.1349	9.98037	6.44412	6.34587	5.44128	5.35831	10.8898	10.7237	5.07433	4.99696	11.2354	11.0641	9.13177	8.99254	9.67906	9.53148	13.1696	12.9688
10.0682	9.93164	5.44819	5.37427	5.69663	5.61934	10.8937	10.7459	4.98133	4.91375	11.2616	11.1088	9.01535	8.89303	9.12252	8.99875	13.0876	12.91
9.93732	9.72874	5.53979	5.42351	5.68015	5.56092	10.7136	10.4887	5.09022	4.98338	11.1469	10.9129	9.26024	9.06587	9.12399	8.93248	12.9442	12.6725
9.56949	9.73521	5.7136	5.81254	5.29114	5.38277	10.7574	10.9437	5.48943	5.58449	10.8467	11.0345	8.28192	8.42533	7.25895	7.38465	12.9346	13.1586
9.83581	10.1289	6.23147	6.41714	5.77676	5.94888	11.0039	11.3318	5.28925	5.44685	10.8572	11.1807	8.80337	9.06567	9.45673	9.7385	12.8945	13.2787

RAB7A		RAB11A		RAB11A		LAMP1		LAMP1		LAMP1		M6PR	
211961_s_at		234998_at		200864_s_at		201553_s_at		213728_at		201552_at		200900_s_at	
10.99294	10.9929	9.097529	9.09753	9.425798	9.4258	12.74539	12.7454	10.11692	10.1169	8.280172	8.28017	7.168081	7.16808
11.09117	11.0177	8.824088	8.76562	8.455332	8.3993	12.99282	12.9067	9.883682	9.81819	8.131713	8.07783	6.969058	6.92288
11.41369	11.376	8.954215	8.92465	8.524943	8.4968	13.25339	13.2096	10.15597	10.1224	8.759005	8.73009	6.845203	6.8226
11.30981	11.2871	8.881155	8.86335	9.361292	9.34252	13.02044	12.9943	10.15251	10.1321	8.532188	8.51508	7.271422	7.25684
10.88789	10.9143	9.035235	9.0572	9.730219	9.75387	12.79368	12.8248	10.62754	10.6534	8.970098	8.9919	7.117226	7.13452
11.3126	11.1923	9.33277	9.23347	9.26987	9.17124	13.0276	12.889	10.1393	10.0314	8.29989	8.21158	6.62416	6.55368
11.1377	11.0753	9.27561	9.22363	10.4025	10.3442	13.0292	12.9562	10.783	10.7226	9.50479	9.45152	7.24119	7.20061
11.2368	11.1913	9.04706	9.01042	8.94736	8.91113	12.9172	12.8649	10.1677	10.1266	8.34825	8.31444	6.97317	6.94493
11.2354	11.0641	9.13177	8.99254	9.67906	9.53148	13.1696	12.9688	10.4978	10.3378	8.61286	8.48154	6.93638	6.83062
11.2616	11.1088	9.01535	8.89303	9.12252	8.99875	13.0876	12.91	10.2018	10.0634	8.37259	8.25899	6.98753	6.89273
11.1469	10.9129	9.26024	9.06587	9.12399	8.93248	12.9442	12.6725	9.94776	9.73896	8.21137	8.03902	6.90314	6.75825
10.8467	11.0345	8.28192	8.42533	7.25895	7.38465	12.9346	13.1586	10.6735	10.8583	8.52416	8.67177	6.96117	7.08172
10.8572	11.1807	8.80337	9.06567	9.45673	9.7385	12.8945	13.2787	10.5929	10.9085	8.81979	9.08258	7.11857	7.33067

SM98206	healthy	12.4515	6.56892	6.59166	5.24119	5.25934	11.3306	11.3699	6.15729	6.17861	10.2455	10.2809	5.90492	5.92537
SM98207	healthy	12.6081	6.51539	6.45675	4.99497	4.95001	11.4483	11.3452	6.20031	6.14451	9.5748	9.48862	5.28586	5.23828
SM98209	healthy	12.3013	6.26085	6.35923	5.12216	5.20265	11.604	11.7863	6.68259	6.78761	10.0325	10.1902	5.05586	5.13532
SM98211	healthy	12.3149	6.96896	7.07066	4.97306	5.04563	11.4311	11.598	5.82812	5.91318	9.91483	10.0595	5.03633	5.10982
SM98212	healthy	12.3945	6.67315	6.72707	4.95337	4.99339	11.689	11.7834	6.31405	6.36507	9.67882	9.75703	4.85893	4.89819
SM98213	healthy	12.3317	6.81786	6.90792	5.01327	5.0795	11.7091	11.8638	6.10844	6.18913	10.0266	10.1591	5.83555	5.91264
SM98217	healthy	12.2957	7.34211	7.46087	5.40605	5.49349	10.7914	10.9659	6.34037	6.44293	9.89648	10.0566	4.38447	4.45539
SM98218	healthy	12.1682	6.5028	6.67724	4.91451	5.04634	10.3606	10.6385	5.90576	6.06417	9.71635	9.97699	5.3978	5.54259
SM98219	healthy	12.0809	6.80188	7.03484	5.06769	5.24126	10.9506	11.3256	6.07187	6.27983	9.6842	10.0159	4.70557	4.86673
SM98221	healthy	12.3416	6.72916	6.81263	5.19098	5.25537	9.96874	10.0924	6.11026	6.18604	9.3408	9.45666	4.92527	4.98636
SM98222	healthy	11.919	6.10116	6.3958	5.04632	5.29002	10.2611	10.7567	6.27727	6.58042	9.73071	10.2006	4.81185	5.04423
SM98223	healthy	12.0081	6.21526	6.46706	5.15958	5.36861	10.4403	10.8633	6.41679	6.67675	9.86735	10.2671	4.9292	5.1289
SM98225	healthy	12.1863	6.56191	6.72794	5.24383	5.37651	11.1513	11.4335	6.3214	6.48134	9.23147	9.46505	4.70824	4.82737

