UNIVERSITI PUTRA MALAYSIA

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ABSTRACT

Effect of *Blomia tropicalis* Allergen (Blo t 21) on Junctional Protein Expression in Human Bronchial Epithelial Cells

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Introduction: The airway epithelium acts as a physical barrier that primarily regulated by junctional proteins which restrict the passage of inhaled substances such as house dust mite (HDM) allergens. The epithelial barrier disruption through the degradation of junctional proteins by HDM allergens displaying proteolytic activity has been extensively reported in the literature. However, little is known on the other biological functions of HDM allergens such as the lipid binding protein on the effect of junctional protein expression. Blo t 21, a group 21 HDM allergen is one of the major allergens found in the HDM species *Blomia tropicalis* which displays structural homologies with the lipid binding protein Der p 5. Blo t 21 has been recognised as a major sensitizing protein with Ig-E binding frequency reaching more than 50% in HDM sensitized population. Despite the high sensitization frequency of this allergen, the effect of the Blo t 21 on the epithelial barrier function is still unknown. **Objective:** This study aimed to investigate the effect of Blo t 21 on the expression of junctional protein specifically occludin in a human bronchial epithelial cell line, namely 16HBE14o-. **Methodology:** 16HBE14o- cells were used as these cells express the principal intercellular tight junctional proteins. The cells were induced with recombinant Blo t 21 at the concentration of 20 μg/mL for 24 hours. The cell viability was determined using lactate dehydrogenase (LDH) activity assay. The effect of Blo t 21 on the protein expression of occludin was then assessed via Western Blot and the band intensity was analyzed by using ImageJ software. **Results:** No significant death of 16HBE-14o cell viability was observed between control and Blo t 21-induced groups. Blo t 21 (20 μg/mL) was shown to downregulate the protein expression of occludin in 16HBE14o- cells, as indicated by a significant reduction of the band intensity of occludin in Blo t 21-induced group ($p < 0.05$). **Discussion:** Based on the findings from LDH assay, no significant death was observed in cells induced with 20 μg/mL Blo t 21. This finding indicates that the regulatory effect on occludin expression was not arisen from the cytotoxicity exerted by Blo t 21 on 16HBE14o- cells. In term of protein expression, Blo t 21 significantly downregulated the protein expression of occludin by 54% as compared to the normal group. This finding is in line with an earlier study which proved that HDM allergen that was independent of proteolytic activity could

exerted regulatory effect on tight junctional protein expression at gene level. **Conclusion:** In short, the present study signifies that Blo t 21 may capable of downregulating the expression of occludin. This finding further strengthens the speculation that non-proteolytic HDM allergens may interfere with the epithelial barrier integrity through the regulation of tight junctional protein expression. Nonetheless, the effects of Blo t 21 on the expression of other junctional proteins and the mechanisms underlying the regulatory effect of Blo t 21 should be further dissected in the future.

Keywords: human airway epithelium, 16HBE14o-, house dust mite (HDM), Blo t 21, junctional protein, occludin

ABSTRAK

Kesan Hama *Blomia tropicalis* (Blo t 21) kepada Ungkapan Protein Simpangan dalam Sel Epitelia Bronkial Manusia

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Pengenalan: Epitelium saluran pernafasan bertindak sebagai perlindungan fizikal melalui kawalan selia oleh protein simpangan yang menghalang laluan masuk oleh bendasing melalui penarikan nafas seperti hama rumah. Gangguan kepada pelindungan epitelium melalui degradasi protein simpangan oleh hama rumah yang menunjukkan aktiviti protease telah dilaporkan secara meluas di dalam literatur. Namun begitu, fungsi biologi lain seperti protein pengikat lipid terhadap kesan ungkapan protein simpangan masih tidak diketahui. Blo t 21, iaitu hama rumah kumpulan 21 adalah salah satu allergen utama dijumpai dalam hama rumah spesies *Blomia tropicalis* yang menunjukkan struktur homolog seperti protein pengikat lipid Der p 5. Blo t 21 ialah protein pemekaan utama dengan kekerapan mengikat IgE menjangkau lebih dari 50% dalam populasi sensitif terhadap hama rumah. Walaupun Blo t 21 mempunyai frekuensi pemekaan yang tinggi, kesan Blo t 21 terhadap fungsi perlindungan epitelium masih belum diketahui. **Objektif:** Kajian ini bertujuan untuk mengkaji kesan Blo t 21 terhadap ungkapan protein simpangan khususnya occludin dalam sel epitelia bronkial manusia, 16HBE14o-. **Metodologi:** 16HBE14o- sel digunakan kerana sel-sel ini mengekspresikan protein persimpangan ketat antara sel. Sel diinduksi dengan rekombinan Blo t 21 pada kepekatan 20 μg / mL selama 24 jam. Sitotoksisiti sel ditentukan menggunakan ujian aktiviti lactate dehidrogenase (LDH). Kesan Blo t 21 pada ungkapan protein occludin kemudian dikaji melalui pengujian immunoblot dan intensiti jalur dianalisis dengan menggunakan perisian ImageJ. **Hasil:** Tiada kematian 16HBE-14o sel yang signifikan antara sel normal dan sel diinduksi dengan Blo t 21. 16HBE14-o sel yang diinduksi oleh Blo t 21 (20 μg/mL) menunjukkan penurunan ungkapan protein occludin yang signifikan ditunjukkan oleh penurunan intensiti jalur occludin ($p<0.05$). **Perbincangan:** Berdasarkan penemuan dari ujian LDH, tiada kematian sel yang signifikan oleh sel yang diinduksi oleh 20 μg/mL Blo t 21. Penemuan ini menunjukkan bahawa kesan terhadap ungkapan protein occludin tidak timbul dari sitotoksisiti aktiviti oleh Blo t 21 pada 16HBE14o- sel. Dari segi ungkapan protein, Blo t 21 secara signifikan menunjukan penurunan ungkapan protein occludin

sebanyak 54% dibandingkan dengan sel normal. Penemuan ini sejajar dengan kajian sebelumnya yang membuktikan bahawa alergen hama rumah yang tidak menunjukkan aktiviti proteolitik mampu memberikan kesan pengawalseliaan terhadap ungkapan protein simpang pada peringkat gen. **Kesimpulan:** Ringkasnya, kajian ini menunjukkan bahawa Blo t 21 mungkin mampu menyebabkan penurunan terhadap ungkapan protein occludin. Penemuan ini memperkuatkan spekulasi bahawa hama rumah alergen yang tidak menunjukkan aktiviti proteolitik boleh mengganggu integriti perlindungan epitelium melalui pengawalan ungkapan protein persimpangan ketat. Walaupun begitu, kesan Blo t 21 terhadap ungkapan protein simpang lain dan mekanisma yang menyebabkan kesan pengawalseliaan Blo t 21 terhadap ungkapan protein simpang harus dikaji dengan lebih lanjut pada masa hadapan.

Kata Kunci: epitelium saluran pernafasan, 16HBE14o-, hama rumah, Blo t 21, protein persimpangan, occludin

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TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION

AJs	Adheren Junctions
APCs	Antigen Presenting Cells
ATCC	American Type Culture Collection
BCA	Bicinchoninic Acid Assay
Blo t	*Blomia tropicalis*
BSA	Bovine Serum Albumin
BSC	Biosafety Cabinet
CCD	Charge-coupled device
CO2	Carbon Dioxide
DCs	Dendritic cells
Der p	*Dermatophagoides pteronyssinus*
DMSO	Dimethyl Sulfoxide
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
EMEM	Eagle's Minimum Essential Medium
FBS	Fetal Bovine Serum
HCL	Hydrochloric acid
HDM	House Dust Mite
HRP	Horseradish peroxidase
IgE	Immunoglobulin E
IL-13	Interleukin-13
IL-4	Interleukin-4
IUIS	International Union of Immunological Societies
JAMs	Junctional adhesion molecules
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MDCK	Madin-Darby canine kidney
MHC	Major Histocompatibility Complex

NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide hydride
NMR	Nuclear Magnetic Resonance
PAMPs	Pathogen-Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PRRs	Pattern Recognition Receptors
PVDF	Polyvinylidene difluoride
RIPA	Radioimmunoprecipitation assay
rpm	Resolution per minute
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard Error of the Mean
TBST	Tris buffered saline with Tween
TER	Transepithelial electric resistance
Th2	Type 2 T Helper Cell
TJs	Tight Junctions
TLR-2	Toll-like receptor-2
WHO	World Health Organization
ZO-1	Zonula occludens-1

CHAPTER 1

INTRODUCTION

1.1 Background of Study

The human respiratory system is lined with epithelial cells that operate as a protective barrier against the external environment. Through respiration, air contaminants or allergens are introduced into the human respiratory system. However, the epithelium lining of human airway system is highly regulated with the presence of junctional protein complexes that could prevent the invasion of the inhaled environmental agents such as allergens. The junctional system is composed of numerous junctional protein groups, namely adherens junctions, gap junctions and tight junctions. Tight junction is the most apical of the intercellular junctional complexes which act as a barrier to regulate the permeability of ions and small molecules across the cell. A tight junction is composed of transmembrane proteins including occludin, junctional adhesion molecules (JAMs) and claudin. Of these, occludin plays an important role in regulating the epithelial permeability of the airway system as it is responsible in sealing the gap to allow selective transport of substance across the cells.

The junctional proteins complexes however are vulnerable to disruption after being exposed to the most common source of allergen, house dust mites (HDMs). These allergens may interact with the immunological cells of the human body's defensive mechanism, thereby triggering a variety of immunological reactions such

as inflammation. The HDM allergens with proteolytic activity (Group 1, 3, 6, and 9) had been proven to be able to degrade junctional proteins in the epithelial barrier, thus allowing the passage of the allergens into the airway submucosa. Disruption of the epithelial barrier by the HDM allergens will result in allergic sensitization and can lead to asthma if the exposure towards the allergens is prolonged.

Previous studies mainly focused on the roles of the proteolytic allergens of HDM in epithelial barrier disruption. However, the roles of lipid binding HDM allergens (Group 2, 5, 7, 13, 14, and 21) in allergic asthma are still unknown and there are limited studies about these groups of HDM allergens on epithelial barrier disruption. Sensitization rates to *B. tropicalis* have increased over the last decades, resulting in increased research efforts on *Blomia* allergens. Numerous studies have been conducted on the *B. tropicalis* mite, highlighting its role as a major asthma sensitizing agent in tropical areas. Blo t 21, a group 21 HDM allergen which has been suggested to be the member of lipid binding protein families is one of the major allergens found in the HDM species *Blomia tropicalis*. Blo t 21 has been identified as a significant cause of allergic diseases with up to 50% IgE reactivity. This allergen displays sequence homologies with fatty acid binding proteins but its effect on junctional protein in the human airway epithelial barrier remains to be elucidated. Thus, this study aims to understand the effect of lipid binding HDM allergens particularly Blo t 21 on tight junctional protein expression of occludin in order to get a better grasp of the pathogenesis of HDM-induced allergy which may help for better diagnosis and treatment of allergic diseases.

1.2 Hypothesis

Blo t 21 may play a role in the alteration of airway epithelial permeability by regulating the tight junctional protein expression particularly occludin in human bronchial epithelial cells (16HBE14o-).

1.3 Objectives

1.3.1 General Objective

To investigate the effect of Blo t 21 on junctional protein expression in a human bronchial epithelial cell line (16HBE14o-).

1.3.2 Specific Objectives

1) To optimize the positive control group of the 16HBE14o- cells induced with lipopolysaccharides (LPS) using two different concentrations (1 μg/mL and 10 μg/mL) by Western Blot assay.

2) To determine the non-cytotoxic concentrations of *Blomia tropicalis* allergen (Blo t 21) in the human bronchial epithelial cells (16HBE14o-) via lactate dehydrogenase (LDH) activity assay.

