EFFECT OF ETANERCEPT AND GOLD NANOPARTICLES ON THE EXPRESSION OF TNFR2^{+} REGULATORY T CELLS AND CD103^{+} DENDRITIC CELLS IN PERIPHERAL BLOOD MONONUCLEAR CELLS OF ASTHMA PATIENTS

SUHANA BINTI AHMAD

UNIVERSITI SAINS MALAYSIA

2020

EFFECT OF ETANERCEPT AND GOLD NANOPARTICLES ON THE EXPRESSION OF TNFR2^{+} REGULATORY T CELLS AND CD103^{+} DENDRITIC CELLS IN PERIPHERAL BLOOD MONONUCLEAR CELLS OF ASTHMA PATIENTS

by

SUHANA BINTI AHMAD

Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

ACKNOWLEDGEMENT

In the name of Allah, the Most Generous and the Most Merciful

First and foremost, gratitude and appreciation are for Allah, the All Mighty God for His Compassion and Blessings for granting me health, strength, and motivation for the completion of my PhD. Thanks to Him, my three years of PhD is surrounded with these people who keep motivated, inspired and encouraged me for my goal. **Dr. Rohimah Mohamud**, my main supervisor. I'm highly grateful for her guidance and continuous supports. Her one-of-a-kind optimism, although sometimes scared me, kept me aim higher and moving forward in my study. I would like to thank **Prof. Lim Jit Kang** and **Dr. Ramlah Kadir**, my co-supervisors for always available whenever I need their assistance and guidance. I would like to give sincere thanks to the staff of Department of Immunology especially **Mr. Jamaruddin** and **Mr. Azlan** for their assistance in the acquisition on flow cytometer, and **Dr. Diyana, Mrs Maziah** and **Miss Fadzilah** and **nurses in Klinik Pakar Perubatan HUSM** for their help in sample collection. Not to forget, my friends, **Nor Azrini, Siti Khadijah, Farhana, Nur Ellene, Mastura, Husna, Farah Izati, Sarah,** and **Hidayah**, for always be there for my ramblings, overtime lab works, and keep motivating each other. Last but most importantly, I owned my family big time, especially my ***Umi*** and ***Abah*** for their relentless support and my **siblings**, who always willingly taking care of their only nephnew during this completion of PhD. And to my precious son, **Muhammad Imran**, thank you for your understanding even in your very own 'stimulating' way.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

AHR	Airway hyperresponsiveness
ALDH	Aldehyde dehydrogenase
APCs	Antigen presenting cells
AuNPs	Gold nanoparticles
cDC	Conventional DC
COPD	Chronic obstructive pulmonary disease
DCs	Dendritic cells
DLS	Dynamic light scaterring
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immusorbent assay
ETA	Etanercept
FBS	Foetal bovine serum
FMO	Fluorescence-minus-one
Foxp3	Forkhead box transcription factor 3
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horse radish peroxidase
ICS	Inhaled corticosteroid
IFN	Interferon
IgE	Immunoglobulin E
IL	Interleukin
KO	Knockout
LPS	Lipopolysaccharides

MDSC	Myeloid-derived suppressor cells
MLN	Mesenteric lymph node
OVA	Ovalbumin
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered solution
pDC	Plasmacytoid dendritic cells
PDI	Polydispersity index
PRR	Pattern recognition receptor
RCT	Randomized control trial
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SIT	Allergen-specific immunotherapy
Teffs	Effector T cell
TEM	Transmission electron microscopy
TGF	Transforming growth factor
Th	T helper
TLR	Toll-like receptor
TLSP	Thymic stromal lymphopoeitin
TMB	3,3′,5,5′-Tetramethylbenzidine
TNF	Tumor necrosis factor
TNFR1	Tumor necrosis factor receptor 1
TNFR2	Tumor necrosis factor receptor 2
Tregs	Regulatory T cell

KESAN ETANERCEPT DAN NANOPARTIKEL EMAS KE ATAS EKSPRESI SEL T PENGAWALSELIA TNFR2^{+} DAN SEL DENDRITIK CD103^{+} DI DALAM SEL MONONUKLEAR DARAH PERIFERAL PESAKIT ASMA

ABSTRAK

Toleransi imun oleh sel T pengawalselia (Tregs) adalah salah satu daripada pelbagai mekanisma yang digunakan untuk mengekalkan homeostasis dan melindungi perumah terhadap pelbagai rangsangan alam sekitar. Belakangan ini, sebahagian daripada Tregs yang mempunyai pengekspresian TNFR2 (TNFR2^{+} Tregs) dikenalpasti sebagai populasi sel yang lebih menekan dan proliferatif; manakala pengawalaturannya dilaporkan terganggu dalam masalah pernafasan seperti asma. Nanopartikel emas (AuNPs), yang sangat berpotensi dalam terapi imun, telah dilaporkan melindungi manusia daripada ciri-ciri utama asma. Oleh itu, kajian ini ingin merungkai kesan AuNPs terhadap pengekspresian TNFR2^{+} Tregs, dan juga pada CD103^{+} sel dendritik (DCs), yang telah ditunjukkan mendorong Tregs dalam pesakit asma. Kajian ini juga ingin mengkaji kesan AuNPs terhadap populasi sel kajian dengan TNF yang juga dilaporkan penting dalam perkembangan asma menggunakan etanercept, salah satu anti-TNF. Kaedah *flow cytometry* lima warna digunakan untuk menentukan pengekspresian populasi sel kajian dalam pesakit asma dan individu bukan asma (n = 6) dan ujian ELISA digunakan untuk menentukan tahap TNF-α dan IL-10. Stimulasi sel mononuklear darah periferal (PBMCs) pesakit asma dengan AuNPs selama 24 jam memperlihatkan AuNPs tidak memberi kesan signifikan kepada populasi sel kajian. Manakala, sebagai anti-TNF, etanercept dilihat

mengurangkan secara signifikan TNFR2^{+} Tregs (p = 0.0210) dan sel T efektor TNFR2^{+} (Teff) (p = < 0.0001) namun memperlihatkan kadar peningkatan untuk Tregs sahaja. Yang mengejutkan, etanercept ditunjukkan dengan ketara meningkatkan tahap TNF-α, mempersoalkan mekanisma utama etanercept sebagai antagonis TNF. Dalam analisis korelasi, populasi Tregs termasuklah TNFR2^{+} Tregs dikawal selia secara songsang oleh CD103^{+} DCs (r = - 0.6853, p = 0.0170), menunjukkan subset DCs ini tidak terlibat dalam regulasi homeostasis imun, terutamanya dalam keadaan asma. Secara keseluruhan, keputusan kajian menunjukkan AuNPs boleh digunakan sebagai pembawa formulasi berpotensi untuk terapi imun kerana kemampuannya untuk dikenalpasti oleh sel penyaji antigen seperti DCs tanpa mengakibatkan tindak balas imun.