5.58141	5.60073	6.39801	6.42016	8.64287	8.67279	5.4213	5.44007	9.44187	9.47456	7.28368	7.3089	10.773	10.8103	5.84174	5.86196	8.45959	8.48888
5.3709	5.32256	6.43476	6.37685	9.40705	9.32238	5.27835	5.23084	9.92589	9.83655	6.60834	6.54886	10.4467	10.3526	5.68862	5.63741	7.87932	7.8084
5.53172	5.61865	6.3962	6.49671	8.61256	8.74791	5.32648	5.41018	9.2796	9.42543	7.60762	7.72717	10.4651	10.6296	5.82815	5.91973	8.51557	8.64939
5.51167	5.5921	6.32388	6.41616	9.50481	9.64352	5.38883	5.46747	9.26619	9.40141	6.98857	7.09056	10.4145	10.5665	5.38352	5.46208	8.18732	8.3068
5.67694	5.72282	6.35119	6.40251	9.12814	9.20191	5.37977	5.42324	9.4132	9.48926	7.38999	7.44971	10.471	10.5556	5.81863	5.86565	8.32193	8.38917
5.489	5.56151	6.30339	6.38666	8.95954	9.0779	5.36028	5.43109	9.17191	9.29307	7.1361	7.23036	10.6458	10.7864	5.92882	6.00714	8.21827	8.32683
5.86397	5.95882	6.4146	6.51835	8.9784	9.12362	5.3063	5.39213	9.36785	9.51938	7.74474	7.87001	10.6322	10.8041	5.75584	5.84894	8.69887	8.83958
5.47827	5.62522	6.20237	6.36874	8.18718	8.4068	5.53247	5.68088	9.26059	9.509	7.04912	7.23821	10.399	10.678	5.96782	6.12791	8.36816	8.59263
5.5357	5.72529	6.33617	6.55318	8.63391	8.92962	5.43579	5.62196	9.22404	9.53996	6.97468	7.21356	10.5388	10.8997	5.94042	6.14387	8.01938	8.29404
5.91391	5.98726	6.41479	6.49436	8.53446	8.64031	5.63004	5.69987	9.70571	9.82609	7.03044	7.11764	10.5429	10.6736	6.10567	6.1814	8.44097	8.54566
5.7038	5.97925	6.28641	6.59	8.14144	8.53462	5.37262	5.63207	9.78046	10.2528	6.91588	7.24987	10.8468	11.3706	5.76828	6.04684	8.05969	8.44892
5.76511	5.99867	6.56049	6.82628	8.54902	8.89537	5.61536	5.84286	10.3749	10.7952	7.26694	7.56135	10.5816	11.0103	5.84424	6.08101	8.24861	8.58279
5.99057	6.14214	6.62845	6.79616	8.5604	8.777	5.45689	5.59496	9.20278	9.43563	6.82236	6.99498	10.6314	10.9004	6.10743	6.26196	8.26542	8.47455

11.131	11.1696	5.32474	5.34317	9.39699	9.42953	6.99883	7.02306	9.4837	9.51654	9.3385	9.37083	4.11125	4.12548	11.6529	11.6932	11.3086	11.3478
10.9969	10.8979	5.38724	5.33875	9.15309	9.07071	6.76672	6.70581	8.22185	8.14785	8.91506	8.83482	4.38955	4.35004	11.3572	11.2549	11.2486	11.1473
11.0822	11.2564	5.45341	5.53911	9.09908	9.24207	6.94639	7.05555	8.92414	9.06438	8.93	9.07033	3.9849	4.04752	11.7187	11.9028	11.4007	11.5799
10.8171	10.975	5.32705	5.40479	9.46801	9.60617	6.75708	6.85569	8.74623	8.87387	9.18005	9.31402	4.14397	4.20445	11.3097	11.4747	11.0174	11.1781
10.9913	11.0801	5.2533	5.29575	9.34105	9.41653	6.3189	6.36996	8.74322	8.81387	8.86045	8.93205	4.18764	4.22148	11.2411	11.3319	11.1095	11.1993
11.0807	11.2271	5.54345	5.61668	9.35743	9.48105	6.8363	6.92661	8.69708	8.81197	9.12874	9.24934	4.26871	4.3251	11.4846	11.6364	11.0477	11.1936
10.5566	10.7273	5.73489	5.82765	10.3653	10.533	6.50746	6.61272	9.09502	9.24213	9.30846	9.45903	3.99058	4.05513	10.9132	11.0898	11.446	11.6312
10.4807	10.7619	5.37703	5.52126	9.9574	10.2245	6.72791	6.90838	9.33248	9.58282	9.18523	9.43162	4.21033	4.32327	11.3417	11.646	11.7863	12.1025
10.5711	10.9331	5.61172	5.80392	10.0584	10.4029	6.79486	7.02758	8.54353	8.83614	9.27841	9.59619	4.17547	4.31848	11.2411	11.6261	11.7444	12.1466
10.1697	10.2958	5.48758	5.55564	10.4744	10.6043	6.9428	7.02892	8.96975	9.08101	9.35513	9.47116	4.14865	4.2001	11.0818	11.2192	11.7397	11.8853
9.96953	10.451	5.36618	5.62533	10.1713	10.6625	6.85186	7.18275	8.64684	9.06442	9.01712	9.45259	4.09399	4.2917	10.7709	11.2911	11.4887	12.0435
9.62004	10.0098	5.29499	5.50951	10.3712	10.7914	7.03065	7.31549	8.6196	8.96881	8.81305	9.1701	4.2353	4.40689	10.8938	11.3352	11.784	12.2615
10.7204	10.9917	5.42208	5.55927	9.74177	9.98826	6.87724	7.05125	8.82106	9.04425	8.69018	8.91006	4.19245	4.29853	11.1289	11.4105	11.5233	11.8149

6.34349	6.36545	9.42781	9.46045	5.11822	5.13594	10.1103	10.1453	7.70879	7.73548	5.56666	5.58593	9.73743	9.77114	6.35079	6.37277	7.49559	7.52154
6.2201	6.16412	9.60965	9.52315	4.65378	4.6119	9.82786	9.7394	7.11367	7.04965	4.94489	4.90039	9.26772	9.18431	6.36689	6.30958	6.8865	6.82452
6.32639	6.4258	9.49915	9.64842	4.67509	4.74856	10.4113	10.5749	7.91856	8.043	5.33093	5.4147	9.26831	9.41395	5.86998	5.96222	7.24414	7.35798
6.37491	6.46794	9.50256	9.64123	4.84125	4.9119	9.65198	9.79283	7.49833	7.60776	4.7286	4.79761	8.99955	9.13089	6.89853	6.9992	6.93406	7.03525
6.14183	6.19146	9.50143	9.57821	5.17384	5.21565	9.62041	9.69815	7.36365	7.42316	5.04242	5.08316	9.34666	9.42218	6.0864	6.13558	6.89393	6.94963
6.19283	6.27464	10.0459	10.1786	5.01092	5.07711	10.0663	10.1993	7.65726	7.75841	4.98864	5.05455	9.17803	9.29927	6.10572	6.18638	7.11206	7.20601
5.96004	6.05645	9.29314	9.44346	4.65521	4.73051	9.63437	9.79021	7.37607	7.49538	4.85215	4.93063	9.72704	9.88438	5.71211	5.8045	6.45461	6.55901
6.35364	6.52407	8.92733	9.1668	5.41333	5.55854	9.66796	9.9273	7.10868	7.29937	4.74546	4.87276	9.23846	9.48628	6.59424	6.77113	6.91793	7.1035
6.09322	6.30191	9.42585	9.74868	4.7315	4.89355	9.85873	10.1964	7.13262	7.37691	5.01439	5.18613	9.61867	9.9481	6.15689	6.36776	6.9322	7.16962
6.50597	6.58666	9.12151	9.23464	4.87534	4.93581	9.77043	9.89161	7.10048	7.18855	5.50868	5.57701	9.33676	9.45257	6.49121	6.57172	6.84556	6.93047
6.53761	6.85333	9.08168	9.52026	4.97724	5.2176	9.02185	9.45754	7.12561	7.46973	5.04451	5.28813	9.70818	10.177	6.47727	6.79007	6.06931	6.36242
6.1571	6.40655	9.14902	9.51968	5.12482	5.33244	9.76578	10.1614	7.1963	7.48785	5.06941	5.27478	9.68807	10.0806	6.61342	6.88136	6.50824	6.77191
6.21595	6.37323	9.45406	9.69327	4.95209	5.07739	9.8185	10.0669	7.26905	7.45297	4.72735	4.84696	9.62136	9.86479	6.47165	6.63539	6.55625	6.72213

