THE EFFECTS OF TIGER MILK MUSHROOM (*Lignosus rhinocerotis)* ON AIRWAY INFLAMMATION IN MURINE MODELS OF ALLERGIC ASTHMA

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by

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TABLE OF CONTENT

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ACRONYMS, ABBREVIATIONS AND SYMBOLS

AHR	Airway hyper-responsiveness
$Al(OH)_3$	Aluminium hydroxide
ANOVA	One-way analysis of variance
ASM	Airway smooth muscle
BALF	Bronchoalveolar lavage fluid
ddH_2O	Deionized water
ECM	Extracellular matrix proteins
FCS	Fetal calf serum
HCL	Hydrochloric acid
H&E	Haematoxylin and eosin
HDM	House dust-mite
IgE	Immunoglobulin E
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-13	Interleukin-13
i.n	Intranasal
i.p	Intraperitoneal
KCL	Potassium chloride
KH_2PO4	Potassium dihydrogen phosphate
LR	*Lignosus rhinocerotis*
LN	Lymph node
Mch	Methacholine
NaCl	Sodium chloride

OVA	Ovalbumin
PBS	Phosphate buffer saline
RT	Room temperature
SEM	Standard error of the mean
TGF-β	Transforming growth factor
TBS	Tris buffer saline
TMB	Tetramethylbenzidine
Th	T-helper

KESAN CENDAWAN SUSU RIMAU (*Lignosus rhinocerotis)* KE ATAS KERADANGAN SALUR UDARA PADA MODEL ALERGI ASMA

ABSTRAK

Alergik asma merupakan satu penyakit yang dikaitkan dengan keradangan pada saluran udara dan pemodelan semula struktur pada paru-paru. Ubat-ubatan semasa diakui keberkesanannya, namun kebanyakannya adalah berasaskan steroid dan mempunyai kesan sampingan. Oleh itu, produk yang berasaskan bahan semulajadi harus diterokai untuk dijadikan sebagai satu alternatif dalam menguruskan asma. Sklerotium pada *Lignosus rhinocerotis* (Cooke) Ryvarden (cendawan susu rimau) telah digunakan sebagai ubat tradisional bagi merawat pelbagai penyakit termasuk asma. Namun begitu, kajian saintifik yang dilakukan untuk melihat kesan cendawan ini adalah terhad. Oleh itu, kajian ini dilakukan untuk menguji tahap keberkesanan ekstrak *L. rhinocerotis* (LRE) terhadap keradangan, pembentukan semula struktur dan hipergerakbalas (AHR) pada saluran pernafasan pada model murin asma. LRE disediakan melalui kaedah pengekstrakan air panas dengan menggunakan soklet selama 24 jam. Kajian ini dijalankan pada dua jenis model haiwan yang berbeza; model ovalbumin (OVA) dan model hama habuk rumah (HDM). Untuk membentuk model kronik, mencit betina Balb/C disensitasi dengan dua suntikan intraperitoneal pada hari 0 and 7 dan diikuti dengan sedutan OVA, untuk tiga hari seminggu selama 2, 6 dan 10 minggu. Setelah itu, LRE (kepekatan 125, 250 dan 500 mg/kg) dan dexamethasone (3mg/kg) diberikan kepada mencit secara oral. Manakala, satu kumpulan mencit lain telah dibiarkan selama 2 minggu tanpa sebarang alergen (minggu ke 10-12). Cecair lavaj bronkoalveolar (BAL)

digunakan bagi melakukan analisis sitokin, serum untuk analisis immunoglobulin E (IgE), tisu paru-paru bagi analisis histologi dan nodus limfa bagi analisis subset sel. Sementara itu, analisis untuk kajian AHR dilakukan dalam model-HDM, yang bergerak balas terhadap kepekatan metacholin. Pada hari 0, mencit disensitasi, diikuti dengan intranasal HDM pada hari ke 7-11, dan LRE diberikan sejam selepas setiap cabaran. Pada hari ke 14, mencit dibius dan trakeotomi dilakukan. Kajian ini mendapati LRE (kepekatan 125, 250 and 500 mg/kg) mengurangkan penghasilan sel-sel goblet dan penyusutan ketebalan dinding salur pernafasan ($p<0.05$). Manakala 500 mg/kg LRE ($p<0.05$) menunjukkan kesan yang paling efektif dalam mengurangkan pengekspresian TGF-β1 dan activin A. Disamping itu, kajian juga mendapati dos LRE yang berbeza memberikan corak pengurangan berbeza terhadap sel keradangan di dalam cecair BAL, paras IgE dan sitokin Th2 (interleukin-4, IL-5, IL-13). Sebaliknya, analisis sitometri aliran telah menunjukkan bahawa LRE tidak mengurangkan peratusan sel T $CD3^+CD4^+$ dan $CD3^+CD8^+$ secara signifikan ($p>0.05$). Untuk kajian AHR pula, kumpulan-LRE telah mengurangkan aras kerintangan saluran udara pada kepekatan metacholin 8-32 mg/ml ($p<0.05$). Secara kesimpulannya, kajian ini menunjukkan bahawa LRE berpotensi untuk mengurangkan keradangan, pembentukan semula struktur dan AHR pada model asma; menjadikan ia sebagai alternatif yang berpotensi bagi pengurusan penyakit alergik asma.