EFFECT OF ETANERCEPT AND GOLD NANOPARTICLES ON THE EXPRESSION OF TNFR2$^+$ REGULATORY T CELLS AND CD103$^+$ DENDRITIC CELLS IN PERIPHERAL BLOOD MONONUCLEAR CELLS OF ASTHMA PATIENTS

ABSTRACT

Immune tolerance by regulatory T cells (Tregs) is one of the various mechanisms, which is employ to maintain homeostasis and protect the host against various environmental stimuli. Recently, a subset of Tregs expressing tumor necrosis factor receptor 2 (TNFR2$^+$ Tregs) is identified as a more suppressive and proliferative cell population and its regulation is reported to be impaired in respiratory dysfunction condition such as asthma. Gold nanoparticles (AuNPs), a highly potential tool in immunotherapies, are shown to be protective against key features of asthma. Hence, the current study would like to elucidate the effects of AuNPs on the expression of TNFR2$^+$ Tregs, as well as on CD103$^+$ dendritic cells (DCs), which have been shown to induce Tregs in asthmatic individuals and whether a TNFR2 agonist, etanercept, can modulate the effects of AuNPs. A five-color flow cytometry assay was used to determine the expression of cell population of interest in asthmatic and non-asthmatic controls (n=6) and traditional ELISA assay to determine the level of TNF-α and IL-10. Stimulation of peripheral blood mononuclear cells (PBMCs) of asthmatic individuals with AuNPs for 24 hours does not induce significant effects on the cell population of interest. Meanwhile, etanercept as anti-TNF is shown to significantly decrease TNFR2$^+$ Tregs (p = 0.0210) and T effector cells TNFR2$^+$ (Teff) (p = < 0.0001) with an increased pattern

of Foxp3^{+} Tregs only. Surprisingly, etanercept is shown to significantly increased the level of TNF-α, question the principal mechanism of etanercept as TNF antagonist. Correlation analysis in the current study demonstrated inverse association of CD103^{+} DCs with Tregs including TNFR2^{+} Tregs (r = - 0.6853, p = 0.0170), indicates to the independency of this subset of DCs in the regulation of immune homeostasis, particularly in asthma. Results indicated to the potential of AuNPs as potential formulation carrier for immunotherapies due to its capability to be effectively uptake by antigen-presenting cells such as DCs without inducing immune response.

CHAPTER 1

INTRODUCTION

1.1 Background of research

The lung is constantly exposed to a diverse stimuli including air pollution particulates and microbial products which resulted in an immune activation or suppression depending on the nature of stimuli (e.g. doses, characteristics), exposure route/time and the lung microenvironment (e.g. host immune system). Thus, the lung must maintain a state of immune tolerance to these stimuli to retain pulmonary homeostasis and prevent immunopathology such as asthma. Asthma is a chronic obstructive pulmonary disease that implicated nearly 300 million people worldwide. The immunobiology of asthma is when both the innate and the adaptive immune responses act together to cause the bronchial hyperresponsiveness, airway narrowing and remodelling as well as mucus overproduction. One of the mechanisms to maintain immune homoestasis in the lung is through the induction of tolerance. Regulatory T cells (Tregs) have been established as the key player in the maintenance of tolerance and in asthma the phenotype and function of this subset of cell is severely dysregulated. Tregs expressing TNFR2 ($CD25^{+}FoxP3^{+}TNFR2^{+}$ Tregs) have been shown to have a more potent immune regulatory function than the TNFR2-negative Tregs and have a higher capacity to expand *in vivo*. This subset of Tregs is strictly regulated by membrane-bound TNF, which is preferentially expressed on Tregs, lead to increase proliferation. Other than Tregs, $CD103^{+}$ dendritic cells (DCs), which constituted as the major DCs in the lung, is shown to promote tolerance although

some contrasting observations are available as well. Hence, induction of these two cells population to promote tolerance serves as a new potential in asthma therapy. Current study aims to investigate the phenotype of TNFR2 expressing Tregs and $CD103^+$ DCs in asthma patients and their responses in the presence of external stimuli, for instance nanoparticles. Nanoparticles such as gold is demonstrated to possess desirable characteristics including versatile surface functionalization, customizable size and shape and low cytotoxicity, select them as potential tool to target cell population of interest.

1.2 Statement of problem

Pathogenesis of asthma is associated with the dysregulation of immune homeostasis in the airways in terms of augmentation of inflammation and reduction of tolerance. Although the intervention of asthma managed to control the symptoms, however it does not modify the course of the disease and continues to impose great burden in terms of productivity and medical costs and even morbidity. Immune-modifying therapies such as allergen-specific immunotherapy (SIT) have been proposed as a potential in asthma therapy and the success of nanoparticles such as AuNPs, as a tool in immunotherapy, excites the idea. AuNPs have been well established to possess advantageous properties to be a highly potential tool in immunotherapy. This study aims to investigate the effect of AuNPs on the regulation of TNFR2 expressing Tregs and $CD103^+$ DCs and whether the presence of TNF antagonist such as etanercept would modulate the effect of AuNPs. It has been shown that TNF is responsible to the inflammatory cascades in asthma through the increased of TNF levels in asthma patients. This study will not only contribute significantly to the understanding of these

two cell populations that might play an important role at the homeostatic level and during airway inflammation in healthy and asthmatic individuals respectively, but also to the modulation of these cells upon exposure to nanoparticles. This might include the chances of targeting $Foxp3^+$ Tregs expressing TNFR2 using nanoparticles to be translated as a biological therapy to maintain and restore immune homeostasis, as now it is being highly investigated in human to selectively expand Tregs into homogenous progeny with potent suppressive function.

1.3 Hypotheses

This study hypothesized that the expression of TNFR2 on Tregs in PBMC of asthmatic and non-asthmatic controls would distinctly be affected by both stimuli, AuNPs and etanercept, regulated by DCs including $CD103^+$ DCs (Figure 1.1). AuNPs will be uptake by the APCs such as DCs and induce the release of mediators that would affect the expression of cell population of interest. Meanwhile, etanercept would neutralize TNF in the system, thus provides an insight on the mechanism of AuNPs on the cell population of interest.