7.49559	7.52154	9.85236	9.88647	5.59614	5.61551	5.33691	5.35539	10.9284	10.9663	5.36721	5.3858	11.3938	11.4332	8.68755	8.71763	8.84228	8.87289
6.8865	6.82452	9.36212	9.27786	5.39843	5.34984	5.58604	5.53576	10.333	10.24	5.01117	4.96606	11.2901	11.1885	8.89955	8.81944	8.27216	8.19771
7.24414	7.35798	9.55631	9.70649	5.39571	5.4805	5.76766	5.8583	10.6361	10.8032	5.41676	5.50188	11.0307	11.2041	8.70809	8.84494	8.63595	8.77166
6.93406	7.03525	9.50142	9.64007	5.0984	5.1728	5.72072	5.8042	10.674	10.8297	5.16718	5.24259	11.3573	11.5231	8.32135	8.44278	8.0014	8.11816
6.89393	6.94963	9.73351	9.81216	5.35191	5.39515	5.60175	5.64701	10.4117	10.4958	5.26626	5.30881	10.9064	10.9946	8.40113	8.46901	8.49756	8.56623
7.11206	7.20601	9.43623	9.56088	5.32366	5.39399	5.44418	5.5161	10.6869	10.8281	5.31595	5.38617	11.1943	11.3422	8.60902	8.72275	8.85453	8.9715
6.45461	6.55901	9.25859	9.40835	5.70285	5.7951	5.53081	5.62027	10.9467	11.1237	5.41666	5.50428	10.8518	11.0273	8.22075	8.35372	7.52675	7.6485
6.91793	7.1035	9.32028	9.57029	5.48106	5.62809	5.90832	6.06681	10.9136	11.2063	5.37079	5.51486	10.6583	10.9442	8.62656	8.85797	8.90729	9.14622
6.9322	7.16962	9.42053	9.74318	5.76162	5.95895	5.88072	6.08213	10.8394	11.2106	5.20954	5.38796	10.7297	11.0972	8.46161	8.75142	9.11698	9.42923
6.84556	6.93047	9.6364	9.75592	5.88472	5.95771	5.7535	5.82486	10.7725	10.9061	5.19294	5.25734	10.8589	10.9936	8.7266	8.83484	9.15579	9.26935
6.06931	6.36242	9.33586	9.78672	5.40453	5.66553	5.67693	5.95109	10.565	11.0752	5.21673	5.46867	10.6441	11.1581	8.2522	8.65072	6.5199	6.83476
6.50824	6.77191	9.24173	9.61614	5.11795	5.32529	5.83787	6.07438	10.722	11.1564	5.08428	5.29026	10.5737	11.0021	7.62194	7.93073	6.81779	7.094
6.55625	6.72213	9.34453	9.58096	5.57272	5.71372	5.96584	6.11678	10.5709	10.8383	5.05479	5.18268	10.5246	10.7909	8.633	8.85143	8.37656	8.5885

8.2.4 Study 4

Series: GSE3212, Platform: GPL80, Sample number: 30 (15 healthy, 15 asthmatic).

		RPL27		FPR1		CDH1		TJP3		TJP1		TJP2		CAV1		CLTC		VCFS	
		200025_s_at		205119_s_at		201130_s_at		35148_at		214168_s_at		202085_at		212097_at		200614_at		204482_at	
GSM38345	healthy	12.78261		9.378199	9.378199	5.610025	5.610025	7.041684	7.041684	6.676078	6.676078	9.097273	9.097273	6.424399	6.424399	12.74997	12.74997	7.653015	7.653015
GSM38346	healthy	12.49789		8.483521	8.676786	5.648162	5.776834	7.214026	7.37837	6.61541	6.766117	7.580822	7.753522	6.091476	6.230247	11.59015	11.85419	8.205788	8.392726
GSM38348	healthy	12.49868		10.07849	10.30743	5.781577	5.912914	7.109904	7.271416	6.560618	6.709652	8.543819	8.737904	6.595662	6.745492	12.91155	13.20486	7.559673	7.731402
GSM38350	healthy	12.71498		10.66593	10.72266	5.572904	5.602545	7.138341	7.176308	6.33013	6.363798	9.96578	10.01878	6.409109	6.443197	13.23783	13.30824	7.862492	7.90431
GSM38351	healthy	13.19885		11.3432	10.98548	5.556336	5.381111	6.639508	6.430123	6.601297	6.393117	9.860026	9.549079	6.363476	6.162796	13.18623	12.77039	7.273111	7.043745
GSM38353	healthy	12.68302		10.51055	10.59308	5.572904	5.616664	6.448312	6.498946	6.899433	6.953609	9.884599	9.962215	6.60888	6.660774	13.27306	13.37728	7.003573	7.058567
GSM38355	healthy	12.98669		10.69035	10.52235	5.826692	5.735129	6.863834	6.755972	6.371367	6.271244	9.795573	9.641641	6.116507	6.020389	13.15755	12.95079	7.318052	7.203053
GSM38356	healthy	13.334		10.44755	10.01552	5.739667	5.50232	6.217188	5.960095	6.531796	6.261693	9.890332	9.481347	6.353837	6.091093	13.12199	12.57937	7.119983	6.825557
GSM38358	healthy	13.16615		10.2244	9.926559	5.515717	5.35504	6.700699	6.505503	6.358831	6.173593	9.884772	9.596821	6.217224	6.036112	13.09719	12.71566	7.291073	7.078678
GSM38362	healthy	12.57931		9.794183	9.952474	5.698968	5.791073	6.40463	6.50814	6.480881	6.585623	10.02598	10.18801	6.38358	6.48675	13.24449	13.45854	7.14286	7.258301
GSM38368	healthy	13.00664		9.820031	9.650885	5.670314	5.572645	6.033783	5.929854	6.564659	6.451585	9.424811	9.262473	6.753332	6.637009	13.26995	13.04138	6.936605	6.817125
GSM38371	healthy	13.13057		11.84986	11.53584	5.405365	5.262123	6.397538	6.228003	6.4122	6.242277	9.932028	9.668829	6.491389	6.319367	13.18796	12.83847	7.104494	6.916225
GSM38373	healthy	13.23251		10.83468	10.46631	5.424746	5.240309	6.267079	6.054003	6.447378	6.228172	9.977044	9.637832	6.449537	6.230258	13.1674	12.71972	7.214061	6.968788
GSM38385	healthy	12.99862		11.70689	11.51235	5.402371	5.312596	6.852204	6.738336	6.635947	6.525672	9.009857	8.860133	6.38358	6.277499	13.02665	12.81018	7.847029	7.716629
GSM38377	healthy	12.75867		10.60498	10.62488	5.552685	5.563106	6.44675	6.458849	6.432381	6.444453	10.38888	10.40838	6.131496	6.143003	13.24028	13.26513	7.221083	7.234635