3) To determine the effect of Blo t 21 on expression of tight junctional protein, occludin, in human bronchial epithelial cells (16HBE14o-) using Western Blot assay.

CHAPTER 2

LITERATURE REVIEW

2.1 Human airway system

The human airway system is essential for respiration. It ensures that the whole body can be delivered with oxygen and carbon dioxide can be exhaled. The respiratory system is anatomically divided into two parts: the upper respiratory tract and the lower respiratory tract (Figure 1). From the neck to the thorax, the trachea extends and is divided into the main bronchus that entered both sides of the lungs. The main bronchus then branches into bronchioles, eventually terminating in alveoli, which are responsible for gas exchange.

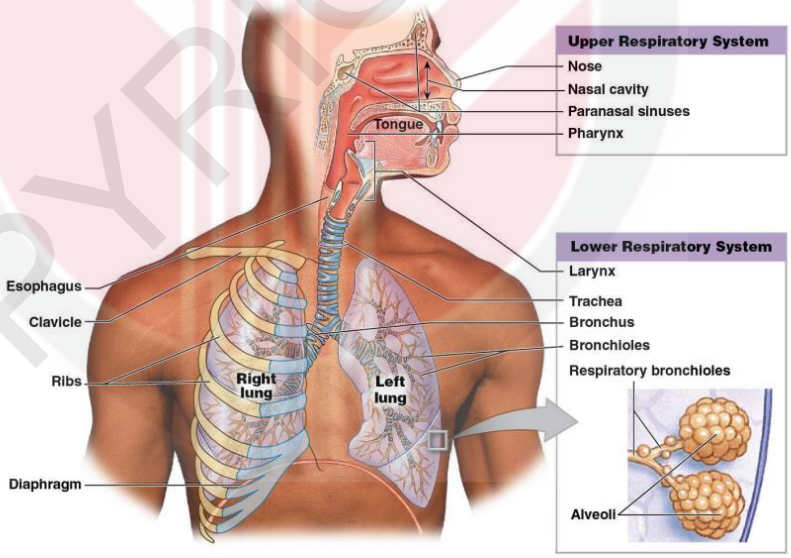

Figure 1. Human respiratory system. The upper and lower respiratory tract of a human. (Adapted from: Martini et. al., 2018).

Functionally, the human airway systems are separated into two zones which are the conducting and respiratory zones. The conducting zone's core roles

are to provide a passage for inhaled and exhaled air, to remove debris and pathogens and to humidify the inhaled air. The respiratory zone, as opposed to the conducting zone, contains structures that involved in gas exchange directly in which the terminal bronchioles connect to a respiratory bronchiole leading to an opening of a cluster of alveoli (Figure 2).

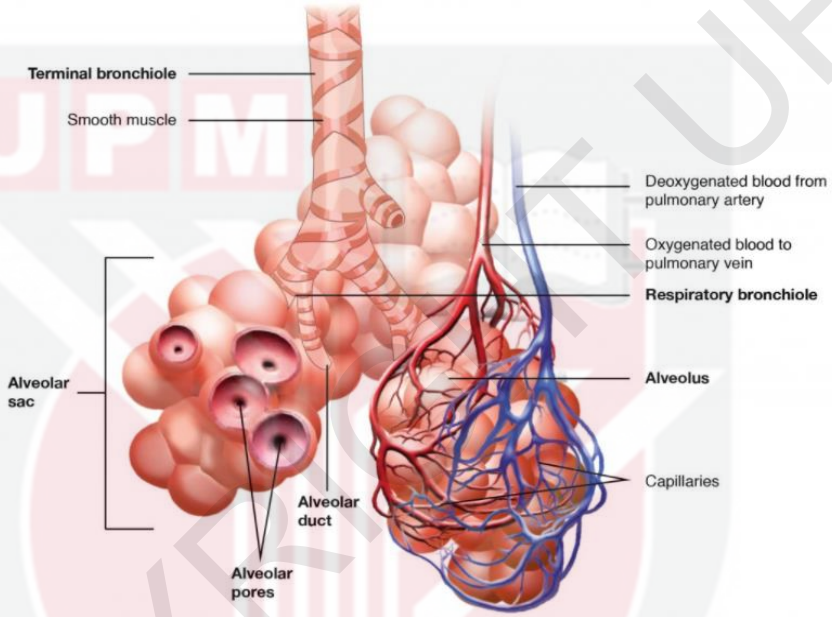

Figure 2. The respiratory zone of human airway system. The respiratory zone that plays a role in gas exchanges between oxygen and carbon dioxide in the alveoli. (Adapted from: The Respiratory System, 2020)

The respiratory organs are the second organ after the skin in terms of constantly being exposed to allergens or pollutants in the external environment. This is due to the fact that an individual breathes around 15,000 liters of air every 24 hours, increasing the exposure to allergens from the inhaled air (Harkema, Nikula & Haschek, 2018). As a result, it is critical that the respiratory system to be well-equipped with a defense mechanism.

2.1.1 Defense mechanism of airway system

The human respiratory system is well-equipped with an impressive array of defense systems that operate as a barrier to prevent invaders that is harmful from gaining an entry to the lungs. These defensive mechanisms are classified into three layers: chemical, physical, and immunological barriers, as shown in Figure 3.

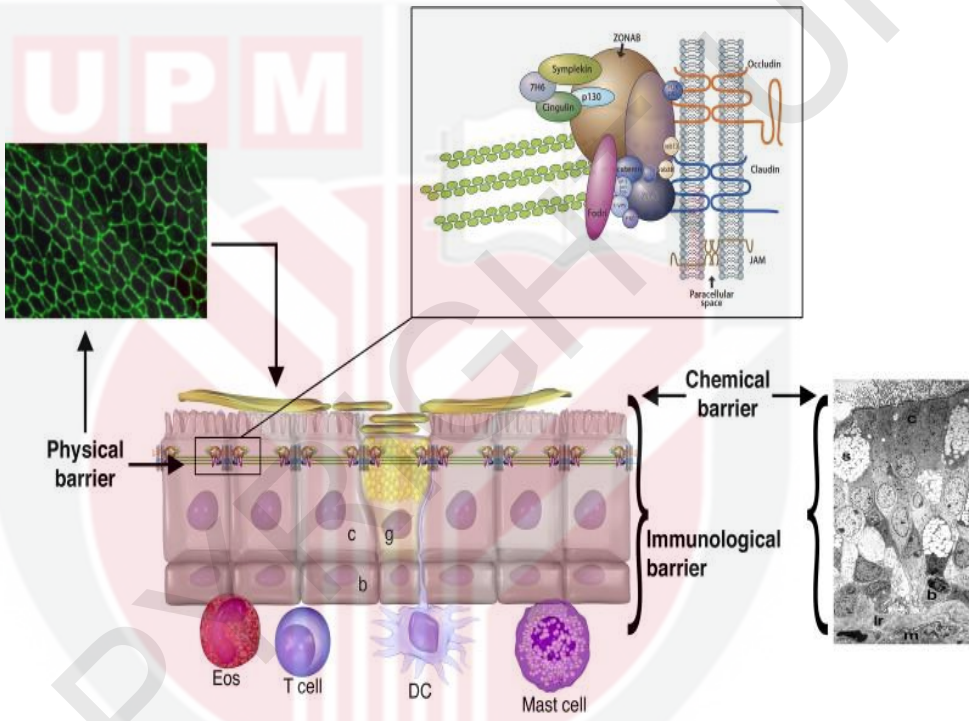

Figure 3. The defense mechanism of human airway system. The three layers of protection of the respiratory system namely physical, chemical, and immunological barrier. (Adapted from: Swindle, Colllins, & Davies, 2009)

Physical barriers of the respiratory system are provided by the closely packed epithelial cell lining. These epithelial cells lining plays a critical role in preventing invaders from entering susceptible tissues and jeopardising its integrity. The epithelial structural integrity is mediated by the junctional protein

complexes that are found on the cell membrane which connect to the neighboring cells. These proteins include tight junctions, desmosomes, and gap junctions. These proteins create an impermeable barrier by providing strength and stability to the epithelial barrier thus preventing the entry of pathogens and toxins inhaled (Förster, 2008). Additionally, the epithelial lining, which is composed of ciliated cells, aids to propel out inhaled particles from the respiratory system, preventing them from accumulating in the lungs (Potaczek et. al, 2020).

The respiratory system's chemical barrier is formed by mucin, a secretion of the bronchial epithelium. The viscosity and acidity of this secretion make it impossible for the invaders to adhere to the underlying cells. Mucin is the primary component of mucus that traps and detoxifies inhaled particles before they are cleared from the airway by cilia beating in a process known as mucociliary clearance (Swindle et al, 2009). Mucins also associate with secretions of antimicrobial components that are involved in innate defense (Thornton et al, 2008). The most abundant antimicrobial peptides are lysozyme and lactoferrin. Lactoferrin is an iron chelator that inhibits bacteria's growth, whereas lysozyme cleaves the bacterial cell wall's glycosylated link (Gohy et al., 2016).

The immunologic barrier, which is composed of humoral and cellular components, plays a role in activating or inhibiting the innate and adaptive immunity for the protection of the internal environment (Borzutzky & Morales., 2020). Immunoglobulins released make up the humoral component, whilst

macrophages, T cells, dendritic cells (DCs), mast cells, B cells, and plasma cells make up the cellular component. When pathogens gain an entry to the airways, the homeostasis is disrupted thus the immunologic barrier is triggered by releasing cytokines and chemokines to provide an extra layer of protection in addition to the physical and chemical barriers.

2.1.2 Epithelial lining as the first line of defense

The first line of defense against invaders from the inhaled air is the epithelial cells lining. The hair-like projection of the epithelial linings illustrated in Figure 4 aids in mucociliary clearance which sweeps out the foreign materials. Additionally, the epithelial cells also facilitate the clearance of the foreign materials by the secretion of antimicrobial peptides, chemokines, and cytokines (Georas & Rezaee., 2014). On top of that, another barrier to the external environment is created by the formation of junctional protein complexes between adjacent epithelial cells which composed of tight junctions (TJs) and adherens junctions (AJs). Epithelial TJs and AJs help in controlling the epithelial permeability by establishing cell-cell contact and cell polarity, as well as paracellular transport of ions and macromolecules (Lu et al., 2014). Thus, it forms a protective barrier that prevents allergens from accessing the cellular compartments of the respiratory systems.

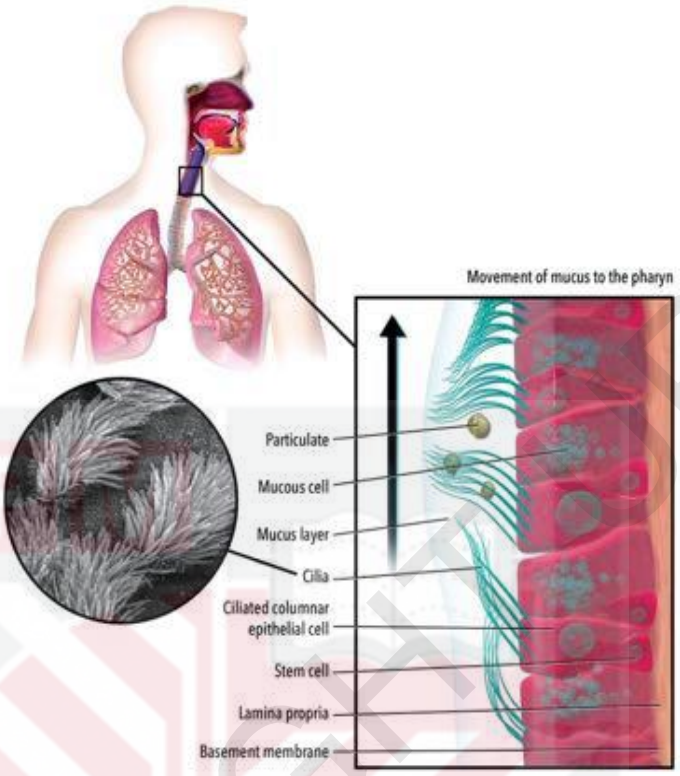

Figure 4. Airway Epithelial Lining. The epithelial lining of the respiratory system is equipped with cilia that propels out foreign particles. (Adapted from: *Blausen.com staff,* 2014).