THE EFFECTS OF TIGER MILK MUSHROOM (*Lignosus rhinocerotis)* ON AIRWAY INFLAMMATION IN MURINE MODELS OF ALLERGIC ASTHMA

ABSTRACT

Allergic asthma is associated with chronic airway inflammation and progressive airway remodelling. Current medications are effective, but these drugs are mostly steroid-based medications and have side effects. Hence, natural products should be explored as an alternative for the management of asthma. The sclerotium of *Lignosus rhinocerotis* (Cooke) Ryvarden (Tiger Milk mushroom) is used traditionally to treat various illnesses including asthma. However, limited studies described the effect of this mushroom scientifically. Thus, this study was carried out to evaluate the effect of *L. rhinocerotis* extract (LRE) on airway inflammation, remodelling and airway hyper-responsiveness (AHR) in murine models of asthma. LRE was prepared by hot water extraction method using soxhlet. This study was conducted in two different types of animal models; Ovalbumin (OVA)-challenged model and house dust mite (HDM)-challenged model. To established chronic model of asthma, female Balb/C mice were sensitised with two intraperitoneal (i.p) injections on day 0 and 7 and further challenged with OVA-inhalation for three times per week for 2, 6 and 10 weeks. Treatments of LRE (125, 250, 500 mg/kg) and dexamethasone (3 mg/kg) were given orally upon after every challenged. One group of mice were left without any allergen challenged after week 10 until week 12. Bronchoalveolar lavage (BAL) fluid was collected for cytokine analysis, serum for immunoglobulin E (IgE), lungs for histopathological analyses and lymph nodes (LN)

for cell subsets analysis. Meanwhile, AHR study was carried out; HDM-model in response to methacholine (Mch) concentrations. Mice were sensitised on day 0, followed by daily HDM intranasal challenge on day 7-11, and treatment were given one hour prior to challenge. On day 14, the mice were anesthetised and tracheotomy was performed. The findings demonstrated LRE treatments (125, 250 and 500 mg/kg) significantly inhibited the production of goblet cell and thickness of airway smooth muscle ($p<0.05$) throughout the prolonged OVA-challenged. Wheras, 500 mg/kg LRE showed the most effective in reducing the expression of TGF-β1 and activin A in the remodelled airways. Moreover, this study showed that different dosages of LRE showed different patterns of suppression on inflammatory cells in BALF fluid, IgE level as well as Th2 cytokines throughout the weeks. In contrast, flow cytometry analysis revealed that LRE did not significantly reduce the percentages of $CD3^+CD4^+$ and $CD3^+CD8^+$ T-cells ($p>0.05$). For AHR study, LRE-treated group significantly attenuated the level of airway resistance at 8-32 mg/ml Mch concentrations ($p<0.05$). In conclusion, these findings suggested that LRE could suppress airway inflammation, remodelling and AHR in mouse model of asthma; thus suggesting its therapeutic potential as an alternative for the management of allergic asthma.

CHAPTER 1

INTRODUCTION

1.1 Asthma

Asthma presents a significant global health burden and it affects over 300 million people worldwide (Asthma, 2018; Harkness *et al.*, 2015; Park and Tantisira, 2017). This figure was estimated to be increase to 400 million by 2025 (Asher and Ellwood, 2014). Asthma is a disease that characterised by the episodes of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation (Reddel *et al.*, 2015).

Asthma can be triggered by allergens, viruses, air pollutants, oxidants, certain drugs, chemicals, changes in temperature and pressure of the environment, emotional disturbances (panic attacks), hyperventilation and hypocapnia (laughter, crying, screaming), exercise, gastroesophageal reflux, food additives and endocrine factors (menstrual cycle, pregnancy, thyroid disease) (Fahy, 2015). Asthma mechanism involves a complex network of inflammatory airway disease, usually caused by various inflammatory and structural cells leading to airway inflammation, airway obstruction, airway remodelling and AHR (Prado *et al.*, 2014). In general, morphological changes (airway remodelling) in the epithelium (goblet cell metaplasia, hyperplasia, and increased mucin stores), submucosa (myofibroblast hyperplasia, subepithelial fibrosis and increased glandular cell volume), smooth muscle cells (hypertrophy and hyperplasia) and an increase of respiratory blood

vessels, usually occurred in individuals susceptible to exaggerated response to allergens (Lambrecht and Hammad, 2015).

The T helper 2 (Th2) driven inflammation of the eosinophilic airways is responsible for up to 50 % of all cases of asthma, and is generally considered to be the major pathogenetic factor in this disease (Lloyd and Saglani, 2013; Shalaby and Martin, 2010). Asthmatic patients typically have elevated levels of Th2 cytokines in their bronchoalveolar lavage fluid (BALF) and bronchial mucosa (Barrett and Austen, 2009). It is well known that activated Th2 cells orchestrate pulmonary immune responses and mediate the inflammation of lung tissues, as well as AHR (Diamant *et al.*, 2013; Fajt *et al.*, 2013; Lin *et al.*, 2013). Activated Th2 cells also inhibit the inflammation of asthmatic airways (Nagai, 2012). It is noteworthy that mice deficient in the Th1-type transcription factor T-bet develop spontaneous AHR and augmented airway eosinophilia (Finotto *et al.*, 2002). The immunomodulation of the Th1/Th2 imbalance encountered in asthma patients is therefore considered to be a practical strategy for controlling asthma (Nagai, 2012).

1.1.1 Prevalence of asthma

Asthma is a major burden worldwide, for governments, healthcare providers, patients and careers (Vos *et al.*, 2012) and there is considerable variation in asthma prevalence, morbidity and mortality (Gibson *et al.*, 2013). Asthma remains a common cause of death in many countries and many asthma deaths are preventable (Lozano *et al.*, 2012). Asthma has been shown to be underdiagnosed across all countries irrespective of the level of development (WHO, 2018).

Asthma has been listed as the 14th most chronic disorder universally and it affects about 8.4 %, 7.7 % and 4.5 % in children, young adults and global populations respectively (National Health and Interview Survey, 2017). Globally, severe uncontrolled asthma accounted for 5-10 % of total asthma cases and contributes to 50% of total asthma cost (Al-Hajjaj, 2011; Dheda *et al.*, 2015). On the basis of disability-adjusted life years, the asthma impact is considered similar to the other major chronic diseases such as Alzheimer's and diabetes (Croisant, 2014). Even though asthma is not seen to be a major cause of death, but the Global Burden of Disease study has reported a global age-standardised death rate of 8.0 per 100,000 in 2013, which is equivalent to breast cancer (7.4) or pedestrian road injuries (8.0) (Naghavi *et al.*, 2015).

In the United Kingdom (UK), on average of three people will die from asthma every day (Levy *et al.*, 2018). Meanwhile, in the United Stated, 2 % of patients with asthma were admitted to hospital in 2009 and 8.4 % were treated in emergency department (ED) (Akinbami *et al.*, 2011). Meanwhile, more than 25 million people were recorded to be affected, and seven million of these people were children (Sutherland *et al.*, 2014). According to National Centre for Health Statistics (2016), asthma affects 6.3 million children in the US (8.6% of the population <18 years of age) and associated with a substantial social and economic burden.