CLDN4		FCGRT		CUBN		FOLR1		EEA1		RAB4A		RAB5A		RAB9A		RAB11A		LAMP1	
1569421_at		218831_s_at		206775_at		204437_s_at		204840_s_at		203581_at		206113_s_at		221808_at		200863_s_at		201551_s_at	
8.072948	8.072948	9.877483	9.877483	6.218229	6.218229	8.539329	8.539329	5.376142	5.376142	8.879889	8.879889	6.419919	6.419919	9.038836	9.038836	11.41059	11.41059	5.557072	5.557072
8.150254	8.335927	10.39485	10.63166	6.103789	6.242841	8.909182	9.112144	5.431244	5.554974	8.472962	8.665986	6.604353	6.754808	8.496964	8.690535	10.82463	11.07122	5.582352	5.709525
8.121366	8.305855	9.965659	10.19204	6.019599	6.156343	8.336257	8.525627	5.01122	5.125057	8.792801	8.992542	6.202444	6.343342	9.207465	9.416626	11.41621	11.67554	5.845117	5.977897
7.981229	8.023679	10.33216	10.38712	6.07785	6.110176	7.782395	7.823787	5.389569	5.418234	10.08274	10.13637	6.585639	6.620666	10.39244	10.44772	12.12994	12.19445	6.010466	6.042434
7.956502	7.705585	10.41029	10.08199	5.9192	5.732531	8.060051	7.805868	4.976892	4.81994	10.03317	9.716759	6.033996	5.843707	10.20942	9.887458	12.05021	11.67019	5.921417	5.734678
7.877945	7.939804	10.50076	10.58321	5.960812	6.007618	8.313713	8.378994	4.77114	4.808604	9.946224	10.02432	5.823937	5.869668	10.37683	10.45832	12.18927	12.28498	5.867963	5.91404
7.82566	7.702684	10.72747	10.55889	5.908196	5.815352	8.874209	8.734755	5.302538	5.219211	10.32556	10.1633	6.083845	5.988241	10.59882	10.43226	12.18273	11.99129	6.241256	6.143178
7.899073	7.57243	10.74978	10.30526	6.09231	5.840381	9.30348	8.918762	5.73834	5.501048	10.3492	9.921242	5.824337	5.583489	10.59395	10.15587	12.13044	11.62882	6.467728	6.200274
7.865999	7.636856	10.57937	10.27118	5.881838	5.710496	9.417435	9.143098	5.561284	5.39928	10.29557	9.995648	6.219651	6.038468	10.38586	10.08331	12.06811	11.71655	6.229311	6.047846
7.96772	8.096492	9.998629	10.16022	6.08439	6.182724	8.498808	8.636163	4.676172	4.751747	9.646867	9.802777	6.066349	6.164392	10.18778	10.35243	12.04726	12.24197	5.613524	5.704248
8.188688	8.047641	10.31707	10.13936	6.373064	6.263291	8.939905	8.785919	4.733038	4.651513	9.950182	9.778794	6.106249	6.001071	10.43946	10.25964	11.91486	11.70964	6.268232	6.160264
8.03665	7.823679	10.82933	10.54236	5.991672	5.832893	9.374641	9.126213	6.6036	6.428605	10.35481	10.0804	5.90001	5.74366	10.37424	10.09933	11.98686	11.66921	6.044626	5.884443
7.965343	7.694527	10.56152	10.20244	5.927201	5.725681	8.844468	8.543763	6.511578	6.290189	10.25332	9.904718	6.186848	5.9765	10.27149	9.922264	12.06773	11.65744	5.728156	5.533403
8.038043	7.904469	10.65726	10.48016	5.992975	5.893385	9.2098	9.056754	6.149505	6.047314	9.900431	9.735908	6.288216	6.18372	10.09868	9.930861	12.0261	11.82625	5.412979	5.323027
7.704526	7.718985	10.69698	10.71705	6.012321	6.023604	9.349057	9.366602	6.537799	6.550068	10.2011	10.22025	6.102745	6.114198	10.78791	10.80816	12.06415	12.08679	6.277876	6.289658

LAMP1		M6PR		OCLN		CLDN1		RAB7A		FPR1		TJP1		CDH1		TJP2		TJP3	
201551_s_at		200901_s_at		209925_at		222549_at		211960_s_at		205118_at		202011_at		201131_s_at		232017_at		213412_at	
5.557072	5.557072	10.8701	10.8701	5.937805	5.937805	6.48417	6.48417	10.54555	10.54555	5.760989	5.760989	7.417866	7.417866	6.629089	6.629089	8.049579	8.049579	6.035522	6.035522
5.582352	5.709525	10.55294	10.79335	6.190493	6.33152	6.318169	6.462104	9.441128	9.656208	5.429933	5.553633	5.988863	6.125296	6.076332	6.214758	7.823997	8.002237	6.278799	6.421838
5.845117	5.977897	11.1056	11.35788	5.850102	5.982996	6.44227	6.588616	10.389	10.625	5.934284	6.06909	7.18836	7.351654	6.502436	6.650148	8.201641	8.387953	6.146282	6.285904
6.010466	6.042434	11.80157	11.86434	5.246841	5.274747	6.39079	6.424781	10.78069	10.83803	5.770061	5.80075	7.742843	7.784025	6.25622	6.289495	7.900655	7.942676	5.836245	5.867286
5.921417	5.734678	11.81267	11.44014	5.245254	5.079839	6.476647	6.272398	10.80937	10.46848	5.690001	5.51056	8.858985	8.579607	5.822551	5.63893	7.958476	7.707496	5.824115	5.640445
5.867963	5.91404	11.53761	11.6282	5.216426	5.257387	6.272503	6.321756	10.83101	10.91606	5.827544	5.873303	8.184043	8.248306	6.503032	6.554095	8.015435	8.078374	5.721699	5.766627
6.241256	6.143178	11.67896	11.49543	5.454979	5.369257	6.24146	6.143379	10.68424	10.51635	5.794138	5.703086	8.452828	8.319996	6.831027	6.723681	7.910679	7.786367	5.905892	5.813084
6.467728	6.200274	11.73841	11.25301	5.292882	5.074011	6.685629	6.409165	10.53122	10.09573	5.855714	5.613568	8.151944	7.814844	5.577483	5.346843	8.058846	7.725596	5.656402	5.422498
6.229311	6.047846	11.63196	11.29311	5.429036	5.270884	6.371983	6.186362	10.73849	10.42567	5.813981	5.644615	6.936239	6.734181	5.486467	5.326642	8.012575	7.779163	5.855785	5.685202
5.613524	5.704248	11.57576	11.76284	5.419915	5.50751	6.584708	6.691128	10.78777	10.96211	5.801427	5.895188	8.589415	8.728235	7.464327	7.584963	8.271785	8.405471	5.779635	5.873044
6.268232	6.160264	11.49628	11.29827	5.176955	5.087784	6.563601	6.450546	10.6982	10.51393	5.851951	5.751154	7.624939	7.493603	5.954584	5.852019	7.914497	7.778173	5.902891	5.801216
6.044626	5.884443	12.03531	11.71637	5.567744	5.420199	6.514356	6.341726	10.55259	10.27295	5.866655	5.711189	7.680726	7.477187	5.705717	5.554516	8.304959	8.084878	5.928833	5.771719
5.728156	5.533403	11.75043	11.35092	5.460984	5.275315	6.484507	6.264039	10.71243	10.34822	5.794138	5.597142	7.891795	7.62348	6.721385	6.492863	8.297215	8.015116	5.692784	5.499234
5.412979	5.323027	11.71173	11.5171	5.617548	5.524197	6.468326	6.360837	10.05682	9.889702	5.795211	5.698908	6.87299	6.758776	6.129373	6.027517	8.26942	8.132001	6.151907	6.049676
6.277876	6.289658	12.21751	12.24044	5.180851	5.190574	6.275477	6.287254	10.72661	10.74674	5.851951	5.862933	6.950888	6.963933	5.763076	5.773891	8.136667	8.151937	5.555942	5.566369