2.1.3 Tight junctions

Intercellular junctions preserve the structural integrity and essential barrier function of airway epithelium. Figure 5 illustrates the intercellular connections of the airway epithelium. AJs are located just beneath TJs and aid in preserving the epithelial integrity by promoting intercellular adhesion. Desmosomes are in the center of epithelial cells and, by their tight connection with the intermediate filament cytoskeleton, it provides mechanical stability to the epithelium lining. Hemidesmosomes on the other hand are placed at the lowest part of the cell that aid in the epithelial layer's attachment to the basal

membrane. All these intercellular junctional structures act synergistically to regulate the epithelial permeability (Hellings & Steelant., 2020). Disruption of the airway epithelium's normal regulation will definitely have a significant effect on the airway epithelium.

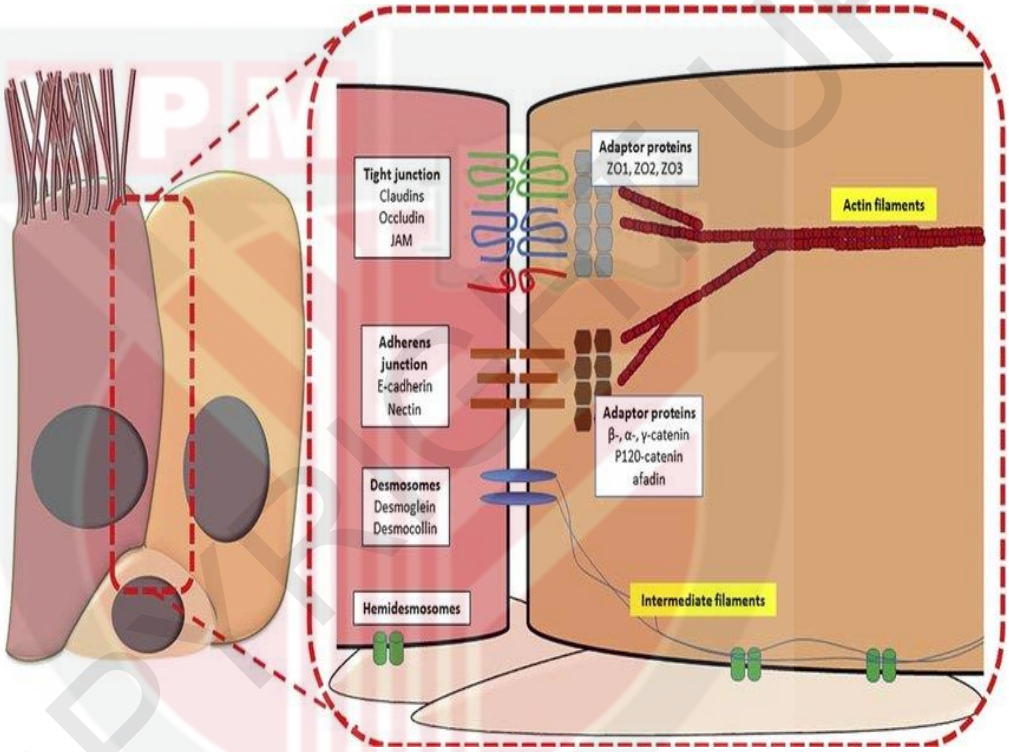

Figure 5. The schematic diagram of intercellular junctions in airway epithelium. Intercellular junctions that help in preventing the entry of the invaders (Adapted from: Reid et. al., 2019)

The airway epithelium's full barrier is directly dependent on the integrity of TJs. TJs is found on the apical end of epithelial cells that facilitates cell-cell adhesion and strictly controls paracellular transport of ions, water, and other substances (Wan et al., 2000). TJs are comprised of transmembrane proteins found in epithelial cells which are occludin, claudin, and junction

adhesion molecules (JAMs). These transmembrane proteins are required to seal the paracellular gaps and regulates paracellular transport between the cells. Additionally, TJs structures are maintained in the cytoplasm by adapter proteins such as zonula occludens-1 (ZO-1) proteins (Bauer et al., 2010). TJs dysregulation has been linked to a variety of pulmonary illnesses such as asthma, prompting considerable research into potential molecular targets for the treatment of the diseases (Sawada, 2013).

2.1.4 Occludin

Occludin is a crucial protein in the functioning of tight junctions. Occludin is needed not just for tight junction formation, but also for tight junction stability and barrier function (Cummins., 2012). Indeed, study has shown that Madin-Darby canine kidney (MDCK) cells deficient in occludin had less complex tight junction strand network and poor barrier function (Yu et al., 2005) whereas occludin overexpression enhances the barrier function as measured by an increase in transepithelial electric resistance (TER) (Medina et al., 2000).

Occludin was initially identified in chick tissues by Furuse et al. in 1993. Occludin with molecular weight of approximately 65 kDa was shown to localized and expressed ubiquitously in epithelial cells and was later validated as the first integral membrane TJs protein (Wang et al., 2014). It is the most sensitive TJs marker and is responsible for sealing the gap and allowing for selective transport of substances across the paracellular spaces (Blasig et al., 2011; Wolburg et al., 2006). Occludin has two extracellular loops and

cytoplasmic domains at the N- and C-terminus (Figure 6). The C-terminal region of occludin is required for direct interactions with ZO-1, while the N-terminus regulates the paracellular permeability (Cummins, 2012).

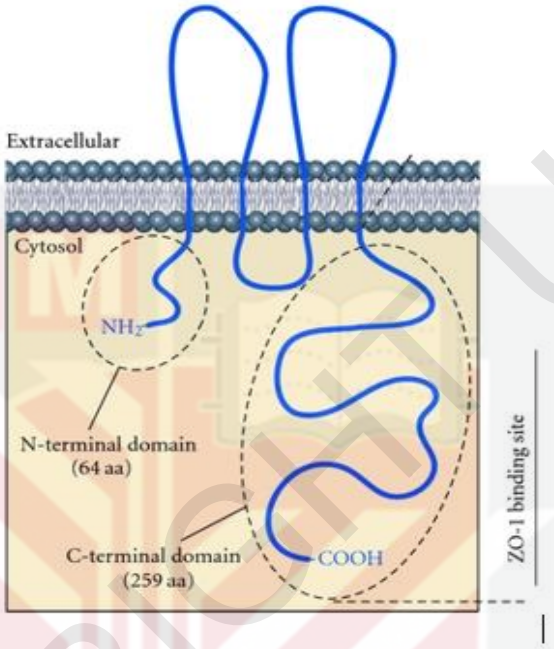

Figure 6. The structure of occludin. Occludin consists of two domains which are the N-terminal and C-terminal. (Adapted from: Dörfel & Huber, 2012).

2.2 Epithelial barrier disruption

Despite the fact that the airway system is equipped with such a well-structured barrier, a number of triggers may impair the airway epithelium barrier's integrity due to its frequent interaction with the external environment (Ganesan et al., 2013). The inhaled allergens, viruses and environmental pollutants may disrupt TJs directly or indirectly. These allergens compromise the epithelial barrier by increasing the airway epithelial permeability, limiting mucociliary clearance, and stimulating the secretion of a variety of inflammatory mediators by airway

epithelial cells. Allergens with protease activity possess the ability to directly cleave the junctional protein thus increasing the permeability of the airway epithelium which will then allow the passage of those allergens to interact with the immunological cells (Frey et al., 2020).

2.2.1 Immunological response of the airway system

As a result of the disruption of the junctional proteins in the epithelial barrier, the epithelium formed a loose gap between the adjacent cells, allowing allergen to pass through the physical barrier and interact with the next level of protection, the immunological barrier. The immunological responses upon the exposure to the allergens are illustrated in Figure 7. The primary exposure to the allergen cause sensitization in which the allergen is taken up and interacts with antigen-presenting cells (APCs), primarily dendritic cells (DCs), and then through the major histocompatibility complex (MHC) II the allergen is presented to the allergen-specific T cells. DCs promote the differentiation of type 2 helper T cells (Th2). When Th2 cells are activated, inflammatory cytokines such as interleukin-4 (IL-4) and interleukin-13 (IL-13) are produced, prompting the B cells to multiply and produce IgE. IgE binds to the Fc receptor on mast cells with a high affinity. When the allergen is re-exposed, it binds to the IgE on mast cells forming a cross-link of the IgE and the Fc receptors complexes which results in the release of mediators such as histamine. These mediators are responsible for the asthmatic reaction characterized by constriction of the airway smooth muscle, vascular leakage, mucus hypersecretion and increased airway reactivity (Taher et al., 2010).

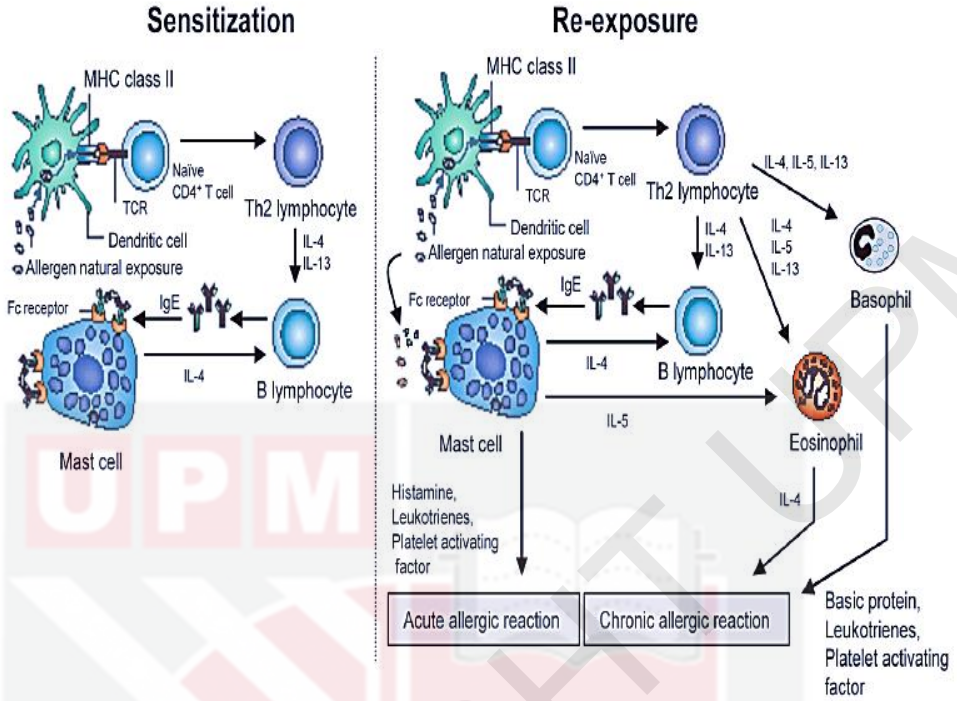

Figure 7. The immunological response of airway system upon the exposure to allergens. Primary exposure to an allergen activates Th2 cells and stimulates IgE production. Subsequently, re-exposure results in the immediate release of mediators via degranulation of mast cell. The eosinophil inflammation will also occur upon subsequent activation of Th2 cells (Adapted from: Taher et al., 2010).