Asthma patients were less likely to be working than those without asthma, and they were more likely to have restrictions on operation at their job, school or home (Sullivan *et al.*, 2011) and when asthma severity worsens, a higher economic burden is noted. Similarly, children with asthma attack have higher rates of school

absent despite available treatments, thus leading to loss of productivity (Bahadori *et al.*, 2009; Bonilla *et al.*, 2005). About 52% of children with asthma had one or more exacerbations that put them at risk for adverse outcomes, including ED visits, hospitalizations, and missed school days (Akinbami *et al.*, 2011).

1.1.2 Asthma in Malaysia

Asthma negatively affects patients' quality of life (QoL) (Heethal *et al.*, 2014) and requires regular visits to ED and hospital admissions. Thus, asthma remains a major public health concern. Hence, optimal asthma care is crucial to reduce the disease burden and to improve patients' QoL (Asthma, 2018; Pedersen, 2010). It requires an optimal process of care, adequate facilities and provision of evidenced-based management according to the guidelines. Through clinical audits, asthma care can be frequently evaluated against research-based standards which are the targets of important quality indicators. The top rated quality indicators for asthma care include asthma control monitoring, controlled medication use, asthma education and pulmonary function monitoring (To *et al.*, 2010)

The International Study of Asthma and Allergies in Childhood (ISAAC III), had estimated that the prevalence of asthma in Malaysia increased from 6.4% to 9.4 % in children aged 6-7 years and from 9 % to 13 % in children aged from 13-14 years (Yadav *et al.*, 2014). According to the latest WHO data published in 2017, asthma death in Malaysia was reported to be 1258 or 0.91% of total death (World Health Rankings, 2017) and the prevalence of asthma among adults is approximately 6.3 % (Chan *et al.*, 2015).

1.1.3 Aetiology of asthma

The aetiology of asthma may be due to genetic, environment or interaction between the both factors and it caused burden to the patients and families in term of economic, causing loss of productivity and premature death (Barnes *et al.*, 2015). Frequent asthma exacerbation is a risk factor associated with progression to severe disease and excessive decline in lung function (Bai *et al.*, 2007). Severe exacerbation is recognised as an indication for evaluating the required level of interventional asthma management to reduce the potential risk of death (Ichinose *et al.*, 2017; Price *et al.*, 2014). In a conceptual model constructed using data from inner-city children, asthma severity was related to a defined allergy pathway that linked allergen sensitisation, allergy inflammation, pulmonary physiology and rhinitis (Liu *et al.*, 2016a). Asthma triggers, occurrences or elements that lead to initiation or worsening of asthma symptoms, have also been associated with severe asthma exacerbations in children (Sala *et al.*, 2011).

Stimulant such as allergens (cockroaches, mold, house dust mites in bedding, carpets and pet dander), irritants and viruses initiate asthma by interrupting the airway epithelial barrier integrity (Heijink *et al.*, 2010; Norimoto *et al.*, 2014; Post *et al.*, 2012; Post *et al.*, 2013). Reducing the integrity of epithelial barrier allows the allergens to reach the submucosa and enable more luminal allergens to be uptake by intraepithelial dendritic cells, and activated the Th2 response, thus will triggered asthma (Georas and Rezaee, 2014; Xiao *et al.*, 2013). Moreover, respiratory infections, usually caused by viruses, are the commonest cause of acute episodes of wheeze or asthma in children of all ages, accounting for up to 90 % of attacks (Dondi *et al.*, 2017; Jartti and Gern, 2017). There is an increased mortality and morbidity

amongst a significant number of older asthmatics due to poorly controlled asthma (Melani, 2013). In the elderly, asthma is superimposed on changes related to ageing, immune function and other diseases common in older age (Hanania *et al.*, 2011). In elderly patients, there are at least two phenotypes, those with long-standing asthma and those with late onset asthma (Hanania *et al.*, 2011).

Factors that further provoke the allergic reactions or irritate the airways are also from the environmental factors. Tobacco smoke exposure correlates with more severe asthma and with disease persistence. Exposure to passive smoke is also common in children with asthma (Kit *et al.*, 2016). Environmental tobacco smoke (ETS) is associated with a higher incidence of upper respiratory tract infections, persistence of asthma, and greater severity of asthma exacerbations (Comhair *et al.*, 2011). Data from many studies suggest that active smoking by adults with asthma leads to steroid resistance and exposure to passive smoking probably has similar effects (Kobayashi *et al.*, 2014). Besides that, smoking during and after pregnancy and delivery is also highly associated with a greater risk of asthma (Bousquet *et al.*, 2007).

In addition to this, psychological stress is also believed to worsen the symptoms, where it alters immune system thus increases the airway inflammatory response to allergens and irritants (Gold and Wright, 2005). Imbalance emotions were reported to increase the respiratory resistance, airway reactivity, shortness of breathing and reduced the peak of expiratory flow rate. Children and adolescents exposed to severe and chronic stressors such as those living in low-income urban communities are at a disproportionately high risk for severe asthma (Chen *et al.*,

2016). Studies have found that children with persistent and severe asthma have higher rates of anxiety and depression when compared with children with mild or severe asthma (Kohlboeck *et al.*, 2013). Besides that, in particular, children with severe asthma are also at increased risk for behavioral problems such as attention problems (Blackman and Gurka, 2007). This comorbidity is important because the management of severe asthma requires careful attention to symptoms and the medical care plan (Booster *et al.*, 2016). Hence, parents of children with severe asthma have been shown to exhibit significantly higher rates of depression, anxiety, and posttraumatic stress disorder. Caregivers' psychological functioning may impact their ability to manage their children with asthma (Booster *et al.*, 2016).