CAV1		OCLN		OCLN		CLDN1		CLDN4		EEA1		EEA1		RAB4A		RAB4A		RAB5A	
203065_s_at		227492_at		231022_at		218182_s_at		201428_at		204841_s_at		225885_at		206272_at		203582_s_at		240990_at	
5.610818	5.610818	7.188352	7.188352	8.216925	8.216925	6.769073	6.769073	6.615737	6.615737	7.823425	7.823425	9.058967	9.058967	8.753049	8.753049	6.440199	6.440199	5.453875	5.453875
5.485024	5.609979	7.24919	7.414335	7.880851	8.060386	7.017154	7.177013	6.490742	6.638609	7.710746	7.886406	7.652956	7.8273	7.927895	8.108502	6.179584	6.320362	5.437439	5.56131
5.627357	5.755191	6.943383	7.101112	8.473409	8.665895	6.4246	6.570544	6.605428	6.75548	7.917902	8.097769	9.564628	9.781903	8.853292	9.054408	6.256562	6.398689	5.147275	5.264203
5.211299	5.239016	7.10947	7.147283	8.365231	8.409723	6.281452	6.314861	6.30262	6.336142	7.877893	7.919793	10.12812	10.18199	9.502118	9.552657	6.471339	6.505758	5.168576	5.196066
5.38468	5.214868	6.793959	6.579704	8.209109	7.950225	6.227622	6.031227	6.770392	6.55688	7.989487	7.737529	10.53109	10.19898	9.764411	9.456479	6.780681	6.566844	5.207175	5.042961
5.532383	5.575825	6.570384	6.621976	8.166723	8.23085	6.084001	6.131774	6.199568	6.248248	7.982132	8.04481	10.55012	10.63296	9.807821	9.884834	7.197775	7.254294	4.94671	4.985553
5.449288	5.363655	6.585825	6.482332	8.017335	7.891347	6.001061	5.906757	6.444632	6.343358	8.014721	7.888774	10.93711	10.76524	9.744469	9.59134	7.719977	7.598662	4.797208	4.721822
5.267848	5.050012	7.042465	6.751245	8.11074	7.775344	6.135321	5.881613	6.478018	6.210139	7.87536	7.549698	10.58045	10.14293	9.961701	9.549764	7.761142	7.440203	5.03557	4.827339
5.418529	5.260683	7.345485	7.131505	8.161044	7.923307	6.138372	5.959557	6.515764	6.325955	7.824527	7.596593	10.75038	10.43722	9.859982	9.572753	7.658465	7.435368	5.127608	4.978237
5.321352	5.407354	7.192916	7.309166	8.247535	8.380829	6.255594	6.356695	6.28525	6.38683	8.153982	8.285764	10.66591	10.83829	9.644523	9.800395	6.866654	6.977631	5.331816	5.417987
5.587968	5.491718	7.003719	6.883083	8.156933	8.016433	6.28585	6.177579	6.363138	6.253536	8.149909	8.00953	10.54977	10.36806	9.532646	9.36845	7.305832	7.179992	5.241124	5.150848
5.885356	5.729394	7.044345	6.85767	8.300976	8.081	6.159196	5.995977	6.218453	6.053664	8.225275	8.007305	10.78455	10.49876	9.826437	9.566036	7.541261	7.341418	5.461446	5.316718
5.399071	5.215507	6.560535	6.337482	8.085741	7.810832	6.2194	6.007945	6.276698	6.063295	8.285473	8.003773	10.80972	10.44219	9.693235	9.363672	7.466488	7.212633	5.838378	5.639878
5.380346	5.290937	7.745589	7.616875	8.072018	7.937879	6.284996	6.180553	6.369264	6.263421	7.869519	7.738745	10.00951	9.843177	9.417063	9.260572	6.653312	6.542749	5.683126	5.588685
5.474935	5.48521	6.79268	6.805428	7.965417	7.980366	6.040739	6.052076	6.404613	6.416632	8.096281	8.111475	11.0201	11.04078	9.856393	9.87489	7.744511	7.759045	5.243335	5.253175

