Novel Pathobiological Roles for CD4 T Cells in Asthma

Soroor Farahnak

Department of Experimental Medicine, Faculty of Medicine

McGill University

Montreal, QC, Canada

December 2017

A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of Doctor of Philosophy

Acknowledgement

This thesis would not have been accomplished without the contribution and support of many individuals. I am most particularly grateful to my supervisor, Dr. James Martin, for offering me the opportunity to pursue my studies in his lab and nourishing courage and curiosity in me for science by generously providing me all possible facilities and keeping faith in my potential.

I am incredibly grateful and blessed with having a loving and supportive family who bestowed upon me everything in their power to pave my way for following my dreams.

I am also grateful for having many incredible colleagues throughout my study; Toby McGovern, Michael O'Sullivan, Karim Hamdy Shalaby, Sana Siddiqui, Nobuaki Hirota, Tracy Nghiem, Saba Al Heialy, Leora Simon, Michael Chen, Yumiko Ishii, Anna Wang, Racheal Kim, Satoshi Ano, Yoichiro Hamamoto, Sonia Bassini, Alice Panariti, Benoit Allard, Joyce Yang, Emily Nakada and Niusha Khazaei.

I thank Dr. Elizabeth Fixman and Dr. Irah King for the generous advices and supports.

I am truly thankful for the support from Persia, Mitra Shourian, Damien Montamat-Sicotte and Haleh Allameh Haery for uplifting my spirit by their gracious friendships.

.

Contribution of Authors

This thesis is structured based on a manuscript format, consisting of one published (chapter 3) and one under preparation manuscript (chapter 2).

Chapter 2. Pathophysiological Role of HB-EGF Synthesized by CD4 T Cells

Soroor Farahnak. Designed and conducted experiments and interpreted data.

Leora Simon. Assisted with acquiring data for lung mechanics.

Michael Chen. Assisted with acquiring data.

Elizabeth Fixman. Provided DO.11.10 mice.

James G. Martin. Contributed to designing experiments and interpretation of data.

Scientific contribution. The novelty of this work is finding physiological role for the synthesis of HB-EGF by CD4 T cells in lung function in the context of allergic asthma by modulating immune response and mechanical properties of the lungs.

Chapter 3. Basic Fibroblast Growth Factor 2 is a Determinant of CD4 T Cell-Airway Smooth Muscle Cell Communication through Membrane Conduits

Soroor Farahnak. Designed and conducted experiments, interpreted data and wrote the manuscript.

Toby K. McGovern. Assisted with data interpretation.

Racheal Kim. Assisted with acquiring data.

Michael O'Sullivan. Assisted with preparing primary human airway smooth muscle cells from tissue.

Minhyoung Lee. Assisted with acquiring data.

Saba Al Heialy. Contributed with acquiring data.

Brian Chen. Assisted with acquiring data.

Joyce Yang. Assisted with conducting experiments.

Haruka Yoshie. Assisted with acquiring data.

Anne Marie Lauzon. Provided human lung tissue for primary airway smooth muscle cell culture.

James G. Martin. Contributed to designing experiments and interpretation of data.

Scientific contribution. This study provides a mechanism for lymphocyte-derived membrane conduit formation by CD4 T, facilitating communication with airway smooth muscle cells. Besides, it clarifies structural and functional properties of such structures.

Abstract

Clinical studies and experimental animal models conclusively indicate a pivotal role for CD4 T cells in the initiation and exacerbation of allergic asthma. Despite established well-defined functions for their various mediators, dictating immunological and structural alterations, there are emerging regulatory mechanisms by which CD4 T cells can potentially exert their pathophysiological properties. Although the expression of heparin binding epidermal growth factor (HB-EGF) by CD4 T cells was initially shown in 1994, there have been no investigations to explore the physiological significance of its synthesis. Here, we studied the role of HB-EGF synthesized by CD4 T cells in a murine model of experimental acute allergic asthma, induced by Ovalbumin, hypothesizing that this growth factor might facilitate the allergic response in the lung, based on the fact that its upregulated expression was observed upon CD4 T cell activation. We found that membrane-bound HB-EGF on CD4 T cells mediates interaction with dendritic cells, which subsequently affects the immune response to allergen. We also found that CD4 derived HB-EGF at the time of allergen challenge augments features of allergic pulmonary disease by mediating eosinophilic inflammation and bronchoconstriction. Therefore, the immunoregulatory role of HB-EGF in allergic asthma provides more insight into the mechanisms by which T_H2 driven airway diseases are regulated.