1.1.4 Types of asthma

Generally, asthma can be divided into few types; allergic (extrinsic), non-allergic (intrinsic), nocturnal, exercise-induced, steroid-resistant and occupational asthma (Paliwal, 2012). Allergic asthma is considered as the most common type of asthma, which usually induced by sensitisation to environmental allergens such as HDM, grass weed, fungal spore and animal dander (D'Amato *et al.*, 2015). A recent study indicated that different types of aeroallergens (airborne substance) such as pollen or spores and specific sensitisation profiles are related to different clinical manifestations of allergic respiratory disease (rhinitis with/without asthma), different clinical symptoms and different level of severity (Valero *et al.*, 2017). Initiation of immune response often begins with the activation and differentiation of specific Th2 cells that triggered by allergen and IgE is produced (Del Giacco *et al.*, 2017). While, non-allergic asthma (intrinsic) has a range of triggers including weather conditions, exercise, infection and stress (Paliwal, 2012).

According to Yawn (2008), in nocturnal asthma, patients experience asthma when they are sleeping, while direct exposure to wood dust, chemical fumes or other irritants leads to occupational asthma. Meanwhile, the steroid-resistant asthma is caused by steroid overdose that leads to a severe asthmatic attack that unresponsive to medication and requires ventilation (Paliwal, 2012). Whereas, occupational asthma accounts for an important percentage of work-related respiratory illnesses (Malo *et al.*, 2004). It has been reported that at least 9–15% cases of asthma in adults are due to occupational exposures (Boule *et al*., 2007). Isocyanates are widely used in various industrial and consumer products, and they are a major cause of chemical-induced occupational asthma throughout the world (Bello *et al.*, 2007). According to a review by the Global Alliance against Chronic Respiratory Disease, severe asthma is classified into three categories; (1) untreated severe asthma, (2) difficult-to-treat severe asthma and (3) treatment-resistant severe asthma (Bousquet *et al.*, 2010).

1.1.5 Symptoms of asthma

Asthma is the most common chronic respiratory disease affecting people from childhood through to adulthood (Fuchs *et al.*, 2017). It is a characterised by variable expiratory airflow limitation, classically presenting with episodes of wheeze, shortness of breath, chest tightness and/or cough (Reddel *et al.*, 2015) and cough is the prominent symptom in the elderly asthmatics (Jones *et al.*, 2011). People with asthma have an immune system that permanently tends to overact. This tendency goes mostly unnoticed until the mucous membranes that line the insides of the bronchi come into contact with specific triggers. The immune system cells in the membranes lining the bronchi are activated, the muscles surrounding the airways tense up, the membranes lining the airways become inflamed and swollen, and very viscous mucus is often produced. The muscles tensing up, the swelling of the mucous

membranes and the extra mucus production cause the airways to become narrower which can result in an asthma attack (Turner *et al.*, 2012).

Asthma symptoms are tending to be worsening at night, which is concordant with the cycle of endogenous cortisol levels. Asthma episodes are resulting from the airway narrowing that occurred through 3 main mechanisms; swelling, secretions and muscle constriction of the bronchi and it ranges broadly in severity and could resolve spontaneously or with minimal treatment, whereas others can lead to emergency room visits, hospitalization or death (Dougherty and Fahy, 2009).

1.1.6 Pathophysiology of asthma

Exposure to aeroallergens such as dust, smoke, animal dander and house dust mite (HDM) has been related with the induction of asthma cardinal features such as airway inflammation, airway obstruction and AHR (Hammad *et al.*, 2009; Holgate, 2008b). Asthma either allergic or non-allergic endotype depends on the cellular and molecular characterisation of the inflammatory cascade. Allergic asthma is usually eosinophilic, while non-allergic asthma may present with neutrophilic or paucigranulocytic phenotype and most of the non-allergic asthma is severe and steroid-resistant (Lötvall *et al.*, 2011). Fundamental studies *in-vitro* and *in-vivo* have uncovered several molecular and cellular events that implicated in the development of asthma. These are including increasing production of immunoglobulin E (IgE) by B cells, imbalance paradigm of Th1/Th2, upregulation of Th2 cytokines, airway cellular infiltration, damage of mitochondria, increase the production of reactive oxygen species (ROS) and reactive nitrite species (RNS) (Aguilera-Aguirre *et al.*, 2009).

1.1.6 (a) Eosinophilic asthma (Mild-to-moderate asthma)

Th2-mediated eosinophilic airway inflammation is associated with varying degree of asthma severity, ranging from mild-to-moderate to severe uncontrolled disease (Pelaia *et al.*, 2015). Analysis of sputum or bronchoscopy sampling suggested that eosinophilic airway inflammation is believed to play essential roles in the pathogenesis of asthma via the secretion of various inflammatory mediators together with cytotoxic eosinophils-derived granule proteins and lipid mediators in the airway (Amin *et al.*, 2016). These mediators promote persistent inflammation that ultimately leading to the asthmatic exacerbations and declined lung functions which is generally observed in biopsies of asthmatic patient's lung (Acharya and Ackerman, 2014; Trautmann *et al.*, 2002). Increasing evidences established that eosinophils essentially contribute to airway remodelling in asthma (Nakagome and Nagata, 2011).

Exposure of the airway to aeroallergens elicit a series of biological events involving interaction of the allergens with toll-like receptor (TLR), release of epithelial IL-25, IL-33, thymic stromal lymphopoietin (TSLP) which enhance the activation and development of dendritic cell and Th2 cells (Wilson *et al.*, 2012). Activated dendritic cells recognise and present fragments of allergen peptides via major histocompatibility complex (MHC) II to naïve T lymphocytes (Th0) (Kallinich *et al.*, 2007). The distinction of Th0 into Th1 or Th2 are depends on the cytokines milieu. Th2 cells literally induced the expression of GATA-3, an important transcription factor that enriches the development of Th2 production especially IL-4, IL-5, IL-9 and IL-13 (Kaiko *et al.*, 2008). Additionally, IL-4 and IL-13 stimulate the production of IgE from B lymphocytes, that influence the secretion of eosinophil. The Th2 secreting cytokines (IL-4, IL-5 and IL-13), granulocyte macrophage colony-

stimulating factor (GM-CSF) and potent chemoattractant such as eotaxin (CCL11), eotaxin 2 (CCL24) and chemokine ligand 5/RANTES (CCL5) (Wenzel, 2013). Meanwhile, IL-9, primarily secreted by Th9 (a subset of Th2 cells) stimulates the infiltration, maturation and degranulation of mast cell airway, whereas IL-5 enhances the induction of pulmonary eosinophil homing (Fulkerson and Rothenberg, 2013b). IL-4 functions by enhancing migration of eosinophil by activating airway vascular cell adhesion molecule 1 (VCAM-1).