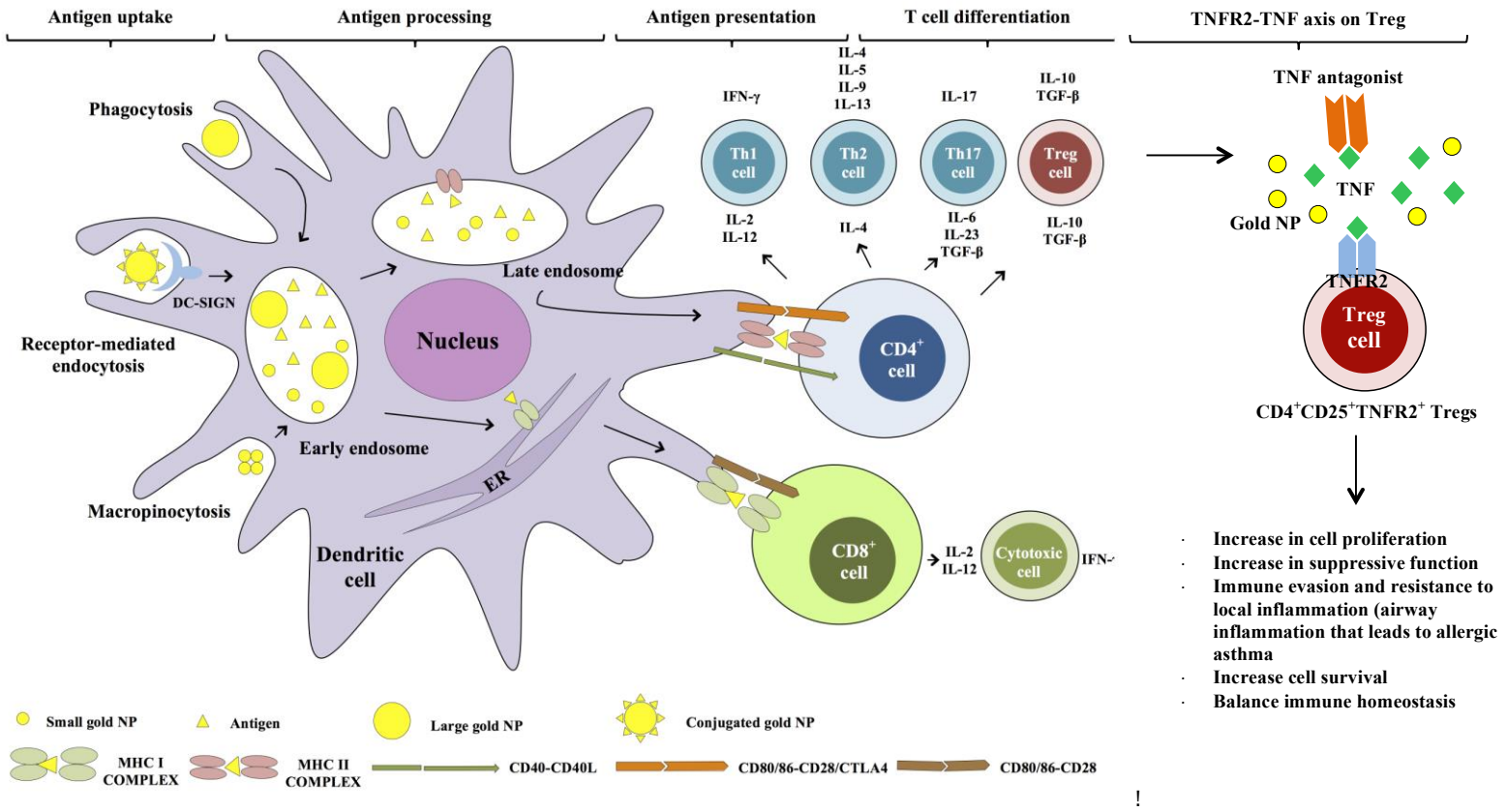


Figure 1.1 Framework of the study. TNF produced by immune cells (dendritic cells, effector T cells) in asthma condition bind to TNFR2 that expressed on Treg and effector T cells in an autocrine and paracrine manner. AuNPs affect the cellular uptake and phenotype of DCs such as $CD103^+$ DCs and both $CD4^+CD25^+TNFR2^+$ Treg and $TNFR2^+$ effector cells, and the addition of TNF antagonist (e.g. etanercept) differently regulates the effects of AuNPs.

1.4 Research questions

The aforementioned hypothesis on the expression of TNFR2 on Tregs and $CD103^+$ DCs on PBMC of asthmatic and non-asthmatic individuals of this study are motivated for answering the following research questions:

a. Do external stimuli such as AuNPs play a role in the secretion of TNF in PBMC of asthmatic and to what extent this would affect cell population of interest?
b. Do neutralization of TNF by anti-TNF such as etanercept would differently regulate the effect of AuNPs to PBMC of asthmatic?
c. What are the associations of these effects with clinical characteristics?

1.5 Objective

1.5.1 General objective

To determine the effects of AuNPs and etanercept on the expression of TNFR2 expressing Tregs and $CD103^+$ DCs in PBMC of asthmatics and non-asthmatic controls

1.5.2 Specific objectives

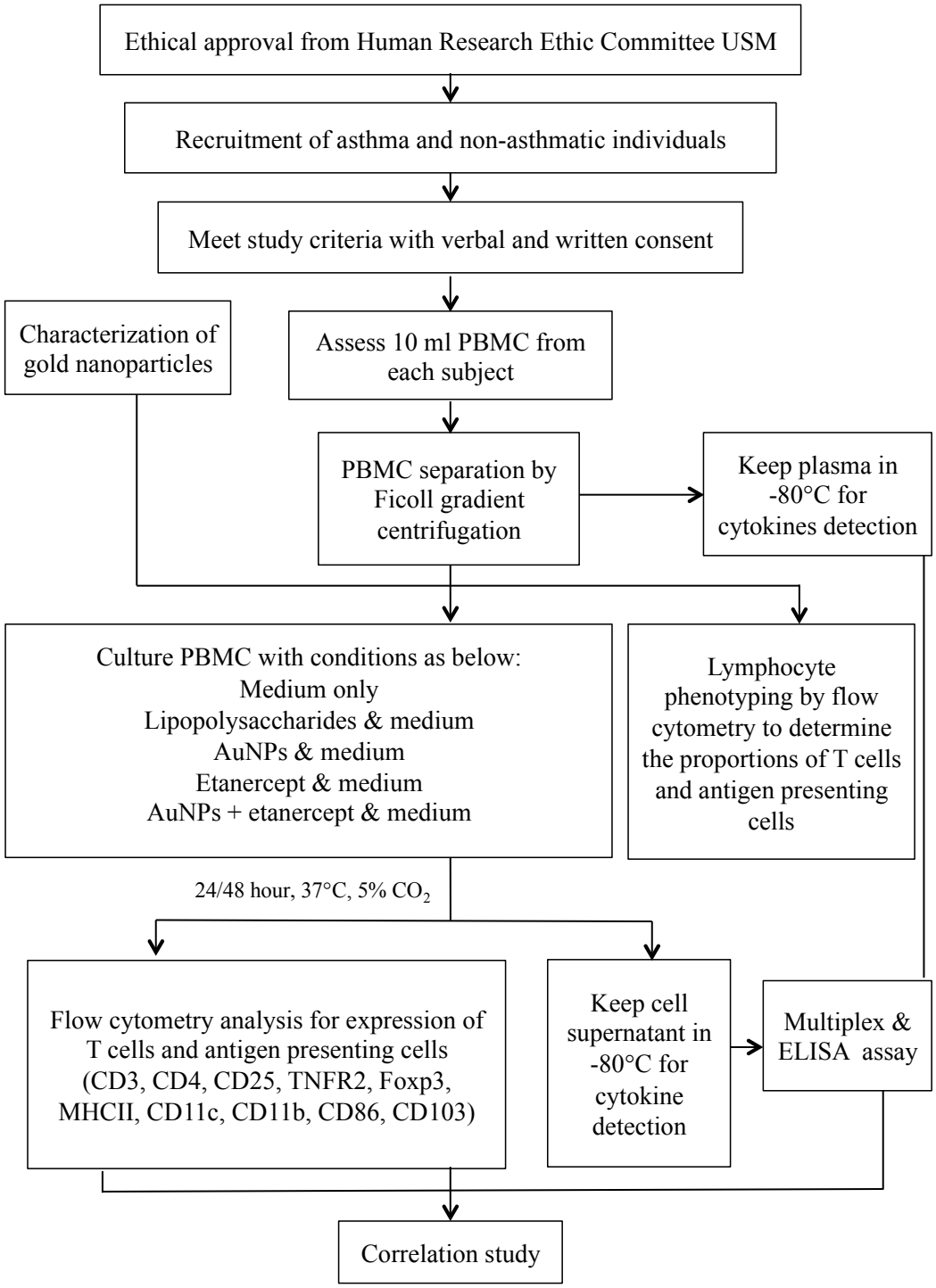
a. To determine the proportion of lymphocytes and DCs subsets in PBMCs of asthmatic and non-asthmatic controls