2.3 House dust mite

House dust mites (HDMs) are the major allergen that may impair the airway epithelial barrier and are the primary cause of allergy sensitization. The three HDM species, *Dermatophagoides farinae, Dermatophagoides pteronyssinus, and Blomia tropicalis*, are regarded to be the most three common allergen sources worldwide (Lahiani et al., 2018). The HDM allergens from the World Health Organization (WHO) and Allergen Nomenclature Subcommittee of the International Union of Immunological Societies (IUIS) are classified into four major families based on their allergenic proteins: proteases, proteins with lipid

affinity, non-proteolytic enzymes, and non-enzymatic components (Jacquet, 2013) as shown in Table 1.

Table 1. Groups of allergens in house dust mite.

Group	Biochemical function	Protein families
1	Cysteine protease	Proteases
2	Lipid binding protein	Proteins with lipid affinity
3	Trypsin-like serine protease	Proteases
4	Amylase	Non-proteolytic enzymes
5	Lipid binding protein	Proteins with lipid affinity
6	Chymotrypsin-like serine protease	Proteases
7	Lipid binding protein	Proteins with lipid affinity
8	Glutathione-S-transferase	Non-proteolytic enzymes
9	Collagenolytic-like serine protease	Proteases
10	Tropomyosin	Non-enzymatic components
11	Paramyosin	Non-enzymatic components
12	Chitinase	Non-proteolytic enzymes
13	Lipocalin	Proteins with lipid affinity
14	Vitellogenin/apolipophorin-like	Proteins with lipid affinity
15	Chitinase	Non-proteolytic enzymes
16	Gelsolin	Non-enzymatic components
17	Calcium binding protein	Non-enzymatic components
18	Chitinase	Non-proteolytic enzymes
19	Antimicrobial peptide	Non-enzymatic components
20	Arginine kinase	Non-proteolytic enzymes
21	Lipid binding protein	Proteins with lipid affinity
22	Lipid binding protein	Proteins with lipid affinity
23	Peritrophin-like protein domain	Non-enzymatic components
24	Biquinol-cytochrome c reductase binding protein	Non-proteolytic enzymes
25	Triosphosphate isomerase	Non-enzymatic components
26	Myosin light-chain	Non-enzymatic components
28	Heat Shock Protein	Non-enzymatic components
29	Cyclophilin	Non-enzymatic components
30	Ferritin	Non-enzymatic components
31	Cofilin	Non-enzymatic components
32	Inorganic pyrophosphatase	Non-enzymatic components
33	Alpha-tubulin	Non-enzymatic components
37	Peritrophin-A domain containing protein	Non-enzymatic components
38	Bacteriolytic enzyme	Non-proteolytic enzymes
39	Troponin C	Non-enzymatic components

The role of HDM allergens with proteolytic activity in epithelial barrier disruption via junctional protein delocalization has been extensively documented in the literature. It was found that mite proteases may cleave tight junctional proteins in order to gain an entry to DCs through occludin and zonula occludens-1 degradation (ZO-1) (Herbert et al., 1995; Wan et al., 2000; Wan et al., 2001).

Interestingly, a study conducted by Post et al. in 2012 revealed that HDM extracts with the lowest protease activity induce the most significant disruption of the epithelial barrier in comparison to the extract with the highest protease activity. The results demonstrated that the HDM protease activity is not essential for the disruption of epithelial barrier function, and this raised the interest to identify the effect of other protein families of the HDM allergens such as the lipid binding protein which might be responsible for epithelial barrier function to be impaired.

2.3.1 Lipid binding protein of house dust mites

The HDM allergens in groups 2, 5, 7, 13, 14, and 21 are proteins that, due to their structural homologies, exhibit lipid binding properties (Thomas et al, 2010). Despite their biological function in the mite is still unclear, they can facilitate allergen sensitization through activation of patent recognition receptors (PRRs) such as toll-like receptor (TLR)-2 and transporting pathogen-associated molecular patterns (PAMPs) derived from microbial lipids (Jacquet & Robinson, 2019).

The first study on the effect of lipid binding protein on the epithelial barrier disruption at gene level was done by Wang et al. in 2011. It was found that Der p 2

which displays structural homology of lipid binding protein increases the permeability of the lung alveolar epithelium by downregulating the expression of the tight junction protein. This finding suggests that HDM allergen with structural homologies exhibiting lipid binding properties could exert regulatory effect on tight junctional protein expression.

2.3.2 *Blomia tropicalis*

Blomia tropicalis was identified for the first time in 1970s and was previously classified as a grain storage dust mite due to its extensive prevalence in grain storage facilities. However, due to its widespread prevalence in the dusts of many subtropical and tropical houses, it is now classified as a house dust mite (HDM) (Santos et al., 2017). Additionally, several studies have reported the allergy sensitization linked to *B. tropicalis* (Baqueiro et al., 2006; Jeevarathnum et al., 2015), and even IgE responses to *B. tropicalis* at an early stage of life have also been documented (Zakzuk et al., 2013). However, HDM species *B. tropicalis* becomes the least studied HDM allergen even though it is common to be found in the subtropical and tropical regions. As a result, advancements in understanding of *B. tropicalis* and its allergen profile are critical for diagnosing and developing clinical therapies Blomia allergy (Santos et al., 2017).

The identification and characterization of novel HDM allergens aid in the diagnosis and treatment of allergy disorders linked with HDM (He et al.,

2019). The World Health Organization (WHO) and Allergen Nomenclature Subcommittee of the International Union of Immunological Societies (IUIS) have designated the following 14 allergens from *B. tropicalis* as shown in Table 2 (Santos et al., 2017). In the group of *B. tropicalis* allergens, Blo t 5 and Blo t 21 are the primary allergens that were discovered in *Blomia tropicalis* mites (Gao et al., 2007).

Table 2. The *Blomia tropicalis* allergens

Allergen	Biochemical function	IgE reactivity (%)
Blo t 1	Cysteine protease	2 – 93
Blo t 2	Unknown	18 – 57
Blo t 3	Trypsin	9 – 57
Blo t 4	Amylase	1 – 63
Blo t 5	Unknown	12 – 98
Blo t 6	Chymotrypsin	0 – 77
Blo t 8	Glutathione-S-transferase	10 – 100
Blo t 10	Tropomyosin	10 – 38
Blo t 11	Paramyosin	5 – 87
Blo t 12	Unknown	0 – 70
Blo t 13	Fatty acid-binding protein	7 – 11
Blo t 19	Antimicrobial peptide	10
Blo t 21	Unknown	57 - 93

2.3.2.1 Blo t 21

Blo t 21 was discovered and characterised by Gao et al. in 2007 with IgE binding frequency reaching more than 50% in HDM sensitized population. It is identified as one of the major *B.tropicalis* allergen that is mainly found in the mite's midgut

and hindgut and in faecal particles (Gao et al., 20017). Blot 21 has been suggested as a paralog of group 5 mite allergens that developed via gene duplication due to the significant sequence similarity between Blot 21 and Blot 5, which shares over 40% structural similarity. Despite their similarity, these allergens do not exhibit a high degree of cross-reactivity (Santos et al., 2017).

The Blo t 21 structure is revealed by the three-dimensional NMR which composed of three anti-parallel α-helices arranged in a helical bundle. The structure is similar to Der p 5, one of the main allergens found in *Dermatophagoides pteronyssinus* mite species (Tan et al., 2012). Der p 5 was discovered to bind to lipid ligands and activate airway epithelial cells via a TLR-2 dependent mechanism (Pulsawat et al., 2018). Thus, it is important to note that Blo t 21 allergen displays structural homologies with the lipid binding protein of Der p 5 although the physiological function of Blo t 21 is still unknown as it suggests that Blo t 21 may act as a lipid binding protein that could have the potential to cause the impairment of epithelial barrier integrity.

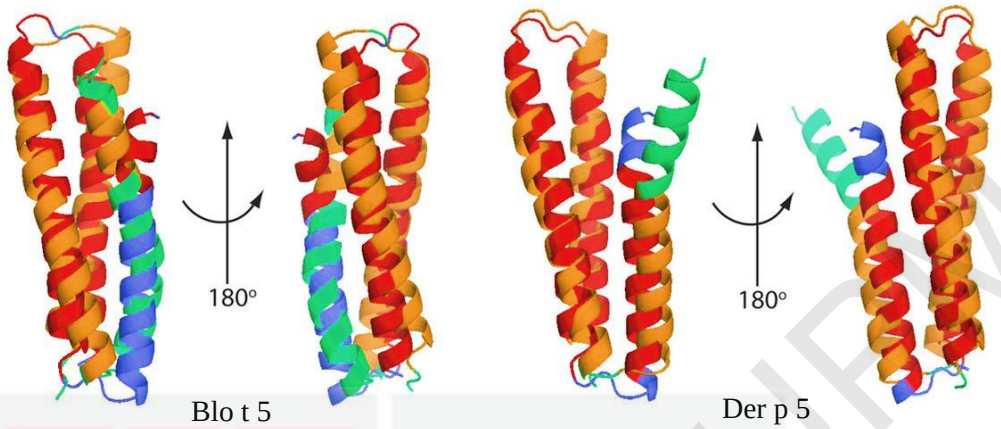

Figure 8. The Blot 21 structure superimposed over Blot 5 and Der p 5 structure. Blo t 21 (query structure) is shown by a blue color, while the Blo t 5 or Der p 5 (target sequences) are indicated by a green color. Orange (Blo t 21) and red colored (Blo t 5 or Der p 5) indicate pairs with a high degree of structure similarity (Adapted from: Tan et al., 2012).

2.4 16HBE14o- cell line

Dr Dieter C. Gruenert isolated the 16HBE14o- cell line from a one-year-old male heart-lung transplant patient. The cells were initially primary cells before being transformed with the pSVori- plasmid by calcium phosphate transfection (Cozens et al., 1994). The 16HBE14o- cell line has been extensively employed in the research of the airway epithelium's barrier function, as it closely mimics the *in vivo* airway bronchial epithelium. 16HBE14o- exhibits significant characteristics such as the ability to express major tight junctional proteins and they form polarized cell making it a reliable *in vitro* model for airway epithelial barrier studies (Callaghan et al., 2020; Forbes, 2000).

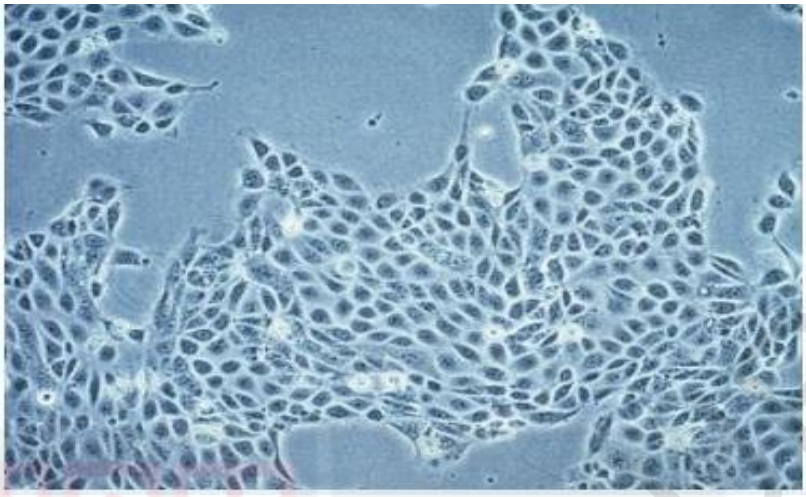

Figure 9. The 16HBE14o- human bronchial epithelial cell line. The 16HBE14o- cell line is a good in vitro model for airway epithelial barrier studies as it retains the characteristics of normal differentiated bronchial epithelial cells. (Adapted from: Sigma Aldrich, n.d.)