CD4 T cells interact with immune and structural cells by direct contact. Previous data have shown that rodent CD4 T cells trigger airway smooth muscle proliferation by mechanisms that involve HB-EGF. One novel mechanism of communication employed by CD4 T cells is the generation of membrane nanotube (MNT)-like structures. Herein, we have demonstrated a molecular mechanism for formation of MNT-like structures through the synthesis of basic fibroblast growth factor 2 (FGF2b) by airway smooth muscle acting on the FGFR1 expressed on the CD4 T cells. The tubular structures mediated mitochondrial transfer from smooth muscle cells to CD4 T cells. The significance of this finding for T cell function is not established. Similarly, the consequences of the interaction for airway smooth muscle function requires further exploration but may contribute to aberrant function of airway smooth muscle in asthma.

Résumé

Des études cliniques et des modèles animaux expérimentaux indiquent de manière concluante que les cellules T CD4 jouent un rôle central dans l'initiation et l'exacerbation de l'asthme allergique. Malgré des fonctions bien définies pour leurs différents médiateurs, dictant des altérations immunologiques et structurelles, de nouveaux mécanismes de régulation sont découverts par lesquels les lymphocytes T CD4 pourraient potentiellement exercer leurs propriétés physiopathologiques. Bien que l'expression du facteur de croissance de type EGF l'héparine, HB-EGF, par les lymphocytes T CD4 ait été initialement montrée en 1994, aucune étude ne s'est penchée sur la signification physiologique de sa synthèse. Ici, nous avons étudié le rôle de HB-EGF synthétisé par les cellules T CD4 dans un modèle murin d'asthme allergique aiguë, induite par l'ovalbumine, en supposant que ce facteur de croissance pourrait faciliter la réponse allergique dans le poumon. Cette hypothèse étant supporté par le fait que son expression est régulée lors de l'activation des lymphocytes T CD4. Nous avons constaté que HB-EGF lié à la membrane des cellules T CD4 induit une interaction avec les cellules dendritiques, ce qui affecte ainsi la réponse immunitaire à l'allergène. Nous avons également constaté que, lors de l'exposition à l'allergène, HB-EGF dérivé des lymphocytes T CD4 provoque une augmentation des manifestations de la maladie pulmonaire allergique par la médiation de l'inflammation par les eosinophiles et la bronchoconstriction. Par conséquent, le rôle immunorégulateur de HB-EGF dans l'asthme allergique permet de mieux comprendre les mécanismes par lesquels les maladies des voies respiratoires provoquées par le TH2 sont régulées.

Les cellules T CD4 interagissent avec les cellules immunitaires et structurelles par contact direct. Des données antérieures ont montré que les cellules T CD4 des rongeurs déclenchent la prolifération des muscles lisses des voies respiratoires par des mécanismes impliquant HB-EGF. Un nouveau mécanisme de communication employé par les lymphocytes T CD4 est la génération de structures ressemblant à des nanotubes de membranes (MNT). Ici, nous avons démontré un mécanisme moléculaire pour la formation de structures de type MNT à travers la synthèse du facteur de croissance des fibroblastes basique 2 (FGF2b) par le muscle lisse des voies respiratoires agissant sur le FGFR1 exprimé sur les cellules T CD4. Les structures tubulaires ont favorisé le transfert mitochondrial des cellules musculaires lisses aux cellules T CD4. L'importance de cette découverte sur la fonction des lymphocytes T n'est pas établie. Les effets de cette interaction sur

la fonction du muscle lisse des voies respiratoires nécessitent une exploration plus poussée, contribuant possiblement à une fonction aberrante du muscle lisse des voies respiratoires dans l'asthme.