b. To assess the expression of TNFR2 expressing Tregs and $CD103^{+}$ DCs in asthmatic and non-asthmatic controls after exposure to AuNPs and etanercept

c. To assess the cytokine levels of asthmatic and non-asthmatic controls after exposure to AuNPs and etanercept

d. To perform a correlation study between exposure effects (b & c)

1.6 Flowchart of the study



CHAPTER 2

LITERATURE REVIEW

2.1 Asthma

Asthma is a chronic obstructive pulmonary disease characterized by coughing, wheezing, shortness of breath and chest tightness, resulting to difficulty in breathing (Global Inititative for Asthma, 2019). Since the disease is a very common disease, affecting almost 300 million people worldwide and 4.5% of Malaysian population (Institute for Public Health, 2008), it imposed a huge burden to the society in term of the loss of productivity and huge healthcare expenditure. The symptoms of asthma are potrayed as both innate and adaptive immune responses act together to cause bronchial hyperreactivity, mucus overproduction, airway wall remodeling and airway narrowness. Asthma can be divided into several phenotypes, differing in their clinical manifestations, pathophysiology and even demographic location, but mainly asthma is categorized into allergic and non-allergic asthma (Wenzel, 2012). Allergic asthma, which comprised of nearly 50% of asthma patients, characterized by the presence of serum immunoglobulin E (IgE) antibodies and/or positive for common allergen-specific antibodies. This type of asthma usually develops from allergy sensitization occurred in childhood, accompanied by other types of allergy such as eczema and allergy rhinitis, with a positive family history of asthma (Bosnjak et al., 2011). Meanwhile, non-allergic asthma, also called adult-onset asthma as it develops later in life, does not involve any IgE reactivity to allergens and often requires long-term treatment with systemic steroids (Barnes, 2009). Asthma manifestations also can be

induced by vigorous physical activities, smoking, viral infection and obesity. Asthma is clinically controlled with inhaled or oral corticosteroid mostly in combination with short- or long-acting β-agonist that have a broad and non-specific activity to suppress inflammatory symptoms of asthma. However, there are a portion of patients of asthma that remain symptomatic and inadequately controlled with conventional treatments, thus requiring additional individual theraputic options (Jayaram et al., 2006, Green, 2002, Wenzel et al., 1999).

2.1.1 Pathogenesis of asthma

Previously, asthma is considered as T helper type 2 (Th2) disease of the airways through the increase production of IgE antibodies and prominent recruitment of mast cells and eosinophils to the airways (Lambrecht and Hammad, 2015). Recent evidences indicated that some inflammation in asthma is controlled by other subset of T cells including Th17 and Th9 cells (Lee et al., 2016a, Erpenbeck et al., 2003), indicating to heterogenosity of asthma. Although asthma can be induced by different factors leading to different phenotypes, its development mainly divided into sensitization and memory phase, and effector phase (Figure 2.1).

Sensitization occurs upon the first encounter of foreign antigens such as microorganisms, allergens, and pollutants recognized by pattern recognition receptors (PRRs) expressed by airway epithelium. This recognization would leads to the production of pro-inflammatory cytokines [interleukin (IL)-33, thymic stromal lymphopoeitin (TLSP), tumor necrosis factor (TNF), IL-1β] that activates DCs, the antigen-presenting cells (APCs) responsible to modulate further immune responses (Hammad et al., 2009). These cytokines trigger DCs in several mechanisms, including

recruitment to the lung, migration and maturation to the lymph nodes and polarization and maintenance of Th2 cells (Lambrecht and Hammad, 2009). The prominent roles of Th2 cells in asthma are based on the increase level of IL-4 and IL-5 in asthmatic patients, correlating with the degree of eosinophilia in the airway and the abolishment of asthma features in Th2 cytokines-knockout animals (Robinson et al., 1992, Walker et al., 1994, Komai et al., 2003). IL-4 helps to mediate IgE synthesis by B cells, while IL-5 induces airway eosinophilia, where both are the prominent hallmarks of asthma (Steinke and Borish, 2001, Farne et al., 2017). These events would sensitize mast cells through binding of IgE with its high-affinity receptor complex (IgE-FcεRI), providing an initiation point for effector phase upon the next encounter with the same antigen (Reuter et al., 2010).