RAB5A		RAB7A		RAB7A		RAB11A		RAB11A		LAMP1		LAMP1		LAMP1		M6PR	
209089_at		1570061_at		211961_s_at		234998_at		200864_s_at		201553_s_at		213728_at		201552_at		200900_s_at	
12.76072	12.76072	6.31414	6.31414	11.77068	11.77068	8.865151	8.865151	7.456815	7.456815	14.19247	14.19247	9.455517	9.455517	13.08067	13.08067	8.680318	8.680318
12.2293	12.50789	6.612267	6.762902	10.76127	11.00643	8.679773	8.877509	6.448443	6.595346	13.61276	13.92287	8.461277	8.654035	11.27193	11.52871	7.713807	7.889537
12.83201	13.12351	6.183599	6.324069	11.62344	11.88749	8.659516	8.85623	7.276908	7.442214	14.22056	14.5436	9.522701	9.739023	13.37208	13.67584	8.464624	8.65691
12.78601	12.85402	6.068295	6.10057	11.97945	12.04317	8.875542	8.922748	8.673978	8.720112	14.32706	14.40326	9.775572	9.827565	13.60102	13.67336	9.012343	9.060277
12.78488	12.3817	6.475517	6.271304	12.11238	11.73041	9.255436	8.963555	9.205815	8.915499	14.26972	13.81971	9.662457	9.35774	13.45785	13.03344	9.462425	9.164017
12.8258	12.92651	6.235071	6.28403	12.097	12.19199	9.177525	9.249589	9.455201	9.529445	14.3029	14.41521	9.865639	9.943106	13.67607	13.78346	8.921936	8.991993
12.43845	12.24298	6.335708	6.236146	11.62928	11.44653	8.607926	8.472657	9.263151	9.117585	14.25785	14.0338	9.561152	9.410904	13.44016	13.22895	9.326374	9.179815
12.21175	11.70677	6.375299	6.111667	11.765	11.27849	8.962048	8.591449	9.342861	8.956515	14.18606	13.59943	9.720982	9.319	13.45323	12.89691	9.457574	9.066484
12.31752	11.9587	6.160798	5.981329	11.6245	11.28587	8.96511	8.70395	9.115605	8.85006	14.19078	13.77739	9.516893	9.239659	13.4136	13.02285	9.377632	9.104454
12.73063	12.93638	6.253891	6.354965	11.76112	11.9512	9.167757	9.315924	9.691073	9.847697	14.24828	14.47856	9.553127	9.707522	13.57581	13.79522	9.071711	9.218325
12.57176	12.35522	6.566857	6.453746	11.73062	11.52857	8.607224	8.458968	9.143509	8.986016	14.29582	14.04958	9.595887	9.430602	13.69811	13.46217	9.128656	8.971419
12.32868	12.00197	6.308447	6.141273	11.46509	11.16127	8.877244	8.641997	9.302549	9.056032	14.1812	13.8054	9.414442	9.164959	13.31243	12.95965	10.33383	10.05998
12.3262	11.90712	6.255634	6.042947	11.78857	11.38776	8.941715	8.637704	9.458502	9.13692	14.1511	13.66997	9.366695	9.048235	13.2546	12.80395	10.31779	9.966988
11.90289	11.70509	6.262841	6.158767	11.32407	11.13589	9.103302	8.952025	8.253337	8.116185	14.11002	13.87555	9.459312	9.302119	13.21064	12.99111	9.84865	9.684987
12.43555	12.45889	6.27167	6.28344	11.47576	11.49729	8.792455	8.808956	9.98844	10.00719	14.15402	14.18058	9.437588	9.455299	13.35732	13.38239	11.25184	11.27296

GSM38361	asthma	13.18744		11.68443	11.32574	5.67152	5.497417	6.095212	5.908102	6.51443	6.314451	9.984156	9.677664	6.543948	6.343063	12.8835	12.488	6.671033	6.466247
GSM38363	asthma	13.22595		11.2285	10.85212	5.601693	5.413924	5.802233	5.607742	6.295469	6.084444	9.711687	9.38615	6.629468	6.407248	12.91703	12.48405	6.531688	6.312745
GSM38364	asthma	13.27581		11.31994	10.8994	5.6516	5.441641	5.833015	5.616316	6.387546	6.150246	10.23576	9.855498	6.445102	6.205664	12.9062	12.42673	6.646098	6.399193
GSM38365	asthma	13.11683		11.57206	11.2772	5.629593	5.486151	5.708436	5.562985	6.532163	6.365723	10.31649	10.05362	6.528918	6.362561	12.96495	12.6346	6.525643	6.359369
GSM38370	asthma	13.19722		10.74868	10.411	5.552178	5.377748	6.316582	6.118137	6.207287	6.012276	9.77068	9.46372	6.439606	6.237296	13.09083	12.67957	7.039153	6.818008
GSM38372	asthma	12.91253		10.66189	10.55462	5.375207	5.321125	5.721898	5.664327	6.23466	6.17193	9.992372	9.891834	6.529769	6.46407	13.35121	13.21688	6.90812	6.838614
GSM38375	asthma	13.08816		11.06204	10.8038	5.60113	5.47037	5.964201	5.824965	6.457944	6.307181	9.378121	9.159186	6.561203	6.40803	13.0613	12.75638	7.189665	7.02182
GSM38378	asthma	13.18797		9.90958	9.604985	5.627849	5.454864	6.632412	6.428549	6.491691	6.292153	9.493072	9.20128	6.257121	6.064793	13.17452	12.76957	7.174635	6.954105
GSM38379	asthma	12.97957		12.06983	11.88668	5.364747	5.28334	5.849849	5.761081	6.240996	6.146292	9.932911	9.782184	6.333107	6.237005	13.19525	12.99502	6.642059	6.541269
GSM38381	asthma	12.60119		11.82017	11.99035	5.476408	5.555253	5.541929	5.621717	6.617554	6.712828	9.908073	10.05072	6.483141	6.57648	13.37335	13.56588	6.843703	6.942233
GSM38383	asthma	13.1814		10.76656	10.44082	5.541086	5.373446	6.993846	6.782254	6.580094	6.381019	7.66657	7.434625	6.322765	6.131476	13.03434	12.64	8.037836	7.794659
GSM38386	asthma	13.00847		11.01285	10.82164	5.503137	5.407589	6.862333	6.743186	6.445677	6.333764	10.00384	9.83015	6.38358	6.272745	13.07895	12.85187	7.352176	7.224524
GSM38387	asthma	12.85724		11.10413	11.03968	5.64912	5.61633	7.255747	7.213631	6.533193	6.495271	9.032959	8.980527	6.129682	6.094102	12.91535	12.84038	7.638833	7.594493
GSM38388	asthma	13.18611		10.99274	10.65635	5.58292	5.412079	6.749164	6.542636	6.337016	6.143099	10.2822	9.967554	6.138274	5.950439	13.02541	12.62683	7.231169	7.009891
GSM38389	asthma	13.11377		11.07377	10.79412	5.635564	5.493252	7.181627	7.000273	6.601898	6.435183	10.10014	9.845085	6.175926	6.019968	13.15608	12.82386	7.219013	7.036715