2.5 Lactate dehydrogenase (LDH) assay

Cytotoxicity is often determined by the activity of cytoplasmic enzymes produced by injured cells. LDH is an enzyme that was found in every cell and can be used used as a marker of cytotoxicity (Kumar et al., 2018). When cell viability is decreased, the plasma membrane's leakiness increases, resulting in the release of the LDH enzyme into the culture supernatant. LDH is responsible for the conversion of lactate to pyruvate, which results in the reduction of nicotinamide adenine dinucleotide (NAD) to nicotinamide adenine dinucleotide hydride (NADH). The catalyst (diaphorase) then transfers H+ from NADH to the tetrazolium salt (iodonitrotetrazolium (INT)), which is next reduced to red formazan. The amount of the LDH activity is directly proportional to the intensity of the colour produced by the red formazan which is measured spectrophotometrically. This LDH activity in turn correlates directly with the

number of dead or injured cells.

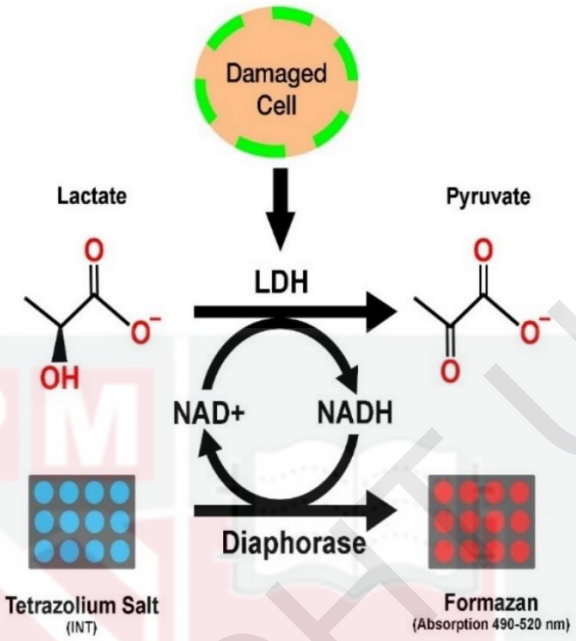

Figure 10. The principle of lactate dehydrogenase (LDH) assay. The LDH cytotoxicity test is a colorimetric technique for determining the cytotoxicity of cells. (Adapted from: Nuohai Life Science., n.d.)

2.6 Western blot assay

Western blotting (alternatively called immunoblotting) was invented in 1979 by Towbin et al. and is a widely used method in research for separating and identifying proteins in a sample. In western blot, the protein in the sample is separated based on the molecular weight by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins are then transferred to a membrane. Following that, the membrane is incubated with antibodies specific to the target protein. The antibody that is not bound to the target protein is washed away. Typically, the transferred protein is incubated with two antibodies which are primary

antibody (specific to the target protein) and secondary antibody (specific to the primary antibody). The secondary antibody is conjugated with an enzyme that produces a detectable signal when coupled with a suitable substrate. The use of a chemiluminescent substrate is commonly used in which it produces light as a result of the enzyme-antibody interaction. The chemiluminescent signal is filmed and recorded using a digital imaging equipment based on charge-coupled device (CCD) cameras. Often, only one band should be seen since the antibodies specifically identify the target protein and the amount of protein present correlates directly to the thickness of the band (Mahmood & Yang., 2012).

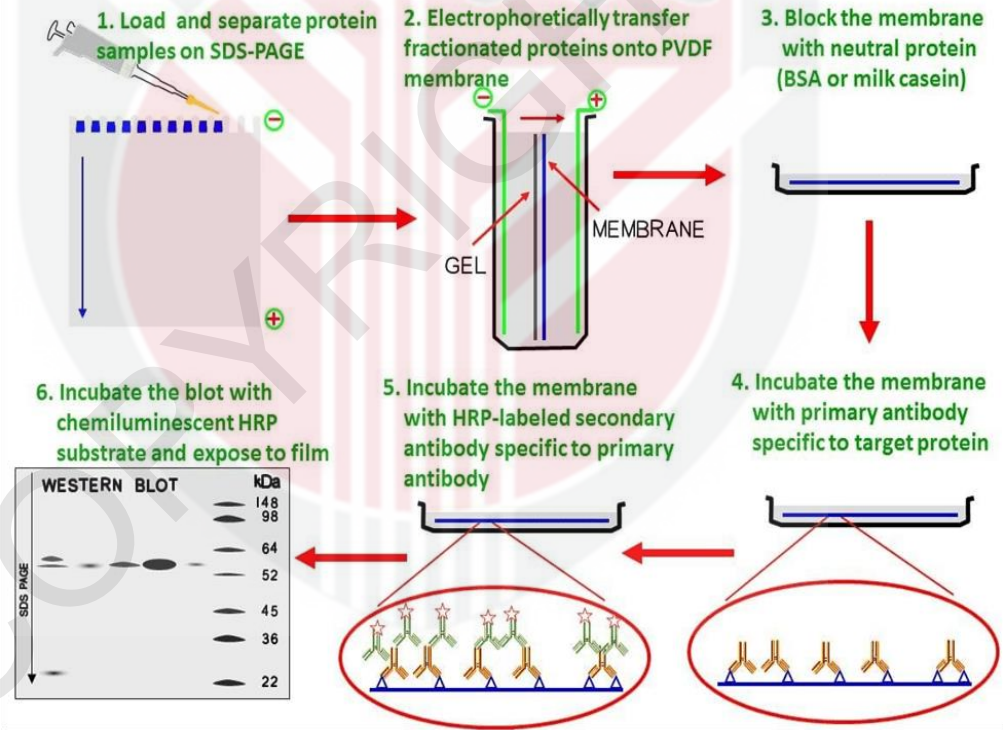

Figure 11. The general procedure of Western Blot assay. The indirect method of western blot assay involving two antibodies. (Adapted from: Bhandari et al., 2021).

CHAPTER 3

MATERIALS AND METHOD

3.1 Materials

3.1.1 Cell line

16HBE14o- human bronchial epithelial cell line was generously gifted by Dr. Dieter C. Gruenert from University of California, San Francisco. The cell line was isolated from the human bronchial surface epithelium of a 1-year-old male heart-lung transplant patient. The cells were first primary cells before being transformed by calcium phosphate transfection with pSVori-plasmid. This cell line is the most well-established model for the study of airway epithelial barrier function as it expresses important intercellular junctional proteins.

3.1.2 Cell culture

Eagle's Minimum Essential Medium (EMEM) was purchased from American Type Culture Collection (ATCC) (Manassas, Virginia, USA). Fetal Bovine Serum (FBS) was purchased from Gibco (Waltham, Massachusetts, USA). Trypsin-EDTA 10X and dimethyl sulfoxide (DMSO) were axquired from Sigma Aldrich (St Louis, Missouri, USA). Tissue culture flask and multi-well plates were acquired from BD Biosciences (Franklin Lanes, New Jersey, USA). L-Glutamine was purchased from Merck Millipore (Berlington, Massachusetts, USA).

3.1.3 Blo t 21 house dust mite (HDM) allergen

Purified proteins of 0.25 mg/mL house dust mite allergen, Blo t 21, was prepared and given by Associate Professor Dr. Alain Jacquet from Chulalongkorn University (Pathumwan, Bangkok, Thailand).

3.1.4 Antibodies

Occludin rabbit monoclonal antibody was acquired from Abcam (Cambridge, UK). HRP-conjugated goat anti-rabbit IgG was purchased from Cell Signalling Technology (Danvers, Massachusetts, USA) while HRP-conjugated mouse monoclonal beta-actin IgG was purchased from Santa Cruz Biotechnology (Dallas, Texas, USA).

3.1.5 Reagents

Phosphate buffered saline (PBS) and lipopolysaccharides (LPS) was purchased from Sigma Aldrich (St Louis, Missouri, USA). Polyvinylidene difluoride (PVDF) membrane and Bicinchoninic Acid (BCA) protein assay kit was purchased from Merck Milipore (Berlington, Massachusettes, USA). WesternBright Sirius enhanced chemiluminescence (ECL) was purchased from Advansta Inc. (Menlo Park, California, USA). Lactate Dehydrogenase Activity Assay Kit was purchased from Sigma-Aldrich (St Louis, Missouri, USA).

3.2 Methods

3.2.1 Preparation of culture media

16HBE14o- cells were cultured in complete EMEM which was prepared by adding 10% FBS and 1% 200 mM L-Glutamine into the basal medium (Refer Appendix A1(a)). The preparation of the complete media was performed in biosafety cabinet (BSC) class II. The complete media was stored at 4°C.

3.2.2 Preparation of trypsinizing solution

The trypsinizing solution was diluted to 1× trypsin-EDTA from 10 × stock solution for the detachment of 16HBE14o- cells.

3.2.3 Preparation of phosphate-buffered saline (PBS)

PBS (100 mL) was prepared by dissolving 1 tablet of PBS in ultrapure water as washing solution for 16HBE14o- cells. The solution was then autoclaved to ensure sterility and kept at 4ºC**.**

3.2.4 Cell culture

16HBE14-o cells were cultured in T25 flasks and incubated in a 5% CO_2 humidified incubator at 37°C. 16HBE14-o cells between passage 80 to 89 were used throughout this study. Daily monitoring of the cells was done using an inverted microscope to check for its confluency or presence of any contamination. The media was changed every 2 days. When the cells reached ~90% confluency, the cells were typsinized and subcultured. The cells were also stored as stock in freezing solution containing 50% FBS, 40% complete EMEM and 10% DMSO at -80°C before being transferred

to liquid nitrogen for long term storage (Refer Appendix A1(b)).

3.2.5 Cell counting

16HBE14o- cells were harvested by removing the used media from the culture flask and the cells were then washed with PBS. 16HBE14o- were incubated with 2 mL of 1 × trypsin-EDTA for 10 minutes at 37°C to allow the cells to be detached from the surface of the flask. Four mL of fresh complete EMEM was added to the cells to stop trypsinization and the cell suspension were transferred from the culture flask to a sterile 15 mL-falcon tube. The cell suspension was then centrifuged at 1200 rpm for 4 minutes at 4°C. The supernatant was discarded and 1 mL of fresh complete EMEM was added to the cell pellet. The cell viability test was carried out using trypan blue exclusion test by performing serial dilution, in which 10 µL of the cell suspension was mixed with 10 µL of 0.4% trypan blue. The solution was then loaded to a hemocytometer and the cells were viewed under a microscope. The number of viable cells (unstained) and non-viable cells (stained) was counted separately. The cell concentration was then calculated by using the formula as below:

$$\left(\frac{Number\ of\ viable\ cells}{4}\right) \times 10^4 \times Dilution\ factor$$

Where,

Dilution factor = 2

3.2.6 Cell induction

16HBE14o- cells were seeded on the 6-well plate with seeding density of 1×10^6

cells/well until they reached approximately 90-100% confluency. The cells were induced with 20 µg/mL Blo t 21 for 24 hours. Cells of the normal group were only fed with complete EMEM and the cells in positive control group were treated with 10 µg/mL LPS. For the lactate dehydrogenase (LDH) assay, an additional group of cells exposed to 1% Triton X-100 was included as positive control to determine the maximum LDH release from the cells. The details of the experimental groups are briefly illustrated in the table below:

Table 3. Induction groups for 16HBE14o- cells

Group	N	Blo t 21	LPS	Triton X-100
Blo t 21 (20 µg/ml)	-	+	-	-
Lipopolysaccharide (10 µg/ml)	-	-	+	-
1% Triton X-100	-	-	-	+

Group N: Normal control, Blo t 21: Blo t-21 induction group, LPS: positive control, Triton X-100: positive control in LDH assay.