In the effector phase, the same antigen encounter would immediately cross links with IgE-FcεRI complex on sensitized mast cells, releasing the mediators of inflammation such as histamine, leukotrines, cytokines, chemokines and proteases (Flint et al., 1985). The release of these mediators contributes to the airway constriction and remodelling, Goblet cell hyperplasia and influx of inflammatory cells including Th2 cells and eosinophils (Casale et al., 1987, Yamauchi et al., 2008, Yu and Chen, 2003). Continuous activation of mast cells and Th2 cells secrete cytokines (IL-4, IL-5, IL-9, IL-13) that further augment the airways tissue injury. While Th2 cells are among the mediator for airway inflammation in the mild to moderate asthma, other T cell subsets starts to appear as the disease becomes more severe (Manni et al., 2014). Th17 cells have been associated with severe asthma that is less responsive to corticosteroid by inducing neutrophils recruitment and increased inflammation of the airways (Wong et al., 2001, Newcomb and Peebles, 2013). Previously, sensitization phase is induced by the production of Th2 cells, mediated by IL-4, instead of Th1

cells by IL-12. In a more severe asthma, polarization of Th1 cells exacerbate the airway inflammations induced by Th2 cells (Rogala et al., 2015). Th9 cells are vital for recruitment and activation of mast cells during the early allergic responses while Th22 cells which are increased in children with asthma, involved in the recruitment of leukocytes and disrupt the integrity of epithelial lining in the lung (Jones et al., 2012, Farfariello et al., 2011).

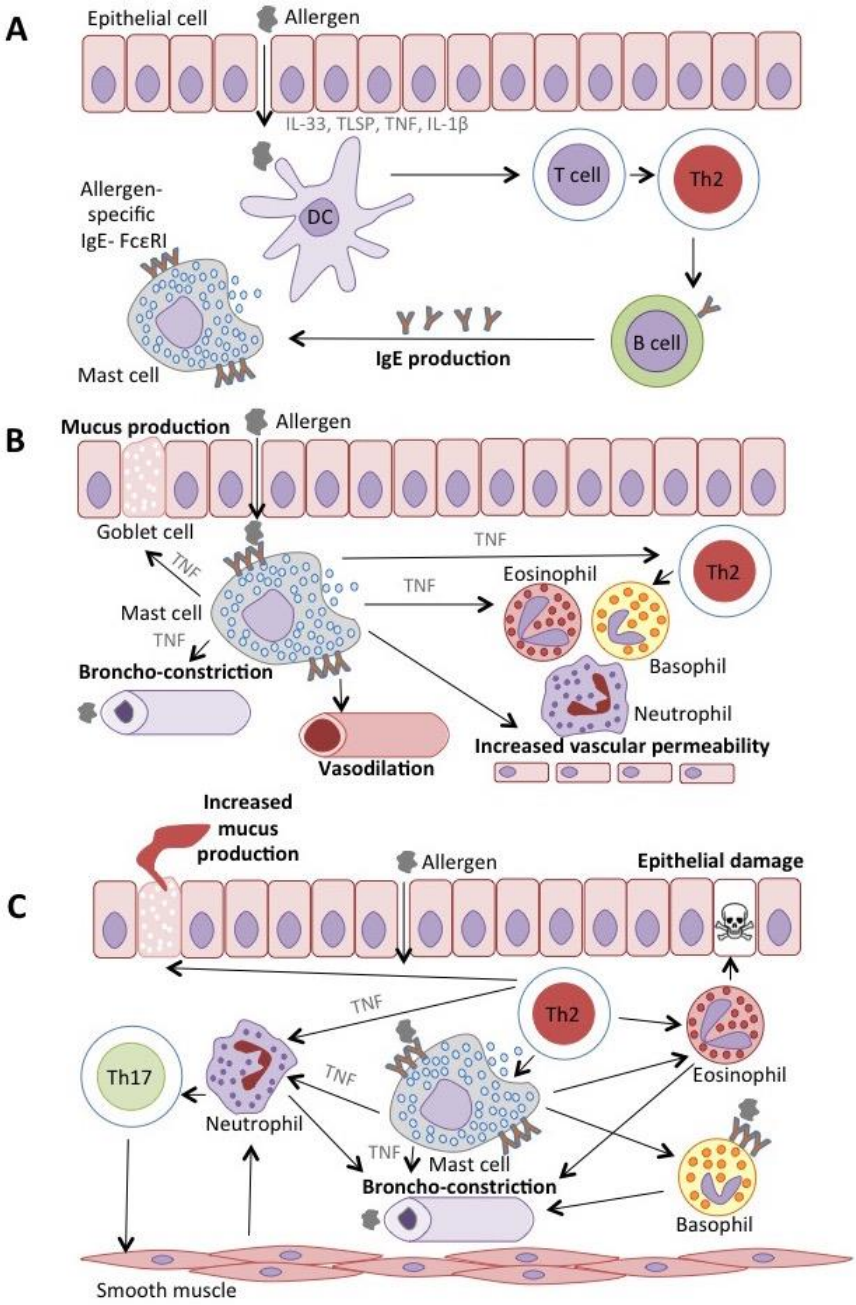


Figure 2.1 Pathogenesis of asthma. **A** First exposure to allergen and other antigenic stimuli induces epithelial cells to release mediators, which activates DCs to induce Th2-mediated responses including sensitization of mast cells. **B** Upon next encounter with the same antigen, sensitized mast cells released mediators that induce inflammatory effects potrayed by the manifestation of asthma. **C** Continuos activation of mast cells and Th2 cells augment the tissue injury and further initiate production of other effector T cells (Th9, Th17), the phenotype for chronic asthma (Ahmad et al., 2018).

2.1.2 Treatment of asthma

Clinically, treatment of asthma is approached based on severity and causing factors. In a common clinical setting, asthma is managed with two types of interventions including inhaled corticosteroids (ICS) and β2-agonist bronchodilators (Global Inititative for Asthma, 2019). Currently, there are combination drugs of ICS and bronchodilators such as Symbicort® and Seretide®, which have been effective in preventing asthma exacerbations (Bousquet et al., 2007). Although 90% of patients were effectively treated with these treatments, there are 5-10% of patients who do not respond well to these treatments that do not only cost more in healthcare, but also increased morbidity from the disease. Generally, treatment of asthma aims to achieve rapid improvement of the symptoms, as no disease-modifying treatment is available yet. Currently, new discovery on the pathophysiological mechanisms of asthma provides several strategies as potential disease-modifying treatment for asthma including allergen-specific immunotherapy (SIT), IgE as therapeutic target, inhibitors of mast cells as well as cytokine-based immunotherapies (Holgate and Polosa, 2008). SIT, for instance, is an immune modifying therapy that aims to induce long-term tolerance through the repeated exposure to allergens. The tolerance acquired through SIT is through the induction of Tregs and tolerogenic DCs that suppress the effector function of mast cells, eosinophils and T cells in asthma (Daniels et al., 2016). Apart from asthma, SIT in several types of allergy such as allergic rhinitis, atopic dermatitis and food allergy have been effective in inducing tolerance, thus reducing the symptoms and drugs intake in these diseases (Casale and Stokes, 2011). However SIT requires years of treatment for sufficient protection from disease, and several other challenges in terms of cost, extract availibity and age of patients, which limit the usage of this intervention in a bigger population (Chini et al., 2014).