8.022038	7.775779	11.34995	11.00153	6.231609	6.040312	10.06016	9.751338	5.671043	5.496954	10.41782	10.09801	5.881225	5.700684	10.59231	10.26715	12.13837	11.76575	6.997662	6.782849
7.955804	7.689125	11.42084	11.03801	6.243714	6.034424	9.51368	9.19478	6.286079	6.075369	10.62515	10.26899	5.854635	5.658387	10.41759	10.06839	11.88113	11.48288	7.365033	7.118156
7.949353	7.654032	11.15058	10.73633	6.230377	5.998916	9.911559	9.543341	6.164918	5.935889	10.33382	9.949914	5.890809	5.671963	10.20814	9.828908	11.99338	11.54782	6.997177	6.737229
8.00645	7.802446	11.31994	11.0315	6.154656	5.997835	9.93995	9.68668	5.892642	5.742497	10.56687	10.29762	6.078046	5.923177	10.46893	10.20218	12.01605	11.70988	7.01829	6.839464
7.761782	7.517934	10.70263	10.3664	5.840147	5.65667	9.208082	8.918797	6.237147	6.041198	10.27846	9.955543	5.956356	5.769228	10.74293	10.40543	12.08409	11.70445	6.392737	6.1919
7.92569	7.845946	10.69279	10.5852	6.089736	6.028464	9.607684	9.511017	6.676338	6.609164	10.42911	10.32418	5.985347	5.925126	10.55041	10.44425	11.73453	11.61646	6.268009	6.204944
7.915232	7.730449	10.82366	10.57098	6.016891	5.876425	9.185183	8.970752	5.94318	5.804435	10.31009	10.0694	5.851457	5.714853	10.10247	9.86662	11.9313	11.65276	6.281211	6.134574
7.810843	7.570758	11.288	10.94103	6.017596	5.832631	9.379455	9.091155	5.813062	5.634384	10.53203	10.2083	6.01512	5.830231	10.54013	10.21615	11.82856	11.46498	6.506843	6.30684
7.763226	7.645423	10.66477	10.50294	5.92078	5.830935	9.163236	9.024189	7.084071	6.976574	10.31776	10.1612	6.183475	6.089644	10.40743	10.24951	12.02862	11.84609	6.30214	6.206508
7.984183	8.099132	10.58322	10.73559	6.011418	6.097965	8.61473	8.738757	6.073584	6.161026	10.48208	10.63299	6.09468	6.182426	10.37469	10.52405	11.733	11.90192	6.736035	6.833015
7.915232	7.675764	10.35358	10.04034	5.936075	5.756485	9.642288	9.35057	6.227148	6.038751	10.22941	9.91993	6.113355	5.928401	10.32483	10.01246	11.95571	11.594	5.66966	5.49813
7.609395	7.477277	11.16831	10.9744	6.002887	5.898662	9.422368	9.258773	6.006883	5.902589	10.53263	10.34976	6.175233	6.068016	10.3658	10.18583	11.94025	11.73294	6.169329	6.062214
7.905491	7.859603	10.95275	10.88918	5.931305	5.896877	8.211665	8.164	6.198917	6.162935	10.05648	9.998102	6.398523	6.361383	10.35742	10.2973	12.18174	12.11103	5.874589	5.84049
7.808354	7.569414	11.07858	10.73957	5.884093	5.704036	9.446543	9.157473	7.009783	6.795279	10.47156	10.15112	6.104759	5.91795	10.34868	10.032	11.88297	11.51935	6.151946	5.963693
7.754172	7.558359	10.27616	10.01666	6.012877	5.861037	9.145533	8.914585	6.722022	6.552274	10.14708	9.890839	6.522236	6.357533	10.11652	9.861056	12.05592	11.75148	5.889688	5.740958

6.997662	6.782849	12.11311	11.74126	5.272329	5.11048	7.128667	6.909832	10.72624	10.39697	6.489736	6.290515	7.599174	7.365896	7.721138	7.484116	7.938678	7.694978	5.994269	5.810258
7.365033	7.118156	12.14345	11.7364	5.354592	5.175106	6.68383	6.459787	10.93859	10.57193	6.134841	5.929201	8.229569	7.953713	6.154251	5.94796	8.094002	7.82269	5.535277	5.349734
6.997177	6.737229	12.0282	11.58135	5.036511	4.849403	6.788869	6.53666	10.74674	10.3475	5.927064	5.706872	7.601264	7.318874	8.484196	8.169005	8.2963	7.98809	5.491072	5.287077
7.01829	6.839464	11.97931	11.67408	4.950327	4.824193	6.653515	6.483983	10.76464	10.49035	6.476919	6.311887	7.977732	7.774459	5.213424	5.080586	8.188111	7.979478	5.791511	5.643943
6.392737	6.1919	11.91675	11.54237	5.343056	5.175196	6.524303	6.319332	10.83933	10.4988	5.867954	5.683604	7.134507	6.910366	5.791971	5.610008	8.106605	7.851924	5.824115	5.641142
6.268009	6.204944	12.13655	12.01443	5.274754	5.221682	6.523248	6.457615	10.88241	10.77291	5.785294	5.727085	7.908918	7.829343	8.493095	8.407642	8.015435	7.934788	5.724001	5.666409
6.281211	6.134574	11.53409	11.26483	5.394143	5.268215	6.615631	6.461187	10.85844	10.60494	5.92818	5.789785	7.988456	7.801963	6.736796	6.579523	8.048728	7.860828	5.817774	5.681956
6.506843	6.30684	12.12425	11.75158	5.397218	5.231322	6.591987	6.389366	10.66316	10.3354	5.603868	5.43162	6.720945	6.514361	7.417466	7.189472	7.860204	7.618602	5.824115	5.645097
6.30214	6.206508	11.97399	11.79229	5.080046	5.002959	6.555602	6.456124	10.50045	10.34112	6.251347	6.156486	7.315401	7.204394	5.92332	5.833437	8.130259	8.006887	5.491085	5.407761
6.736035	6.833015	12.2878	12.46471	5.084076	5.157272	6.604002	6.699081	10.50407	10.6553	5.549867	5.629769	7.82646	7.939139	6.433306	6.525927	8.436415	8.557875	5.70685	5.789012
5.66966	5.49813	11.91859	11.558	5.66796	5.496481	6.344769	6.152814	9.902852	9.60325	5.643785	5.473038	6.052838	5.869715	5.914418	5.735483	7.689312	7.456679	5.886553	5.708461
6.169329	6.062214	11.77454	11.57011	5.14229	5.053007	7.058381	6.93583	10.63415	10.44952	5.772978	5.672745	7.451509	7.322132	7.003825	6.882221	7.96144	7.82321	6.051373	5.946306
5.874589	5.84049	11.9039	11.8348	5.373952	5.342759	6.585078	6.546855	10.40369	10.3433	5.873102	5.839012	6.746286	6.707127	7.170464	7.128843	7.919053	7.873087	6.030962	5.995955
6.151946	5.963693	11.85236	11.48967	5.224741	5.064861	6.466849	6.26896	10.63583	10.31036	5.960386	5.777995	7.966705	7.722919	5.702139	5.52765	8.044974	7.798793	5.970677	5.787971
5.889688	5.740958	11.6713	11.37657	5.450881	5.313232	6.844437	6.671598	10.20102	9.943421	5.802458	5.655931	7.690391	7.496189	9.792763	9.545471	7.905776	7.706135	5.766194	5.620583