Table of Content

CHAPTER 1

Introduction & Literature Review

1.1 Overview of allergic asthma

Asthma is a chronic inflammatory syndrome, with various overlapping phenotypes, induced in susceptible hosts by environmental stimuli; manifested by wheezing, coughing and shortness of breath, airway inflammation, recurrent airflow obstruction and airway hyperresponsiveness [1 1]. Repeated exposure to environmental stimuli and the chronic inflammation it causes is the leading cause of permanent structural alterations and remodeling of the airways resulting in subsequent irreversible airway obstruction. The contributions of genetic makeup and environment give asthma highly heterogeneous clinical features and variable responses to different therapeutic approaches. Although there have been substantial advances in the understanding of asthma genetics, airway biology and immunology, there are no proven therapeutic approaches to curing asthma which continues to be a serious public health problem. According to the World Health Organization, some 235 million people worldwide were affected by asthma in 2011 and approximately 250,000 people die each year from the disease and related complications. Most forms of asthma are readily treatable but severe asthma leads to significant morbidity, hospitalization, loss of productivity and societal costs[2-4].

1.2 Etiology and risk factors of asthma

Much of our understanding of the etiology of asthma comes from studies of occupational asthma in which a well-defined sensitizing agent is identified, and a period of exposure is documented prior to the development of disease. Historically, asthma was categorized as allergic or non-allergic, distinguishable by the presence or absence of a T_H2 immune response and allergen specific immunoglobulin E, induced by high molecular weight agents[5]. Continued exposure following sensitization leads to rhinitis that is frequently followed by asthma. Low molecular weight agents have a less well-defined allergic basis, however, in some cases IgE has been implicated. Non-allergic asthma which is T_H2 and IgE independent often occurs later in life and is often associated with obesity, chronic rhinosinusitis and female sex. Regarding the heterogeneity and complexity of the clinical and pathophysiological manifestation of disease, the classification of asthma goes far beyond the two categories mentioned above into groups based on pathophysiology known as endotypes[6].

Risk factors for asthma are not well defined which reduces the possibilities for prevention and treatment. Genetic predisposition and inheritance affect the risk of asthma development. Positional cloning of single genes causing disease and genome wide association studies have identified multiple genes associated with disease including genes encoding inflammatory mediators; interleukin 4 (IL-4), IL-5, IL-13, thymic stromal lymphopoietin (TSLP), IL-33, toll-like receptor 2 (TLR2) as well as human leukocyte antigen genes, growth, repair mediators such as a disintegrin and metalloproteinase (ADAM33) and regulators of airway smooth muscle cell (ASMC) function (β2 adrenergic receptor)[7,8]. Age is another contributing risk factor; children with refractory wheeze are at greater risk in developing asthma[9]. There is a greater prevalence of asthma in boys before age 13 and in girls after puberty; this indicates that sex is also a risk factor[10]. Life style and hygiene highly influence on the onset of asthma. According to the hygiene hypothesis, improved hygiene, vaccination and antibiotics in industrialized regions have changed the natural pattern of microbial exposure which normally induces a T_H2 to T_H1 shift in immune response. Furthermore, pathogen exposure is crucial for the induction of regulatory T cell development and maintaining balance of the immune response[6,11-13]. Although not well investigated mechanistically, it has been suggested that diets rich in anti-oxidants and vitamin D can be protective[14,15]. Moreover, obesity has been positively correlated with asthma in both adults and children[16]. Potential mechanisms of the contribution of obesity to the clinical features of asthma are via changing physiology and mechanics of the respiratory system as well as the production of cytokines and chemokines (IL-6, IL-8, monocyte chemotactic factor-1) from adipose tissues[16]. Exposure to environmental pollutants and irritants, particularly in the workplace, can cause or exacerbate the course of disease. High molecular weight (plant and animal derived glycopeptides) and low molecular weight agents (reactive dyed and acid anhydrides), can sensitize the immune system and induce forms of allergic response. Pollutants such as ozone, cleaning agents, tobacco smoke, diesel exhaust, nitrogen dioxide and endotoxins, can contribute to allergic responses in a non-atopic manner[17,18].

1.3 Pathophysiology of asthma

Once asthma is induced by exposure to inhaled causative substances, several characteristic features can be identified that define the condition. Key hallmark features of asthma are AHR and airway inflammation, which can lead to airway remodeling, dysregulated physiological repair and adaptation to chronic inflammation and injury. The pathological modification of the airway

reticular basement membrane, epithelial and ASM structures increases the thickness and stiffness of the airways, mucus production, prolongs inflammation, and increases ASMC contractility and thereby AHR. The airway wall modifications include epithelial detachment, goblet cell hyperplasia, increased mucus production, sub-epithelial fibrosis, increased in ASM mass and angiogenesis[19].