2.1.3 Treatment with biologics such as TNF antagonist

In inadequate controlled asthma, addition of biologics such as omalizumab, an IgE-targeted monoclonal antibodies, greatly reduces exacerbations in asthma and streroid use (Rodrigo et al., 2011). Other biologics such as IL-5- and IL-13-specific drugs have also shown reduction of exacerbations in severe asthma in terms of reduce eosinophilia (Pavord et al., 2012, Gauvreau et al., 2011).

In asthma pathogenesis, TNF is shown to play significant role and contributes to the early sensitization and late effector phase. TNF is secreted upon allergen or antigen challenge and continue to be responsible to the manifestations of asthma (Figure 2.1). Due to the significant role of TNF in the pathogenesis of asthma, the prospective of TNF antagonist as a disease-modifying treatment in asthma is anticipated. Currently, there are five TNF antagonists (three monoclonal antibodies; infliximab, adalimumab, and golimumab, a TNFR2 receptor etanercept and certolizumab, the PEGylated Fab antibodies) have been established as therapeutic options for inflammatory diseases in clinical settings.

Etanercept, which comprises of TNFR2 and Fc portion of IgG1, have been shown to attenuate allergic lung inflammation in allergy model (Maillet et al., 2011). Etanercept is also shown to restore the expression and functions of FoxP3 on Tregs derived from allergic asthmatics (Lin et al., 2008). However, etanercept only showed to be well-tolerated therapy in moderate-to-severe asthma with no significant clinical efficacy in a randomized control trial (RCT) (Holgate et al., 2011). In another RCT, etanercept failed to attenuate airway inflammation in mild-to-moderate asthma eventhough it significantly increased the TNFR2 levels (Rouhani et al., 2005). Another anti-TNF monoclonal antibodies, golimumab, demonstrated to be unsuitable for treatment in severe asthma when it shows unfavorable risk-benefit profile in a

RCT (Wenzel et al., 2009). Although the approach of TNF antagonism in asthma is conflicting, current discovery of anti-intuitive effects of TNF mediated by TNFR2 revealed a novel strategy in the treatment of asthma.

2.1.4 Requirement of tolerance to control asthma

Asthma is associated with airway hyperresponsiveness and inflammations induced by allergens, excessive physical activities, smoke particulates and even obesity. Ideally, immune responses provide mechanism that induce and maintain tolerance towards excessive immune responses induced by these factors. One of them are $CD4^{+}FoxP3^{+}$ Tregs, the immunosupressive cells that normally dysregulated in disease conditions including asthma. Although observations in the number of Tregs were found to be conflicting, decreased, normal, or even increased in another study, the expression of FoxP3 protein, a critical marker for Tregs phenotype and function, was diminished (Provoost et al., 2009, Lin et al., 2008). Induction of Tregs from bacterial infection such as *Helicobacter pylori* (*H. pylori*) and *Streptococcus pneumoniae* (*S. pneumoniae*) efficiently protect animal against asthma (Arnold et al., 2011, Thorburn et al., 2010). Enhancement of FoxP3 expression by mean of modified FoxP3 mRNA in allergen-induced animal rebalance the T cells population in the lung including Th2 and Th17 cells, thus attenuating the airway inflammations (Mays et al., 2013, Zhang et al., 2014). Even more, increased Tregs number have been identified to be partially the underlying mechanism of protection against asthma in farm-exposed children (Lluis et al., 2014).

2.2 TNFR2^{+} regulatory T cells

Tumor necrosis factor (TNF) is well-established as pro-inflammatory cytokine that contributes to pathological process in acute and chronic inflammation, autoimmune disease and cancer. In asthma, TNF is shown to be upregulated, particularly in severe asthma, and antagonism with TNF greatly improved the quality-of-life score of the patients (Berry et al., 2006). TNF is also shown to synergize with IL-17 to promote recruitment of neutrophil that underlies severe form of asthma (Manni et al., 2014). Furthermore, a meta analysis study suggested that TNF polymorphisms were significantly associated with asthma susceptibility (Huang et al., 2014). In asthma pathogenesis, TNF is secreted by epithelial barriers, mast cells and DCs, along with other pro-inflammmatory cytokines, to induce Th2 cells that lead to the immune cascades responsible for asthma symptoms (Figure 2.1). In models of airway inflammation, neutralization of TNF significantly decreased leukocytes recruitment such as eosinophils and neutrophils into the lung and airways (Lukacs et al., 1995, Maillet et al., 2011). Role of TNF in the pathogenesis of asthma is mainly regulated by TNFR1 signalling that bears death domain, thus inducing inflammation and apoptosis (Whitehead et al., 2017, Berry et al., 2006). In addition, TNF is involved in the downregulation of FoxP3 expression, thus abrogate the immune tolerance by Tregs in asthma (Lin et al., 2008).

Meanwhile, one of the receptor of TNF, TNFR2, have been directly associated with the proliferation and maintenance of function in suppresive cells such as Tregs, tolerogenic DCs and myeloid suppressive cells (MDSCs) (Ahmad et al., 2018). Thus, targeting this receptor in asthma and other allergic conditions to induce Tregs, suppressing the inflammatory cascade and maintaining homeostasis, is

anticipated (Figure 2.2). Recent studies identified TNFR2, which preferentially expressed on Tregs ($CD25^+$ and $Foxp3^+$ cells), constituted as a maximally suppressive Tregs than the TNFR2-negative population (Chen et al., 2008). Human $CD25^+TNFR2^+$ T cells are shown to have the highest level of regulatory markers of Tregs (CD54RO, CTLA-4, CCR-4, Foxp3), hyporesponsive to TCR stimulation and effectively inhibit the proliferation of Teffs compared to $CD25^+TNFR2^-$ and $CD25^-TNFR2^+$ cells (Chen et al., 2010b).

Previously, it is established that TNFR2 is responsible to maintain the balance of TNF through regulation by DCs thus prevents inflammation (Martin et al., 2014). Recently, studies indicate to the role of TNFR2 in promoting the differentiation and function of induced Tregs through the maintenance of Foxp3 expression, both *in vitro* and *in vivo* (Yang et al., 2019, Tseng et al., 2019). TNFR2, which is preferentially expressed on human and mouse Tregs, are shown to involve in the inhibition of inflammatory responses by TNF through its regulation on Tregs (van Mierlo et al., 2008, Nguyen and Ehrenstein, 2016). In asthma model, impaired TNF-TNFR2 signalling promotes polarization of Th2 and Th17 cells, while inhibit Th1 and $CD4^+CD25^+$ T cells, thus exacerbate the airway inflammation (Li et al., 2017). This group later confirmed that activation of TNF-TNFR2 signalling reduced the infiltration of eosinophils and neutrophils as well as the expression of Th2 and Th17 cytokines, alleviating inflammation in allergen-induced asthma model (Peng et al., 2019). However, there were studies which indicate otherwise. TNF that is promoted by natural killer (NK) cells induce Th2 response in virus-associated asthma model and the induction is via TNFR2 signalling instead of TNFR1 (Choi et al., 2014). Meanwhile, in an obese asthma model, TNFR2 is required in the development

of airway hyperresponsiveness (AHR) and is associated with IL-17A, one of the phenotype in severe asthma (Williams et al., 2013).