7.599174	7.365896	7.721138	7.484116	7.938678	7.694978	5.994269	5.810258	5.638256	5.465174	7.203114	6.981994	8.022822	7.776539	6.297747	6.10442	6.806551	6.597604
8.229569	7.953713	6.154251	5.94796	8.094002	7.82269	5.535277	5.349734	5.561069	5.374661	7.404006	7.155823	7.907319	7.642265	5.77876	5.585055	6.721866	6.496548
7.601264	7.318874	8.484196	8.169005	8.2963	7.98809	5.491072	5.287077	5.543736	5.337784	7.266405	6.996456	8.085418	7.785042	6.100786	5.87414	6.815585	6.562384
7.977732	7.774459	5.213424	5.080586	8.188111	7.979478	5.791511	5.643943	5.71615	5.570502	6.713383	6.542326	8.024622	7.820154	6.079452	5.924547	6.788018	6.615059
7.134507	6.910366	5.791971	5.610008	8.106605	7.851924	5.824115	5.641142	5.439167	5.268288	6.991217	6.771578	8.030355	7.77807	6.166171	5.972452	6.247467	6.051194
7.908918	7.829343	8.493095	8.407642	8.015435	7.934788	5.724001	5.666409	5.538396	5.482672	7.02296	6.952299	8.163694	8.081555	6.245029	6.182195	6.307915	6.244448
7.988456	7.801963	6.736796	6.579523	8.048728	7.860828	5.817774	5.681956	5.457591	5.330182	6.851271	6.691326	8.382694	8.186997	6.359335	6.210874	6.275335	6.128835
6.720945	6.514361	7.417466	7.189472	7.860204	7.618602	5.824115	5.645097	5.468739	5.300644	6.41933	6.222017	8.147564	7.897129	6.068276	5.881753	6.386179	6.189884
7.315401	7.204394	5.92332	5.833437	8.130259	8.006887	5.491085	5.407761	5.757871	5.670498	7.476062	7.362617	7.974297	7.853291	5.958159	5.867747	6.165407	6.07185
7.82646	7.939139	6.433306	6.525927	8.436415	8.557875	5.70685	5.789012	5.489962	5.569002	6.653165	6.748952	8.147564	8.264866	5.991294	6.077551	6.445911	6.538714
6.052838	5.869715	5.914418	5.735483	7.689312	7.456679	5.886553	5.708461	5.352526	5.19059	7.338933	7.1169	7.967013	7.725978	6.19631	6.008846	6.548209	6.350099
7.451509	7.322132	7.003825	6.882221	7.96144	7.82321	6.051373	5.946306	5.423996	5.329822	6.66949	6.553691	7.678397	7.545081	6.279595	6.170566	6.864616	6.745429
6.746286	6.707127	7.170464	7.128843	7.919053	7.873087	6.030962	5.995955	5.270326	5.239734	6.938508	6.898233	7.904961	7.859077	6.258149	6.221824	6.744635	6.705486
7.966705	7.722919	5.702139	5.52765	8.044974	7.798793	5.970677	5.787971	5.340476	5.177054	6.988339	6.774492	7.75467	7.517372	6.150333	5.962129	6.754938	6.548233
7.690391	7.496189	9.792763	9.545471	7.905776	7.706135	5.766194	5.620583	5.307031	5.173015	7.78675	7.590115	8.174444	7.968018	6.113611	5.959227	6.632297	6.464815

7.203114	6.981994	8.022822	7.776539	6.297747	6.10442	6.806551	6.597604	7.614426	7.380679	10.14919	9.837629	9.548434	9.255318	8.385936	8.128506	5.060254	4.904915	11.83588	11.47254
7.404006	7.155823	7.907319	7.642265	5.77876	5.585055	6.721866	6.496548	8.195074	7.920374	10.68068	10.32266	9.875945	9.544902	8.918547	8.619596	5.18465	5.01086	12.21739	11.80787
7.266405	6.996456	8.085418	7.785042	6.100786	5.87414	6.815585	6.562384	8.000975	7.703736	10.38663	10.00077	9.58917	9.232929	8.341461	8.031573	5.268587	5.072857	11.99976	11.55396
6.713383	6.542326	8.024622	7.820154	6.079452	5.924547	6.788018	6.615059	7.686594	7.490739	10.45437	10.18799	9.702057	9.454848	8.413009	8.198645	5.229516	5.096268	12.01322	11.70712
6.991217	6.771578	8.030355	7.77807	6.166171	5.972452	6.247467	6.051194	8.023747	7.771669	10.80248	10.4631	10.15078	9.831874	7.866634	7.619492	5.241124	5.076466	12.49986	12.10716
7.02296	6.952299	8.163694	8.081555	6.245029	6.182195	6.307915	6.244448	8.175228	8.092973	10.87461	10.7652	10.13129	10.02936	8.098994	8.017506	5.170131	5.118112	12.62411	12.49709
6.851271	6.691326	8.382694	8.186997	6.359335	6.210874	6.275335	6.128835	8.127301	7.937567	10.20063	9.962496	9.722399	9.495427	8.210819	8.019135	5.2204	5.098528	12.18757	11.90305
6.41933	6.222017	8.147564	7.897129	6.068276	5.881753	6.386179	6.189884	7.850113	7.608821	10.38628	10.06703	9.830471	9.528308	7.848902	7.607647	5.030689	4.876059	12.18377	11.80928
7.476062	7.362617	7.974297	7.853291	5.958159	5.867747	6.165407	6.07185	7.986854	7.865658	10.77129	10.60784	9.963522	9.812331	8.049503	7.927356	5.206596	5.127589	12.23581	12.05014
6.653165	6.748952	8.147564	8.264866	5.991294	6.077551	6.445911	6.538714	8.454972	8.576699	10.78156	10.93678	9.914241	10.05698	7.802621	7.914956	5.380183	5.457642	12.46527	12.64474
7.338933	7.1169	7.967013	7.725978	6.19631	6.008846	6.548209	6.350099	7.730353	7.496478	9.821711	9.524564	9.287432	9.006449	7.164406	6.947654	5.241124	5.082559	12.10927	11.74292
6.66949	6.553691	7.678397	7.545081	6.279595	6.170566	6.864616	6.745429	7.699594	7.56591	10.55791	10.3746	9.859992	9.688798	7.894535	7.757466	5.25022	5.159063	12.24907	12.0364
6.938508	6.898233	7.904961	7.859077	6.258149	6.221824	6.744635	6.705486	7.840281	7.794772	10.3135	10.25363	9.191849	9.138495	6.844486	6.804757	5.230132	5.199774	12.28801	12.21668
6.988339	6.774492	7.75467	7.517372	6.150333	5.962129	6.754938	6.548233	7.473697	7.244997	10.52499	10.20292	9.749135	9.450806	8.048194	7.801914	4.823463	4.675862	12.08611	11.71627
7.78675	7.590115	8.174444	7.968018	6.113611	5.959227	6.632297	6.464815	7.636128	7.443296	10.51717	10.25159	9.859318	9.610345	7.208339	7.02631	5.238801	5.106508	12.38028	12.06764