3.2.7 Cytotoxicity assay

The supernatant from each well in the 6-well plate after 24 hours of the induction was collected and centrifuged at 13000 x g at 4ºC for 15 minutes. The soluble fraction of the supernatant was used as samples for LDH activity assay. The samples were added into duplicate wells of a 96-well plate prior to LDH Assay Buffer. Different volumes of 1.25 mM NADH standards (0, 2, 4, 6, 8, and 10µL) were also prepared for colorimetric detection as standard curve as instructed in the protocol given (Refer Appendix B1). Master Reaction Mix was set up according to the

protocol and added to each well. Initial measurement of the absorbance was then taken at 450 nm by using ELISA microplate reader (Tecan, Switzerland). The plate was incubated at 37°C and measurements were taken every 5 minutes until the value of the most active sample was greater than the value of the highest standard. The LDH activity of the samples was then determined by using the following equation:

$$\frac{\boldsymbol{B}}{(\boldsymbol{Reaction\ time}) \times \boldsymbol{V}} \times \boldsymbol{Sample\ Dilution\ Factor}$$

Where,

B = Amount of NADH generated between $T_{initial}$ and T_{final}

Reaction Time = $T_{final} - T_{initial}$

V = Volume of sample added to the well

3.2.8 Western blot assay

3.2.8.1 Sample preparation

The attached 16HBE14o- cells from Section 3.2.7 excluding Triton X-100 control group were used for Western Blot assay.

3.2.8.2 Whole protein extraction

16HBE14o- cells were rinsed with cold 1 × PBS. Complete removal of the PBS was ensured before adding RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1% protease and phosphatase inhibitors cocktail) to lyse the cells. The cells were then scraped gently

by using rubber cell scraper. The cell lysates were collected and transferred to a microcentrifuge tube. The cell lysates were then centrifuged at 13000 × g for 15 minutes at 4ºC. The supernatants were collected into new microcentrifuge tubes and stored at -80°C for further use.

3.2.8.3 Protein quantification

Protein in the cell lysates were quantified by using Bicinchoninic acid (BCA) protein assay kit. The quantification of the protein was carried out according to the manufacturer's protocol. The standard graph used in the protein assay was generated by using BSA standard (2 mg/mL) which was prepared by serial dilution of 0, 25, 125, 250, 500 and 1000 µg/mL. The BCA working solution used in the experiment was prepared by adding BCA solution and 4% cupric sulphate at ratio 50:1 respectively. Standard solution (25 µL) or 10 × diluted sample (25 µL) were added to the wells of 96-well plate. BCA working solution (200 µL) was then added to the wells and the plate was incubated at 37°C for 30 minutes. The absorbance reading of the mixtures were then read at 562 nm by using the ELISA microplate reader. Protein concentration was calculated by using the obtained absorbance reading and the standard graph (Refer Appendix B2 and B3).

3.2.8.4 SDS-polyacrylamide gel electrophoresis (PAGE)

Preparation of the polyacrylamide gel used in this experiment is described in the Appendix A2(m). Prior to the experiment, the protein samples were mixed with 2 × Laemli's loading buffer to obtain the final concentration of 20 µg. The samples were then loaded into individual wells of the gel. The protein ladder loaded in a well

which was located at the left end of the polyacrylamide gel was used as protein molecular weight reference. The chamber of the Mini-PROTEAN© vertical electrophoresis system (Bio-rad Laboratories Inc., Hercules, CA, USA) was filled with 1 × electrophoresis running buffer. The preparation of the electrophoresis buffer is further described in the Appendix A2. The electrophoresis was carried out at 50 V until the blue dye reached the resolving gel. The voltage was then increased to 100 V until the dye reached near the bottom of the gel.

3.2.8.5 Wet transfer

The gel was carefully cut by separating the empty wells of the polyacrylamide gel and the section with separated proteins. The gel was then lifted from the glass plate gently. The gel and the methanol activated PVDF membrane were sandwiched between two blotting papers and foam pads. The blotting papers, foam pads, membrane and gel were equilibrated in the transfer buffer before being sandwiched together. The preparation of the sandwich was carried out while being soaked in the transfer buffer. Bubbles trapped in between each layer of the sandwich were eliminated by using the mini roller. The sandwich was then placed in between the cassette holder before being transferred to the wet electrophoretic transfer system (Bio-rad Laboratories Inc., Hercules, CA, USA) which had been filled with the transfer buffer. Preparation of the transfer buffer is further described in Appendix A2. Ice packs was added to the system as cooling agents. The transfer process was carried out at 0.35 A for 1 hour and 30 minutes with constant stirring.

3.2.8.6 Western blot assay

After the wet electrophoretic transfer process, the PVDF membrane was blocked with 5% BSA in 1 × Tris Buffered saline with Tween (TBST) for 1 hour. The membrane was then rinsed three times with TBST for 10 minutes each wash before being incubated with primary antibody at room temperature (1:1000) specific for occludin in TBST containing 5% BSA overnight. The membrane was then rinsed with 1 × TBST three times before being incubated with goat HRP-conjugated anti-rabbit IgG secondary antibody (1:2000) diluted in 5% BSA for 1 hour at room temperature. The membrane was then rinsed again with 1 × TBST three times before visualization.

3.2.8.7 Visualization

After being rinsed with 1 × TBST, the membrane was then incubated with chemiluminescent substrate solution before being visualized using the Fusion FX gel documentation system (Vilber Lourmat, Eberhardzell, Germany). Charge-coupled device (CCD) camera was used to capture the protein bands. The bands intensity was later been quantified by using the ImageJ software and the bands were normalized to the loading control used in this study, which is β-actin.

3.2.9 Statistical analysis

Data of two independent experiments was presented in means ± SEM using GraphPad Prism 9. One-Way Analysis of Variance (ANOVA) followed by a post-hoc comparison using Dunnett test was used to determine statistical significance ($p < 0.05$)

CHAPTER 4

RESULTS

4.1 Optimization of lipopolysaccharides (LPS)-induced group

Western Blot assay was carried out to investigate the effects of different concentrations of LPS on the expression of tight junctional protein, occludin, in 16HBE14o- cells. The optimization on the effect of LPS on occludin expression involves two different concentrations, which were 1 μg/mL and 10 μg/mL. The results of the experiment are as shown in Figure 12.

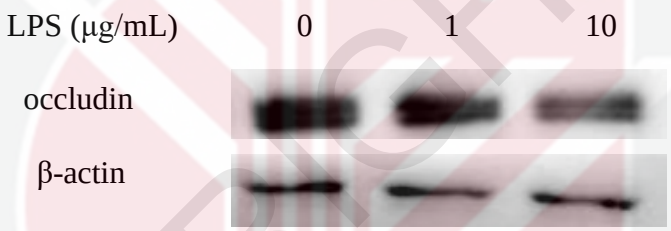

Figure 12. LPS downregulated the protein expression of occludin in 16HBE14o- cells. 16HBE14o- cells were seeded onto 6-well plate until ~90-100% confluency. The cells were induced with 1 μg/mL and 10 μg/mL LPS for 24 hours. The equal expression of the loading control, β –actin, indicated that the protein samples of each group were equally loaded and transferred.

The protein expression of occludin in normal condition was demonstrated as normal group without LPS induction whereas the LPS-induced groups represent the protein expression of occludin which have been exposed to 1 μg/mL and 10 μg/mL of LPS for 24 hours. The protein expression of occludin was reduced in the LPS-induced groups as compared to the normal group. Both LPS concentrations used downregulated occludin expression, in which 10 μg/mL LPS showed more pronounced downregulatory effect in the

expression of occludin. This finding was in consistent with the results from previous studies which has proven that 10 µg/mL LPS significantly downregulated the expression of occludin in 16HBE14o- cells (Ma et al., 2020). Thus, 10 µg/mL LPS was used as the positive control as it displayed significant downregulation of occludin expression.

4.2 Cytotoxic effect of Blo t 21 on 16HBE14o- cells

LDH assay is a colorimetric assay that is used to determine the amount of LDH released by cells as a cytotoxicity indicator. LDH is an oxidoreductase enzyme that catalyzes the conversion of lactate to pyruvate and is released by cells following tissue damage. The absorbance measurement was used to evaluate the LDH activity of the cells, which is an indicator for the presence of cell damage.

LDH activity assay was carried out to determine whether the concentration of Blo t 21 used (20 µg/mL) was cytotoxic to 16HBE14o- cells. The result for the cytotoxic effects of Blo t 21 on 16HBE14o- cells was presented in Figure 13.

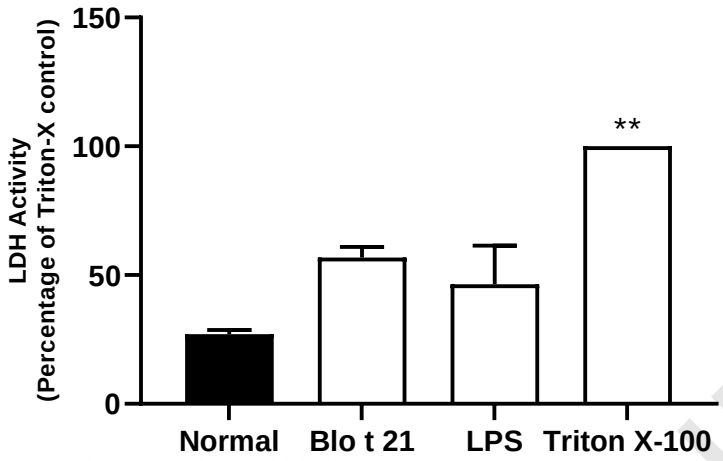

Figure 13. Blo t 21 at 20 µg/mL was non-cytotoxic to 16HBE14o- cells. 16HBE14o- cells were seeded onto 6-well plate until reaching approximately 90-100% confluency before being induced with Blo t 21 at concentration of 20 µg/mL, LPS at concentration of 10 µg/mL and 1% Triton X-100 for 24 hours. The normal group represents cells at normal condition where the cells were only fed with complete EMEM. Results were expressed in mean ± S.E.M of two independent experiments performed in duplicate. ** represents $p \leq 0.01$ significantly different from the normal group.

From Figure 13, the LDH activities of the induction groups of Blo t 21, LPS and Triton-X were compared to the normal group, to determine whether the concentration of Blo t 21 used (20 µg/mL) has cytotoxic effect to 16HBE14o- cells. Normal group represents the cells at normal condition and the Triton X-100 group serves as the positive control group which represents the cells exposed to 1% Triton X-100. Triton-X group was included in the experimental design to prove a significant cell death by quantifying the maximal LDH activity of the lysed cells. The results show that Blo t 21 at the concentration 20 µg/mL and LPS at concentration 10 µg/mL had no significant difference from the normal group, indicating that they did not possess cytotoxic effect on 16HBE14o- cells. Notably, all induction groups showed slight elevation in LDH activity compared to the normal group. Only cells exposed to Triton X-100 showed significant increase in the LDH activity

compared to the normal group. Hence, these results prove that Blo t 21 at concentrations of 20 µg/mL was not cytotoxic to the cells and thus it is suitable to be used in subsequent assay.

4.3 Effect of Blo t 21 on the protein expression of occludin

Previous study has proven that HDM allergens with proteolytic activity could interfere with the epithelial barrier integrity by regulating the junctional protein expression. However, the effect of other non-proteolytically active HDM allergens such as Blo t 21 on the junctional protein expression remains unknown. Hence, Western Blot assay was conducted to study the effect of Blo t 21 on the junctional protein expression particularly occludin in 16HBE14o- cells. The result of the experiment is as shown in Figure 14.