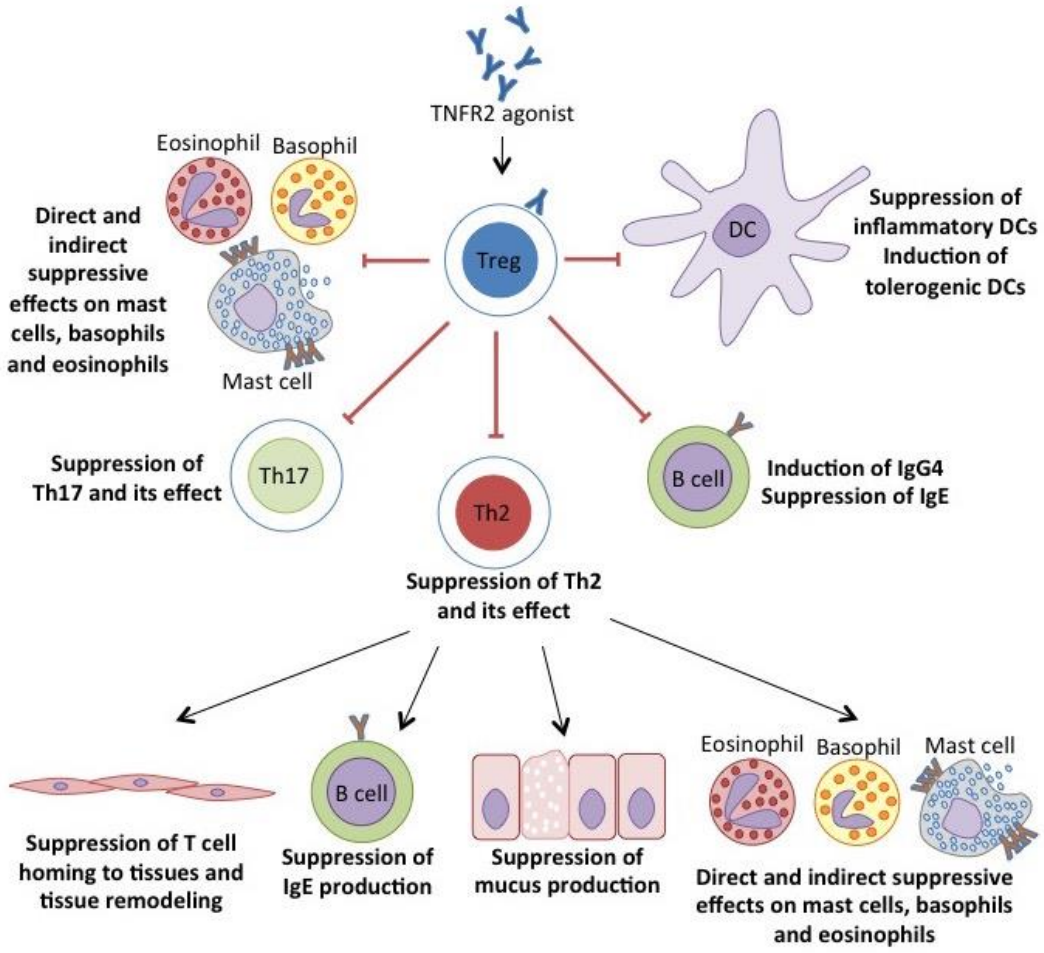


Figure 2.2 Potential of targeting Tregs through TNFR2. Induction of Tregs suppress inflammatory cascade in asthma pathogenesis including induction of tolerogenic DCs such as $CD103^{+}$ DCs (Ahmad et al., 2018).

2.3 $CD103^+$ dendritic cells

It is known that Th2 cells plays a key feature to asthma pathogenesis. However, polarization of this cell population is induced by DCs that demonstrated the absence of DCs in animal transferred with Th2 cells failed to establish characteristics features of asthma (van Rijt et al., 2005). In asthma, DCs, which exist between the airway epithelium and underlying mucosa, would recognize and uptake the antigen to present it to T cells for further immune response (Hespel and Moser, 2012). The prominent role of DCs in asthma is further established by the suppression of Th2 responses and even reverse the airway inflammations through the therapeutic transfer of DCs with immunoregulatory properties in experimental asthma. In another study, constant administration of ovalbumin-induced DCs have been shown to promote the development of Th17 and Th2 cells-associated airway inflammation (Raymond et al., 2011).

In human airways, especially lungs, DCs can be divided into several subsets, depending on the states of the airways, steady or inflamed (Lambrecht and Hammad, 2009). In human, DCs are classified into three major subsets; plasmacytoid DC (pDCs), and two types of conventional DC (cDC1 and cDC2). In non-lymphoid tissues, cDCs consist of two major subsets: $CD103^+$ DCs and $CD11b^+$ DCs where $CD11b^+$ DCs dominate the human blood. Meanwhile, $CD103^+CD11b^+$ DCs are among $CD11b^+$ DCs counterpart that is unique to the intestine (Bain et al., 2017). Although all three subsets come from the same myeloid lineage, it is evidence that each subset bears distinct development and functional specialization (Table 2.1).

Table 2.1 Functional properties of DCs subsets. This table is adapted from Merad et al., (2013) unless cited otherwise

Functional specialization	**$CD103^+$ DCs**	**$CD11b^+$ DCs**	**$CD103^+CD11b^+$ DCs**
Sensing	• Langerin, CD24, Btla, c-kit, CD26, Xcr1, CD36, Clec9A, CD205 • Express double-stranded viral RNA sensor, TLR3, TLR11	• CD24, Btla, c-kit, CD209 • Express high cytoplasmic viral sensor levels • Potent cytokine producers in steady state and upon stimulation	• CX_3CR1, F4/80, CD172a, CD64 • Similar PRR expression profile to $CD103^+$ DCs
Activation of $CD8^+$ T cells	• Superior ability to present microbial antigens and cell-associated antigen to $CD8^+$ T cells • Main source of IL-12 and IL-15	• Able to cross present to $CD8^+$ T cells	NA
Activation of $CD4^+$ T cells	• Induce $CD4^+$ Th1 response	• Predominant role in MHC-II presentation in steady state • Prime Th2 cells	• Induce Th2, Th17 and B cells in mucosa
Tolerance	• Negative selection of developing thymocytes and induction of Treg in thymus • In periphery, participate in deletional tolerance of self-reactive T cells and induce antigen-specific Tregs	• Induce clonal deletion of autoreactive T cell or Tregs differentiation • Express ALDH to induce antigen-specific Tregs	• Able to induce peripheral antigen-specific Tregs differentiation
Cytokine secretion	• IL-12, IL-23	• IL-6, IL-10, IL-23	• IL-17, IL-6, TGF-β, IFN-γ (Wenzel et al., 2015)