The pulmonary eosinophils secrete wide variety of inflammatory mediators, such as cysteinyl leukotrienes (LTC4, LTD4, LTE4) and cytotoxic agent like eosinophil derived neurotoxin, eosinophil peroxidases, eosinophil cationic protein and major basic protein (MBP), that caused airway inflammation and oxidative stress damage that can be seen in asthma (Hall and Agrawal, 2014; Pelaia *et al.*, 2015). Nonetheless, both allergic and non-allergic asthma shows an increase in the level of Th2 cytokines, thus designated the crucial role of IL-4, IL-5 and IL-13 in the pathogenesis of asthma (Brusselle *et al.*, 2013; Lambrecht and Hammad, 2015; Pelaia *et al.*, 2015).

1.1.6 (b) Neutrophilic asthma (Moderate-to-severe asthma)

Apart from airway eosinophilia, asthma may be presented with neutrophilic phenotypes, which is extensively mediated by Th17, a subset of Th cells that secrete IL-17 (Simpson *et al.*, 2014; Wood *et al.*, 2012). This category of asthma is often expressed in severe/uncontrolled asthmatics, steroid insensitive and it may be triggered by allergens or non-allergenic triggers such as microbes, cigarette smoke, diesel exhaust particles and other environmental pollutants (Newcomb and Peebles

Jr, 2013; Polosa and Thomson, 2013; Vroman *et al.*, 2015). Differentiation of thymocytes into Th17 cells depends on related orphan receptor gamma (RORɣt), a transcription factor that requires IL-1β, IL-6 and transforming growth factor (TGF-β) milieu for its upregulation and consequent Th17 differentiation (Vroman *et al.*, 2015). The heterogeneous nature of asthma pathogenesis has rendered a blanket approach to treatment and diagnosis of different asthma endotypes ineffective.

Wenzel and co-workers reported that there is a high neutrophils number in the airway lavage of patients with severe asthma compared to patients with mild and moderate asthma (Wenzel *et al.*, 1997). It is thought to be involved in the progression of more severe irreversible airway obstruction leading to sudden death (Shaw *et al.*, 2007). This statement was supported by the evidence of an excess neutrophils in airway secretions and sub-mucous glands in biopsy sample from patients with fatal asthma (Carroll *et al.*, 2002). There were also evidences stated that mast cells have been linked to the pathophysiology of asthma and often associated with more severe asthmatic phenotypes (Andersson *et al.*, 2016; Galli and Tsai, 2012). Abundant of mast cells are found to be localised within the bronchial smooth muscle bundles in severe asthma and trigger airway remodelling by secreting inflammatory mediators likes amphiregulin (Andersson *et al.*, 2016; Lei *et al.*, 2013). Therefore, if left untreated, asthma can lead to the airway irreversibility such as airway wall remodelling that correlate with the disease severity. Therefore, a proper understanding of asthma pathogenesis is essential for successful treatment discovery and repositioning. Figure 1.1 describes the cellular pathways involved in the pathogenesis of asthma endotypes.