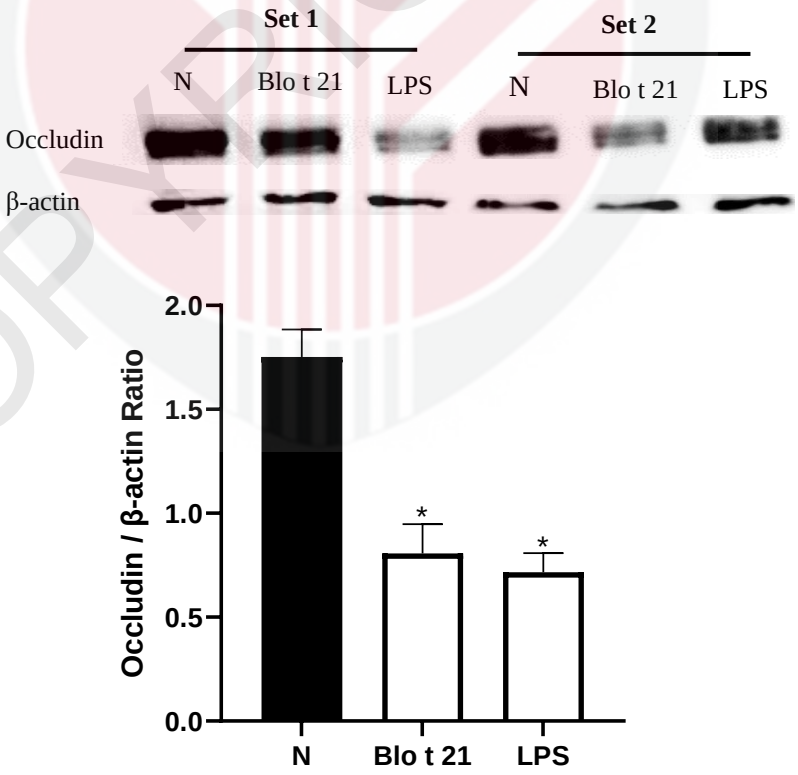

Figure 14. Blo t 21 downregulated the protein expression of occludin. 16HBE14o- cells were seeded onto 6-well plate until reaching approximately 90-100% confluency. The cells were induced with 20 µg/mL Blo t 21 and 10

μg/mL LPS for 24 hours. The cell lysate was collected and SDS-PAGE was conducted. The equal expression of loading control, β –actin, indicated that the protein samples of each group were equally loaded and transferred. The experimental groups were abbreviated as follows: N: Normal group; Blo t 21: Blo t 21-induced group (20 μg/mL); LPS: Lipopolysaccharides-induced group (10 μg/mL). Analysis of protein expression through ImageJ after normalization with β-actin was conducted. Results were expressed in mean ± S.E.M. of two independent experiments. *represents $p \leq 0.05$ significantly different from the normal group.

The protein expression of occludin in normal condition was demonstrated in normal group (N) whereas the Blo t 21 group represents the protein expression of occludin which had been exposed to 20 μg/mL Blo t 21 for 24 hours. The protein expression of occludin in Blo t 21 group was significantly reduced by 54% as compared to the normal group. LPS-induced group (10 μg/mL) which serves as the positive control in this experiment also shows significant reduction of occludin expression by 59.2% as compared to the N group. Thus, this finding shows that the Blo t 21 may disrupt the epithelial barrier integrity by regulating the junctional protein expression specifically occludin.

CHAPTER 5

DISCUSSION

5.1 Introduction

Asthma is one of the most common airway diseases worldwide. One of the most common causes of asthma is the exposure to household allergens such as the HDM. The allergen triggers a cascade of immunological reactions that leads to chronic inflammation airway disorders such as asthma (Milián & Díaz, 2004). The presence of tight junctions is important in preventing the allergens from entering paracellular gaps between the cells. The degree of disassociation of tight junctions has been found to be related to the permeability of the epithelial barrier, resulting in increased allergen entry. It has been well documented that following the exposure of HDM allergens with proteolytic activity into the airway, the epithelial tight junctions are cleaved causing the access of the allergens to the submucosal cells leading to a cascade of inflammatory reactions (Jacquet, 2013). However, the effect of HDM allergens other than proteases on the disruption of the epithelial cells remains unknown even though most of them were found to be highly sensitized in the population of patient with airway disorders such as asthma. Blo t 21 is the major allergen in *Blomia tropicalis* mite species with IgE binding frequency reaching more than 50% in HDM sensitized population. Therefore, determining the impact of Blo t 21 exposure on the epithelium's tight junctional protein expression is critical.

5.2 The optimization of LPS-induced group

Numerous studies have demonstrated tight junction dysfunction in asthmatic airways due to the exposure to HDM allergens (Daan de Boer et al., 2013). LPS, one of the most important inflammatory mediators, inhibits the expression of certain tight junction proteins such as occludin and claudin-1, thus impairing the epithelial barrier integrity (Wu et al., 2020).

In vitro, we stimulated the human bronchial epithelial cell line 16HBE14o- with different concentrations of LPS to explore whether LPS could cause a damage of epithelial tight junctions to make it reliable as our positive control. LPS was chosen as the positive control in this study as it is particularly found abundant in nature thus the exposure to it is a realistic event similar to that of HDM. On top of that, LPS play a role in the activation of TLR-4 which will elicit a robust immunological response (Park & Lee., 2013). TLR-4 activation may impact the TJ complexes by regulating the occuldin expression, resulting in paracellular pathway failure and increase in the TJ permeability (Luo et al., 2012). Decreased in permeability of the endothelial cells due to TLR-4 deficiency which was linked with elevated occludin and ZO-1 expression levels was also reported (Liu et al., 2017).

Ma et al. (2020) demonstrated that exposure to LPS resulted in lung neutrophilic inflammation and epithelial barrier damage by the significant reduction of the occludin expression in 16HBE14o- cells upon exposure to LPS. As shown in Figure 12, LPS exposure at 1 µg/mL and 10

µg/mL decreased the expression of epithelial cell tight junctional protein, occludin in 16HBE14o- cells. However, the donwnregulatory effect of 10 µg/mL LPS was more pronounced than that of 1 µg/mL. These results are consistent with the findings of Ma et al. (2020) which found that 10 µg/mL LPS showed significant reduction of occludin expression in 16HBE14o- cells. Thus, 10 µg/mL LPS was used as our positive control for the subsequent assays.

5.3 The cytotoxic effect of Blo t 21 on 16HBE14o- cells

The cytotoxic effect of Blo t 21 on 16HBE14o- cells was identified using the LDH assay. In this assay, a Triton X-100 group was added into the experimental design as positive control to determine the maximum LDH release from the cells (Aslantürk, 2018). The 16HBE14o- cells were induced with 20 µg/mL Blo t 21 and 10 µg/mL LPS for 24 hours. The cytotoxic effect was determined by the LDH activity of the lysed cells which release the LDH enzyme (Sigma-Aldrich, 2018). From the result in Figure 13, there is no significant difference in the LDH activity of Blo t 21-induced group at concentrations 20 µg/mL when compared to the normal control group, thus indicating that the concentration used is non-cytotoxic. This finding indicates that the regulatory effect on occludin expression was not arisen from the cytotoxicity exerted by Blo t 21 on the 16HBE14o- cells. Notably, the LPS-induced group also showed slight elevation in LDH activity compared to the normal group. Although the tested concentrations of both Blo t 21 and LPS-induced group were shown to be non-toxic to the cells, it is worth noting that

the induction groups had somewhat greater LDH activity than the normal control which might be due to inconsistent cell seeding onto the 6-well plate. Besides that, it is important to highlight that there was only a slight difference in LDH activity between Blo t 21-induced group and Triton-X control group. This might be due to the incomplete cell lysis by 15 minutes of Triton-X incubation, which results in the lack of LDH produced by cells in Triton-X control group. Therefore, longer Triton-X incubation duration might be needed.

5.4 The effects of Blo t 21 on protein expression of occludin in 16HBE14o- cells

Disruption of the epithelial tight junctional proteins may allow allergen entry to interact with the immunological cells that will subsequently enhance the epithelium's pro-inflammatory activity. As in Wan et al. (1999), the HDM allergen with proteolytic activity was proven to possess the ability to cause junctional permeability by directly cleaving the junctional proteins which leads to impairment of the epithelial barrier. However, very surprising results from Post et al. (2011), highlighting that proteases from the HDM extract were not critically needed for epithelial barrier function impairment *in vitro* as the HDM extract with the greatest impact on the disruption of epithelial barrier was the one with the lowest proteolytic activity. This has raised our interest to identify the effect of HDM allergens other than proteases that might also be responsible for the disruption of the epithelial barrier function. Interestingly, previous findings by Wang et al. (2011) revealed that HDM lipid binding allergen, Der p 2 could increase the permeability of lung alveolar epithelium

through downregulation of tight junctional protein expression at gene level. Thus, it has raised our attention in the lipid binding protein allergen of the house dust mite on its regulatory effect on tight junctional protein expression.

At least 30 protein components were identified in the *Blomia tropicalis* extract., and among these, Blo t 21, was the major allergen that frequently detected and demonstrated to have high IgE binding frequency reaching up to 50% in HDM sensitized population (Santos et al., 2017). Although Blo t 21 is one of the most allergenic allergen group in *B. tropicalis*, the exact physiological function of these allergens is unknown at the moment (Carvalho et al. 2013). By using the 3-D NMR, it reveals that Blo t 21 is composed of three anti-parallel α-helices arranged in a helical bundle similar to that of Der p 5, the main allergen found in *Dermatophagoides pteronyssinus* mite species (Kang et al., 2012). Der p 5 was discovered to bind to lipid ligands and activate airway epithelial cells via a TLR-2 dependent mechanism (Pulsawat et al., 2018). Thus, it is important to note that Blo t 21 allergen displays structural homologies with the lipid binding protein of Der p 5 although the physiological function is still unknown. This suggests that Blo t 21 may be consider as a lipid binding protein that could have potential to cause the integrity of the epithelial barrier to be impaired

In this study, we investigate the effects of Blo t 21 on the expression of junctional protein, occludin, in 16HBE14o- cells. Western Blot assay was performed to determine the effect of Blo t 21 at the concentration of 20 μg/mL on the expression of tight junctional occludin upon induction in the

16HBE14o- cells. β-actin acts as a loading control for western blot assay to confirm that protein loaded across the gel is equally same. Figure 14 shows that the β-actin of this assay proven to be equally loaded among induction groups and interestingly, Blo t 21 was found to exert a pronounced effect on the downregulation of the tight junction protein occludin when compared to the normal group. Although the fact that there was inconsistency in the band intensity of the two independent trials as shown in Figure 14, the band still revealed a downregulatory pattern of the Blo t 21-induced group as compared to the normal group in both trials. Blo t 21 significantly downregulated the expression of tight junctional protein, occludin, by 54% as compared to the normal group. This finding is somehow in line with previous study which reported that HDM allergen that was independent of proteolytic activity exerted regulatory effect on tight junctional protein expression at gene level (Wang et al., 2011).

Therefore, the present study shows that the induction of the Blo t 21 lead to downregulation of the occludin expression. These findings provide evidence that proteolytic activity in HDM allergen is not the only factor that is required for the disruption of epithelial barrier. Other biological functions of HDM allergens such as the lipid binding protein may interfere with the epithelial barrier integrity by regulating the junctional protein expression. The underlying mechanism of Blo t 21 which is a lipid binding protein in regulating the junctional protein expression remains unknown. One of the potential regulatory effects of the junctional protein expression might be via activation of TLR-2. In previous study, it was reported that TLR-2 activation

reduced the expression of occludin at both the gene and protein levels in Calu-3 epithelial cell (Ragupathy et al., 2014).

Despite the overwhelming findings of the Blo t 21 on the downregulation of the tight junction occludin expression, there are several limitations that need to be addressed in this study. Firstly, this study used only one concentration of Blo t 21 with only one time point. Due to that, the concentration-dependent and the time-dependent effect on the downregulation of occludin expression could not be determined thus making us unsure whether a lower concentration of Blo t 21 and shorter incubation time could also cause the reduction in the expression of the tight junctional proteins occludin. Other than that, this study only focuses on one tight junctional proteins occludin. There are a few other important junctional proteins such as E-cadherin, Zonula occluden-1 and claudins that are not included in this study which may also demonstrate the reduction in the protein expression upon the induction of Blo t 21 allergens. Lastly, the other limitation in this study is it did not include any study on the gene expression of occludin and only two sets of trials were performed instead of three sets due to limited protein sample. Thus, the results need to be further confirmed by the immunofluorescence assay.