$CD103^+$ DCs, the population of interest in the current study, are the largest population of DCs in the lung. This population of DCs resides in lung mucosa and vascular walls, mediating cell adhesion, migration and signalling that play a role in the homing of T cells in the lung (Sung et al., 2006). Upon allergen challenge, this DCs subset is upregulated and prime Th2 cells, responsible for allergic airway inflammation (Sung et al., 2006, Nakano et al., 2012). Depletion of $CD103^+$ DCs in the lung using allergen-specific cytotoxic T lymphocytes (CTLs) reduces allergic airway inflammation (Daniels et al., 2016). Significant role of $CD103^+$ DCs in asthma is further confirmed by reduced airway hyperresponsiveness and eosinophil recruitment in CD103 knockout mice after aerosol challenge (Fear et al., 2016). Their study also observed a diminished number of Teff and Tregs as well as DCs in the same knockout model, indicating to the role of CD103 in the recruitment of these cells during airway inflammation (Fear et al., 2016). However, some studies reported different findings indicating to the protective role of $CD103^+$ DCs against asthma. In asthmatic condition, CD103 knockout mice augmented lung inflammation when challenge to several antigens (Bernatchez et al., 2015, Blanchet et al., 2010, Conejero et al., 2017). This DCs subset induces protection by the production of IL-12, thus restraining Th2 and Th17 inflammatory responses. Furthermore, this DCs population and its expression on important subsets of T cells such as Tregs is shown to be increased upon antigen exposure (Bernatchez et al., 2015). $CD103^+$ DCs promote tolerance in the airway by upregulate the expression of retinaldehyde dehydrogenase, among the co-factor for FoxP3 induction (Khare et al., 2013). Furthermore, $CD103^+$ DCs isolated from MLN have been shown to promote conversion of naïve T cells into FoxP3 T cells (Coombes et al., 2007). Similar to Tregs, $CD103^+$ DCs is enriched

in the lungs of mice tolerized with *H. pylori* immunomodulators, providing protection against asthma (Engler et al., 2014).

2.4 Targeting $CD103^+$ DCs and $TNFR2^+$ Tregs with gold nanoparticles

As these subsets of cell population are well established to play significant protective role against asthma, targeting these immune cells is one of the most efficient strategies in the application of immunotherapies for asthma. This strategy is explored to deal with several challenges of conventional treatments of asthma such as corticosteroid-sensitive severe asthma, heterogenosity of asthma and other co-morbid factors.

2.4.1 Characteristics of gold nanoparticles

The respiratory tract serves as an important portal of entry to our system that it raised both adverse effect concerns and opportunities for novel immunotherapy applications. Nanoparticles such as AuNPs have been proposed as a highly potential tool in immunotherapies due to its advantageous properties including customizable size and shapes, diverse surface functionality and biocompatibility.

Studies have shown that smaller size and rod-shaped NPs were more readily internalized and efficiently take up by APCs such as DCs compared to bigger size and other-shaped NPs (Fernandez et al., 2015, Niikura et al., 2013, Oh et al., 2011). Since size and shape of NPs are of functional significance in immunotherapies, advances in the synthesis of AuNPs yielded customizable parameters that provide targeting capacity of AuNPs. In addition, AuNPs possessed high surface energy as

well as affinity towards numerous molecules including antibodies, antigenic peptides, nucleic acids, polymers and radioisotopes (Oh et al., 2011, Lee et al., 2016b, Paul et al., 2014, Safari et al., 2012), enabled their functionality to be fine-tuned for targeting capacities. Studies have shown that AuNPs is generally biocompatible and low in cytotoxicty compared to other inorganic NPs (Singh et al., 2010, le Guével et al., 2015).

Applications of AuNPs in the immunotherapies are based on its unique physiochemical properties including localized surface plasmon resonance (LSPR) and colloidal stability. LSPR generated by AuNPs is sensitive to its physiochemical characteristics, thus modification to these characteristics suggest to numerous potential applications, particularly in biomedical imaging (Abadeer and Murphy, 2016, Gao et al., 2014). For colloidal stability that is crucial to avoid aggregation in biological medium, AuNPs can be surface functionalized with a number of compounds such as citrates, amines and thiols, hence effectively stabilize AuNPs for biological applications (Gao et al., 2012, Oh et al., 2013).

2.4.2 Gold nanoparticles in asthma

These manipulative properties of AuNPs have been of interest for targeted delivery of therapeutic agents through its interaction with APCs such as DCs that subsequently modulate further T cells immune responses (Ahmad et al., 2017). AuNPs preferentially accumulate in the lungs and effectively induced immune responses as indicated by increased cells such as macrophages, lymphocytes and monocytes upon exposure to AuNPs (Semmler-Behnke et al., 2008, Lipka et al., 2010, Sung et al., 2011). In apolipoprotein E KO mice, a sensitive model for pulmonary effects, AuNPs is shown to be the least inflammatory and DNA damaging

compared to other carbonaceous particles (Jacobsen et al., 2009). Whereas, in LPS-induced acute pulmonary inflammation model, AuNPs exhibited anti-inflammatory and antioxidative where peptide-conjugated AuNPs reduced the infiltration of inflammatory cells and increased of IL-10 (dos Santos Haupenthal et al., 2020, Wang et al., 2020).

Despite the numerous applications of AuNPs such as photodynamic agent in cancer, anti-microbial agents and as a catalyst, its interaction with the immune system particularly in asthma and allergic condition is indisputably lacking. Like other ambient particulate pollutant (Penttinen et al., 2001, Frampton et al., 2004), environmental exposure to NPs including AuNPs has been associated with pulmonary inflammation and exacerbates the symptoms in existing asthma (Hussain et al., 2011, Klot et al., 2002). However, some studies also indicated to the protective effects of AuNPs against key features of asthma. In asthmatic mice, intranasal application of AuNPs shows inhibition to both inflammatory infiltrates and airway hyperactivity (Omlor et al., 2017, Barreto et al., 2015). Current study aims to investigate the effects of AuNPs on two cell populations of interest, TNFR2 expressing Tregs, which was identified as a superior Tregs, and $CD103^{+}$ DCs, which is shown to promote Tregs.