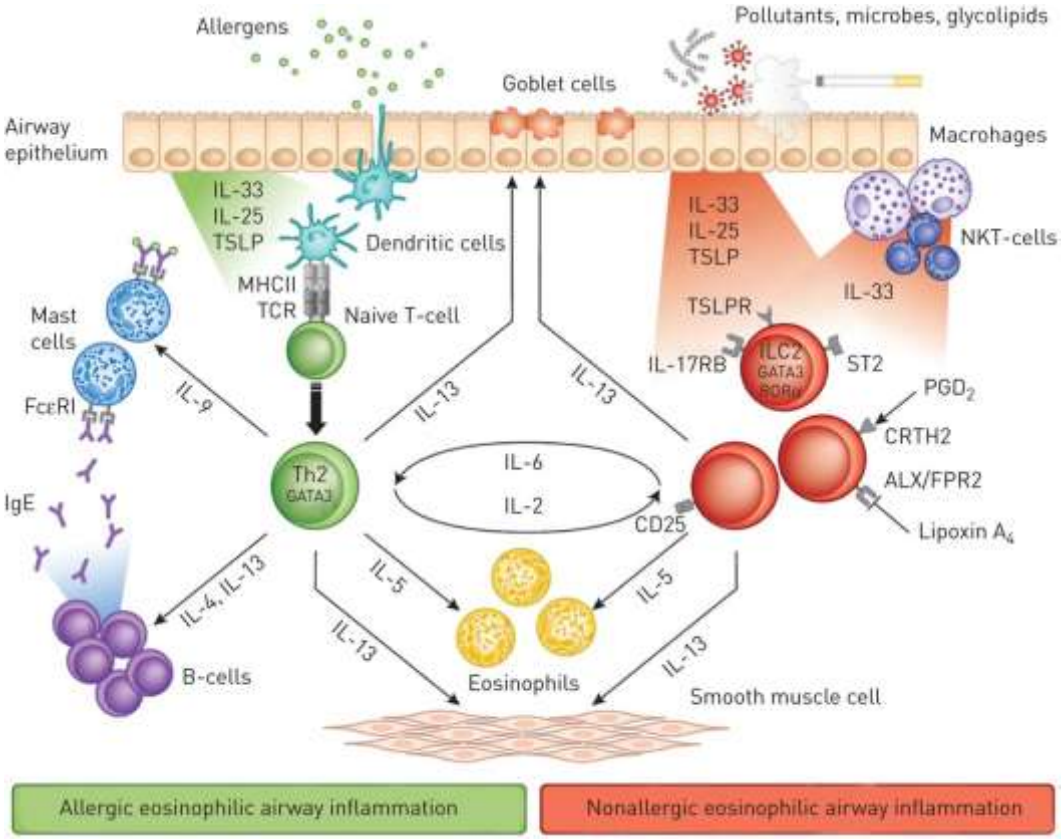

Figure 1.1 Two different pathways lead to eosinophilic airway inflammation in asthma. In allergic asthma, dendritic cells present allergens to $CD4^+$ T-cells, inducing T-helper (Th)2 cells, which produce interleukin (IL)-4, IL-5 and IL-13, and leading to IgE switching in B-cells, airway eosinophilia and mucous hypersecretion. In nonallergic eosinophilic asthma, air pollutants, microbes and glycolipids induce the release of epithelium-derived cytokines, including IL-33, IL-25 and thymic stromal lymphopoietin (TSLP), which activate innate lymphoid cells (ILCs) in an antigen-independent manner via their respective receptors (IL-17 receptor B (IL-17RB), ST2 and TSLP receptor (TSLPR)). Activated ILC2s produce high amounts of IL-5 and IL-13, leading to eosinophilia, mucus hypersecretion and airway hyperreactivity. CRTH2: chemoattractant receptor homologous molecule expressed on Th2 cells; ALX/FPR2: receptor for lipoxin A_4; FcεRI: high-affinity receptor for IgE; GATA3: GATA-binding protein 3; PG: prostaglandin; ROR: retinoic acid receptor-related orphan receptor; NK: natural killer; MHC: major histocompatibility complex; TCR: T-cell receptor. Adapted from Brusselle *et al.*, (2014).

1.1.7 Airway hyper-responsiveness (AHR)

Clinically, asthma presents as a physiological dysfunction of the lung characterised by breathlessness, wheeze and a variable airflow obstruction (Bousquet *et al.*, 2000). This accounts for the dramatic increase in responsiveness of the conducting airways known as airway hyper-responsiveness (AHR). AHR has long been considered a cardinal feature of asthma and its measurement has provided profound insights into the underlying pathophysiology of the disease and considered to be the most important symptom in asthma (Kudo *et al.*, 2013). AHR increased contractility of the airways in response to a variety of stimuli. Direct stimuli (methacoline, histamine, leukotrienes and prostaglandins) act on respective receptors on bronchial smooth muscle cells, which in turn depolarise and contract, thereby constricting the bronchi (O'Byrne, 2010). In contrast, indirect stimuli act on intermediate pathways, such as via mediator release from inflammatory cells and the release of neuropeptides from sensory nerves (Joos and O'connor, 2003).

In general, AHR in allergic asthma patients is most likely caused by chronic inflammation and airway remodelling. A correlation has been observed between the magnitude of AHR and the level of airway inflammation (Meurs *et al.*, 2008). Eosinophils may play a role in AHR with numerous studies demonstrating a direct association between AHR and Th2 cell-driven eosinophilic airway inflammation. AHR had also been shown to be induced in a mouse model of allergic airway disease following blockade of transforming growth factor (TGF)-β1 with a neutralising antibody (Alcorn *et al.*, 2007). This suggests a role for TGF-β1 in suppressing AHR development. AHR is a fundamental abnormality in asthma however at present there

is no clear or consistent association between immunological and structural features of asthma and the increased responsiveness of the airways.

Airway remodelling may play a role in inducing or sustaining AHR. Structural changes that occur as a result of airway remodelling have been found to contribute to AHR, including a reduction of airway diameter, increased contractility of smooth muscle, and epithelial injury (Holgate, 2008a). However, there is evidence that airway remodelling could have a protective effect against AHR (Bates and Maksym, 2011; Grainge *et al.*, 2011). This may be due to increased collagen deposition around the airways causing airway wall thickening and increased airway stiffness which would limit excessive airway narrowing (Meurs *et al.*, 2008). Human studies have shown that asthmatic patients with airway wall thickening have reduced airway reactivity to methacholine (Broide, 2008). Contradictory to this, airway biopsies from asthma patients suggest the degree of smooth muscle thickness and the extent of sub-epithelial fibrosis is related to disease severity and magnitude of AHR (Southam *et al.*, 2007). Although the exact relationship between airway remodelling and AHR is still unclear, it is most likely that airway remodelling changes play some part in sustaining persistent AHR.

1.1.8 Airway remodelling

Airway remodelling can be well-defined as a set of changes in the composition, content and organisation of the cellular and molecular constituents of the airway wall (Bergeron and Boulet, 2006). This could constitute a normal development or repair process during the lung development, aging or as a transient response to airway/lung injury, with restoration of normal structures. However, it can also be a pathological process in response to chronic injury/inflammation, resulting in persistently altered airway structure and function. In severe asthma, the term "airway remodelling" denotes the structural alteration that occurs as a consequence of, chronic inflammation, contributing to airway obstruction.

Airway remodelling refers to a compilation of structural modification characterised by epithelial damage and hyperplasia, deposition of extracellular matrix (ECM) factors below the basement membrane (making the airways less compliant), proliferation of airway smooth muscle, angiogenesis and excess mucus production contribute to the thickening of the airway wall which are associated with an irreversible loss in lung function that tracks from childhood to adulthood (Figure 1.2) (Evans *et al.*, 2009; Halwani *et al.*, 2011; Lambrecht and Hammad, 2012; Martin and Verma, 2012). The structural airway changes are apparent in asthma even in mild disease (Wilson *et al.*, 2013), however, the degree of remodeling often worsens with increasing disease severity (Bonsignore *et al.*, 2015). In addition, the onset of the pathological changes occurs early, during the pre-school years (Saglani *et al.*, 2007). Jeffery (2001b) described that the asthmatic airway walls are generally thickened between 50 and 300% relative to the normal airway walls due to airway remodelling that is attributed to alteration of airway epithelium, basement membrane

and sub-mucosa. This phenomenon leads to luminal narrowing. Furthermore, the epithelium layer of the nasal and lower airways in the asthmatic individuals appears to be more fragile and easily detach from the basement membrane. The consequence of these structural changes may cause partially reversible airway narrowing in mild asthma but mostly irreversible in chronic severe asthma airway together with airway hyperresponsiveness (AHR), mucus hypersecretion and airway oedema leading to a progressive decline in pulmonary function. There is growing support for a role of aberrant airway epithelial repair processes in response to persistent inflammatory response implicated in the development of pathological airways remodeling (Bai, 2010; Ganesan and Sajjan, 2013). Despite evidence that inflammation is involved in airway remodelling, these two processes seem to evolve in parallel, although they do not always correlate well. Changes in structural cells could also influence inflammation and other components of remodelling (Fanat *et al.*, 2009; Larose *et al.*, 2015).