CHAPTER 6

SUMMARY, CONCLUSION AND FUTURE RECOMMENDATION

6.1 Summary

The epithelial barrier is critical in the maintenance of the airway system. This epithelial barrier is primarily regulated by junctional proteins, which connect adjacent epithelial cells. However, the epithelial lining can still be disrupted by environmental allergens during respiration process such as the house dust mite. House dust mites with proteolytic activity are proven to disrupt the epithelial barrier by degrading junctional proteins or delocalizing them from the cell border. However, little is known on the HDM allergens other that proteases on the epithelial barrier function. Blo t 21, a non-proteolytic HDM allergen which may be considered as one of the lipid binding proteins, is one of the major allergens in *Blomia tropicalis* mite species with a IgE binding frequency reaching more than 50% in HDM sensitized population. Despite the high sensitization frequency of this allergen, the effect of the Blo t 21 on the epithelial barrier function is still unknown. Thus, this study was carried out to determine the effect of Blo t 21 on tight junctional proteins expression specifically occludin in 16HBE14o- cells. Blo t 21 was found to be non-cytotoxic to cells at concentration of 20 µg/mL with no significant difference in LDH activity when compared to the normal group. Additionally, it has been demonstrated that Blo t 21 significantly downregulated the expression of tight junctional protein occludin in 16HBE14o- cells.

6.2 Conclusion

In short, the present study signifies that Blo t 21 may be capable of downregulating the protein expression of occludin. This finding further strengthens the speculation that HDM allergens other than proteases may interfere with the epithelial barrier integrity through the regulation of tight junctional protein expression. Nonetheless, the effects of Blo t 21 on the expression of other junctional proteins and the mechanisms underlying the regulatory effect of Blo t 21 should be further dissected in the future.

6.3 Future Recommendation

This study has shown that Blo t 21 plays a role in tight junctional protein disruption by downregulating the protein expression, occludin. Notably, HDM allergen groups 13, 14 and 21 can be considered as lipid binding proteins based on the sequence homology (Thomas et al., 2010). Thus, further study of other subtypes of the lipid binding proteins HDM allergens on the effect of tight junctional protein should be carried out to further support the present study which suggests that lipid binding proteins of HDM allergen may interfere with the epithelial barrier integrity by regulating the junctional protein expression.

As this study can only cover the superficial surface of the issue, deeper understanding on the mechanism involved in the effect of Blo t 21 causing impairment of the epithelial barrier function are needed to be further studied. The lipid binding protein could facilitate the allergen sensitization through the TLR-2 (Jacquet, 2020). Thus, it is recommended to investigate the TLR-2 activation mediated by Blo t 21 in order to identify its underlying mechanism.

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APPENDICES

APPENDIX A: REAGENTS

1) Cell culture reagents

a) 16HBE14o- cells complete EMEM

267 mL	Eagle's Minimum Essential Medium
30 mL	Fetal bovine serum
3 mL	L-glutamine (200 mM)

(Complete medium was stored at 4°C)

b) Freezing solution

400 µL	Complete EMEM
500 µL	Fetal bovine serum
100 µL	DMSO

2) Immunoblotting reagents

a) Radioimmunoprecipitation (RIPA) lysis buffer

NaCl	0.877 g
Tris/HCl, pH 7.4	0.788 g
SDS	0.1 g
Sodium deoxycholate	0.5 g
Triton-X	1 mL

Dissolved to 100 mL ultrapure water and stored at 4°C.

b) 1.5M Tris, pH 8.8

Tris	45.413 g

Dissolved to 250 mL with ultrapure water and the pH was adjusted with hydrochloric acid. Stored at 4°C.

c) 0.5 M Tris, pH 6.8

Tris	15.138 g

Dissolved to 250 mL with ultrapure water and the pH was adjusted with hydrochloric acid. Stored at 4°C.

d) 10% SDS

SDS	10 g

Dissolved to 100 mL with ultrapure water and stored at room temperature.

e) 10% Ammonium persulfate (APS)

Ammonium persulfate	25 mg

Dissolved to 250 µL with ultrapure water.

f) 10X Running buffer

Tris	30.3 g
Glycine	144 g
SDS	10 g

Dissolved to 1 L with ultrapure water. Stored at room temperature.

g) 10X Transfer buffer

Tris	30.3 g
Glycine	144.1 g
SDS	10 g

Dissolved to 1 L with ultrapure water. Stored at room temperature.

h) Transfer buffer

10X transfer buffer	100 mL
10 % SDS	5 mL
Absolute methanol	10 g

Dissolved to 1 L with ultrapure water. Prepared fresh before usage.

i) 10x Tris buffered saline (TBS), pH 7.6

Tris	24.23 g
NaCl	87.66 g

Dissolved to 1 L with ultrapure water and stored at room temperature.

j) Tris buffered saline with Tween-20 (TBST), pH 7.6

10 × TBS	100 mL
Tween-20	1 mL

Diluted to 1 L with ultrapure water and the PH was adjusted by using hydrochloric acid. Stored at room temperature.

k) 2X Laemli buffer

1 M Tris-HCL, pH 6.8	1.25 mL
Glycerol	2 mL
SDS	0.4 g
Bromophenol blue	2 mg

Dissolved to 10 mL with ultrapure water. Stored at room temperature. 10% (v/v) of β-mercaptoethanol was added to the Laemmli buffer prior use.

l) 5% blocking solution

BSA	0.5 g

Dissolved in 10 mL of TBST. Prepared fresh before usage.

m) SDS-Page gels

Table 4. Components of SDS-PAGE gels

Components	10% Resolving gel (mL)	5% Stacking gel (mL)
Ultrapure water	3.85	3.15
40% polyacrylamide	2	0.5
1.5 M Tris, pH 8.8	2	-
0.5 M Tris, pH 6.8	-	1.25
10% SDS	0.08	0.05
10% Ammonium persulfate	0.08	0.05
TEMED	0.008	0.005

APPENDIX B: DATA

1) NADH concentration standard curve for LDH assay

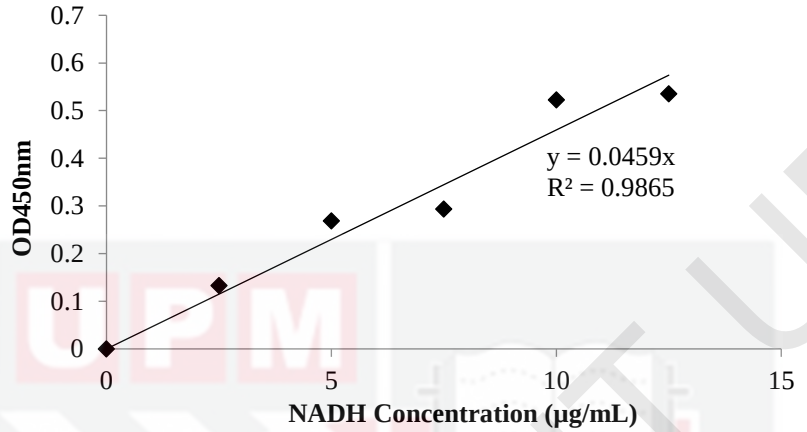

Figure Appendix B1. NADH standard curve. LDH assay was conducted according to the manufacturer's protocol of LDH assay kit. The LDH activity of 16HBE14o- cells were calculated from the NADH assay standard curve.

2) BCA assay standard curve (Set 1)

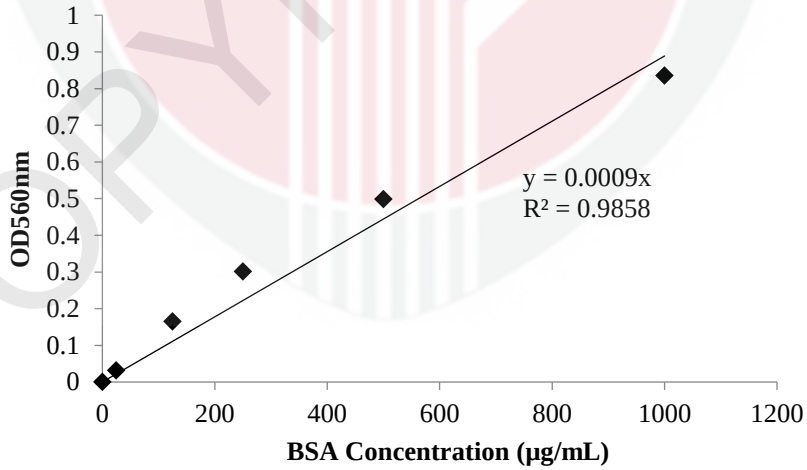

Figure Appendix B2. BCA standard curve of Set 1. BCA assay was conducted according to the manufacturer's protocol of BCA assay kit. The total protein content of 16HBE14o- cells were calculated from the BCA assay standard curve.

3) BCA assay standard curve (Set 2)

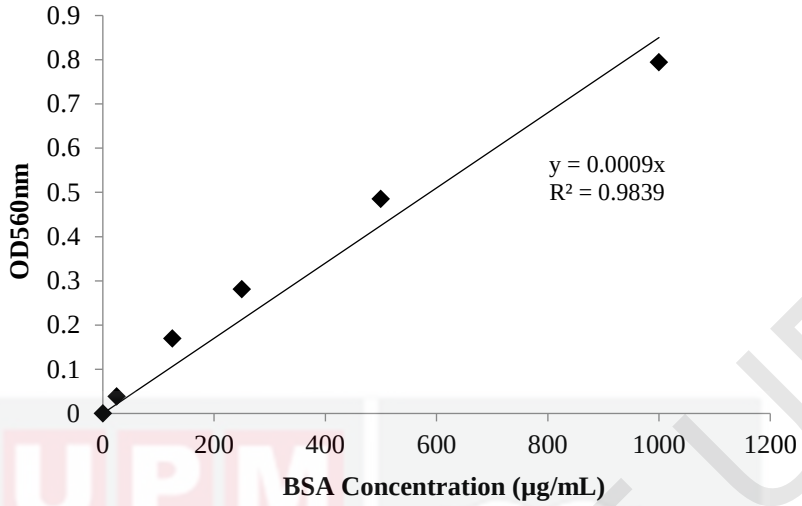

Figure Appendix B3. BCA standard curve of Set 2. BCA assay was conducted according to the manufacturer's protocol of BCA assay kit. The total protein content of 16HBE14o- cells were calculated from the BCA assay standard curve.

4) LDH activity assay

Table 5. Statistical analysis of LDH activity assay by one-way ANOVA

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	52694	3	17565	F (3, 4) = 21.82	P < 0.01
Residual (within columns)	3220	4	804.9		
Total	55913	7			

Table 6. Statistical analysis of LDH activity assay by Dunnett's multiple comparisons test

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Summary	Adjusted P Value
Normal vs. Blo t 21	-9.807	-112.4 to 92.83	ns	> 0.01
Normal vs. LPS	43.65	-58.99 to 146.3	ns	> 0.01
Normal vs. Triton X-100	-170.3	-272.9 to -67.65	**	< 0.01

5) Western blot assay

Table 7. Statistical analysis of protein expression through ImageJ after normalization with β-actin by one-way ANOVA

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	1.319	2	0.6593	F (2, 3) = 21.38	P < 0.05
Residual (within columns)	0.09254	3	0.03085		
Total	1.411	5			

Table 8. Statistical analysis of protein expression through ImageJ after normalization with β-actin by Dunnett's multiple comparisons test

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Summary	Adjusted P Value
Normal vs. Blo t 21	0.9462	0.2671 to 1.625	*	< 0.05
Normal vs. LPS	1.037	0.3575 to 1.716	*	< 0.05