1.1.8 (a) Epithelial layer damage

It is known that chronic inflammation has functional effects that lead to systemic changes in asthma that recognised as the central component that leads to remodeling. The structural changes in cellular phenotypes at the molecular level is primarily initiated by alterations in the airway epithelium, lamina propria and submucosa leading to the thickening of all the components of the airway wall (inner, outer, and total) (Bousquet *et al.*, 2000).

Furthermore, continuous injury to the epithelium lead to its fragility in acute asthma with extensive denudation, disruption, shedding and increased turnover of epithelial cells in severe/chronic asthma (Fixman *et al.*, 2007). Epithelial cell hypertrophy contributes to regular differentiation of these cells into goblet cells that secrete mucus, resulting in goblet cell hyperplasia and mucous gland hyperplasia. Based on Holgate (2008b), shedding of epithelium, goblet cell hyperplasia, reduction of cell layer and upregulation of growth factors are the types of epithelial layer alteration in asthmatic airways wall. Epithelial changes are not only specific to asthma attack; they also can be observed in other airway diseases such as chronic obstructive pulmonary disease (COPD). Epithelial cells revealed a rapid repair mechanism and initiate the signal cascades central to asthma in response to stimuli.

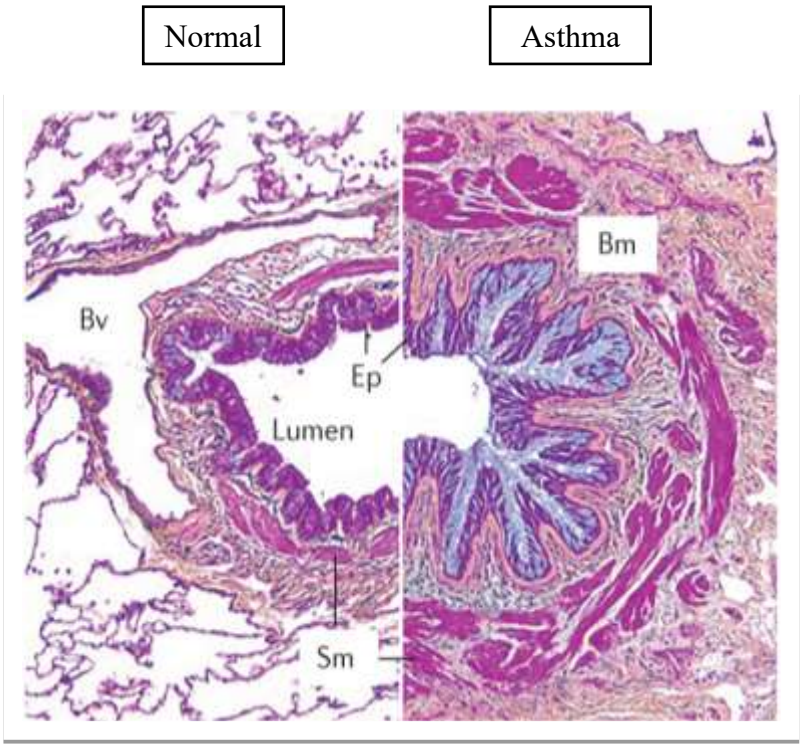

Figure 1.2 The airways in asthma undergo substantial structural remodelling. Histological section of a medium-sized airway from a person without asthma and a patient with severe asthma stained with Movat's pentachrome stain. In asthma, the epithelium (Ep) shows mucus hyperplasia and hypersecretion (blue) and thickening of the basement membrane (Bm). Smooth muscle (Sm) volume is also increased in asthma. Bv=blood vessel. Reproduced, under an open access agreement, from Wadsworth and colleagues. IL-13, asthma, and glycosylation in airway epithelial repair. In: Chang C, ed. Carbohydrates—comprehensive studies on glycobiology and glycotechnology. Wadsworth *et al.* (2012).

1.1.8 (b) Sub epithelial layer thickening

Sub epithelial layer thickening happened due to high deposition of extracellular matrix proteins (ECMs) and considered as one of the features in airway remodelling of asthma (Manuyakorn *et al.*, 2013). This type of thickening is confined to the lamina reticularis (reticular basement membrane-RBM). Immunohistochemistry test revealed that the thickened reticular layer is mostly consisted of collagen I, III, IV and fibronectin (Roche *et al.,* 1989). Apart from that, production of ECMs in the airways are related to the fibroblasts and myofibroblasts, while the inflammatory cells such as eosinophil, mast cells and T cells accumulate in the submucosal layer, where the fibroblasts/myofibroblasts situated. The connection between the structural cells (eg: fibroblasts and epithelial cells), inflammatory cells and acquisition rate of ECMs determines the remodelling and fibrosis of the airways (Manuyakorn *et al.*, 2013). In response to damage happened, the epithelial cells release growth factors such as TGF-β that directly affect the synthesis of ECMs by myofibroblast/ fibroblast (Hostettler *et al.*, 2008).

1.1.8 (c) Airway smooth muscle hyperplasia and hypertrophy

The increased thickness of airway smooth muscle (ASM) layer is one of the main factors contributing to remodelling and hyperreactivity in asthma. Indeed, the amount of ASM mass appears to be directly correlated to both duration and severity of asthma, as well as to AHR to methacholine (Pepe *et al.,* 2005). In patients with asthma, and those subjects suffering from more severe disease, the oversized ASM bundle arises from an enlargement of ASM cell volume (cellular hypertrophy) and especially from ASM cell proliferation (cellular hyperplasia) (Black *et al.*, 2012;

James *et al.*, 2012). The increase of ASM mass, which likely occurs through altered myocyte growth and survival, may be a significant factor inducing dyspnoea in asthma due to its impact on airway narrowing (Al-Muhsen *et al.*, 2011; Prakash, 2013). It is plausible that decreasing excessive ASM would alleviate some of the symptoms exhibited by asthmatic patients.

Increased airways smooth muscle mass has been recognised as the feature of asthma for decades. It is mediated by releasing of cysteinyl leukotriene from eosinophils. Smooth muscle play roles in bronchoconstriction, that triggered by direct and indirect stimuli which contribute symptoms, exacerbations and the remodelling process (Grainge *et al.*, 2011). The increase in smooth muscle mass is associated with increases in growth factors including TGF-β1 and platelet-derived growth factor (Cohen *et al.*, 2000). The muscle itself may also act as a secretory organ in asthma, promoting maladaptive growth and immunologic responses. A recent review of these properties highlighted IL-5, IL-13, TGF-β1, IL-1β and tumour necrosis factor as important mediators in this process (Doeing and Solway, 2013). In fatal asthma, smooth muscle layer in the airways increased more than 50%, compared to normal airways (James, 2005).

1.1.8 (d) Angiogenesis

Angiogenesis happened in airway remodelling where it developed new blood vessels below the basal lamina in the space between the muscle layer and the surrounding parenchyma (Al-Muhsen *et al.*, 2011). This results in increased vascular areas in the airways, changed the blood flow and microvascular permeability and helped in oedema formation (Wiparat *et al.*, 2013). The processes happened will likely

contribute to the thickness of the airway walls even though it is quite difficult to quantify *in vivo*. Among the angiogenic agents responsible for the increased numbers of arterioles and capillaries detectable in asthmatic bronchi, vascular endothelial growth factor (VEGF) plays the most relevant pathogenic role (Hoshino *et al.*, 2001). VEGF is overexpressed in the airways of patients with asthma, and this potent vascular growth factor is produced not only by endothelial cells but also by many inflammatory cells such as eosinophils, macrophages and mast cells (Tseliou *et al.*, 2012).

1.1.8 (e) Goblet cell hyperplasia

A hallmark of airway remodelling in asthma is represented by hyperplasia/metaplasia of mucus producing goblet cells (Fahy, 2002). These structural changes extent throughout all levels of asthma severity, but they are more prominent in severe asthma (Jenkins *et al.*, 2003; Ordoñez *et al.*, 2001). Hyperplastic goblet cells are largely responsible for mucus hypersecretion, which significantly contributes to airway narrowing in asthma. In particular, in asthmatic airways, mucin 5AC (MUC5AC) is the most abundant mucin glycoprotein produced by goblet cells (Rubin *et al.*, 2014). Th2 cytokines, especially IL-13, are powerful inducers of goblet cell hyperplasia and mucus production (Fahy and Dickey, 2010). In this regard, it is noteworthy that airway goblet cell hyperplasia elicited by IL-13 is steroid resistant (Kanoh *et al.*, 2011). IL-13-dependent goblet cell proliferation and mucus hypersecretion are mediated via overexpression of TGF-β2. Indeed, TGF-β2 levels correlate with mucin expression in asthmatic airways (Makinde *et al.*, 2007). In addition to this, other cellular pathways are implicated in mucin production. In fact, mucus secretion is up-regulated by stimulation of the epidermal growth factor (EGF)

receptor, which can be activated by EGF, TGF-α, amphiregulin, epiregulin and β cellulin (Rubin *et al.*, 2014). Mucus hypersecretion could lead to airway obstruction, contributing to the morbidity and mortality of asthma (Curran and Cohn, 2010; Lai and Rogers, 2010).

1.1.9 Asthma medications

Allergy asthma is a chronic inflammatory airway disease and current treatment is mainly used to control the level of allergic inflammation and asthma symptoms. In asthma, adherence to treatment can be quite poor, ranging from <50% in children and 30–70% in adults. This is not only related to unresolved symptoms and affected quality of life (QoL) but also exacerbation frequency and increase mortality (Engelkes *et al.*, 2015). Asthma management aims at reducing symptoms and exacerbations, improving lung function and QoL besides lowering the long term side effects of therapies (Reddel *et al.*, 2015). To achieve this goal, the controller (anti-inflammatory agents) and reliever (symptom reliever drugs) are used. Controllers are taken daily for a long term to keep asthma under clinical control, while relievers are used on as-needed basis that act quickly to reverse the bronchoconstriction and symptoms relievers. For asthma treatment, the medications fall into two large categories; bronchodilators and anti-inflammatory agents. In spite of current available therapies, some of the asthma patients remain ineffectively controlled (Lang *et al.*, 2013) and these drugs have presented a number of side effects and possible resistance with long term use (Barnes, 2010; Louis *et al.*, 2012).

1.1.9 (a) Bronchodilators

1.1.9 (a)(i) Methylxanthines (theophylline).

Bronchodilators are important in the management of asthma as they play an essential role in reversing airway obstruction and provide "bronchoprotection" against bronchospasm due to exercise and other spasmogenic stimuli (GINA, 2018). Since the 1920s, methylxanthines have been used to treat asthma and pulmonary disease but the demand decline after the introduction of β2-agonists (Barnes, 2013). Methylxanthine is a bronchodilator agent that might also improve respiratory muscle function through increases in mucociliary clearance and actions to stimulate respiration (Neame *et al.*, 2015). It could also show anti-inflammatory and immunomodulatory actions that are related with apoptosis of granulocytes (Neame *et al.*, 2015). These potential mechanisms had supported methylxanthine treatment for patients with acute asthma over decades. However, the current international guidelines no longer recommend methylxanthines for acute asthma treatment due to the narrow therapeutic concentration window that can result in adverse effects such as vomiting, arrhythmias, and seizure (Milan *et al.*, 2012).

1.1.9 (a)(ii) Long-acting inhaled β-Adrenergic agonists.

Since 3000 B.C ago, the *β*-adrenergic agonist is one of the oldest classes of treatment to treat breathing problems. The active ingredient such as epinephrine was found to have *α*- and *β*-receptor agonist effects. *β*2-agonists can be classified into short-acting *β*2-agonists (SABAs) and long-acting *β*2-agonists (LABA). Albuterol and terbutaline are examples of SABA which are hydrophilic and give effects up to 4 to 6 hours, while LABA (eg: formoterol, salmeterol, arformoterol, formoterol and indacaterol)