1.3.1 Airway hyperresponsiveness

AHR is a complex interaction between ASM contraction and remodeling of the airways. AHR is defined as exaggerated narrowing of airways in response to bronchoconstrictor agonists and is commonly used as a diagnostic for asthma. AHR is frequently measured from changes in airflow when bronchoconstrictors or bronchodilators are administered and patients are asked to take maximal inspiration followed by forced expiration to calculate the forced expiratory volume in one second (FEV_1). Low concentrations of bronchoconstrictors such as methacholine drop FEV_1 by 20% in asthmatic subjects, while increased FEV_1 from baseline by 12% in response to bronchodilators such as salbutamol in adults are considered diagnostic of asthma[20]. In murine experimental models, invasive and non-invasive techniques are available to measure pulmonary function. Non-invasive approaches (such as Penh) use conscious animals that breathe spontaneously, however the measurements of lung mechanics are variable and nonspecific. Invasive techniques require mechanical ventilators to generate oscillating airflow at a single frequency or multiple frequencies while measuring airway pressure following administration of aerosolized bronchoconstrictors (MCh, serotonin or histamine). Estimates of airway resistance and elastance are acquired in anesthetized, intratracheally cannulated paralyzed mice. A multi-frequency oscillatory flow is used to estimate the large airway resistance (Rn), tissue resistance(G) and elastance (H) of the lung tissue using a more complex mathematical model[21,22].

1.4 Immune response to allergen and airway inflammation

Exposure to environmental protein allergens in normal individuals induces immunologic tolerance. However, genetically susceptible individuals with aberrant immune reactions develop eosinophilia resulting from an allergen-specific memory Th2 response characterized by the presence of serum IgE antibodies. Sensitized subjects react with positive skin-prick tests for the allergenic proteins. Common inhaled allergens are house dust mite, animal dander, fungal spores,

plant and tree pollen. Aeroallergens often have proteolytic properties and may contain traces of bacterial components such as lipopolysaccharides (LPS). Ingested food allergens can also potentially cause asthma by generating a T_H2 biased immune response.

Inhalation of allergens initiates the local inflammatory response in airway mucosa by stimulating pattern recognition receptors on airway epithelial cells (AECs) which are the first barrier at the interface of the airways. The epithelium is the first tissue to respond to the allergen and is central in orchestrating the immune response, producing a variety of cytokines that recruit and activate local dendritic cells (DCs) as well as promoting innate and adaptive immune responses. Upon stimulation, airway epithelial cells release granulocyte monocyte colony stimulating factor (GM-CSF), type 1 interferon, IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) as well as C-C chemokine ligands 2 (CCL2) and 20 (CCL20)[6]. Immature dendritic cells (DC) present in the epithelial layer are specialized for sampling the airway lumen by extending dendrites between epithelial tight junctions and internalizing allergens via micropinocytosis and phagocytosis. Antigen such as house dust mite with proteolytic capacity also can reach submucosal dendritic cells by disrupting epithelial cell tight junctions. Upon uptake of allergen, DCs process them into allergenic peptide fragments and load them on major histocompatibility complex class II (MHCII) molecules. Antigen bearing DCs migrate to airway draining lymph nodes where maturation of DC and antigen presentation to naïve CD4 T cells take place. Activated mature DCs express co-stimulatory molecules such as B7.1/CD80, B7.2/CD86, Inducible T-cell co-stimulator ligand (ICOSL) and OX40 ligand [23] . Presentation of antigenic peptide on MHCII to naïve CD4 T cell receptors (TCR) along with co-stimulation of CD28, ICOS and OX40 prime activation, proliferation and differentiation of CD4 T cells into various subsets that are highly determined by the cytokine milieu[24]. T_H2 differentiation requires high concentrations of IL-4, provided principally by mast cells and basophils, along with low concentrations of IL-12 and interferon gamma (IFN-γ). T_H2 cells express signature cytokines, including IL-4, IL-13, IL-5 and IL-9. IL-5 has a pivotal role in differentiation, maturation, activation and survival of eosinophils. IL-13 and IL-4 induce immunoglobulin class switching in B cells and antigen specific and non-specific IgE production[12,25,26]. Expression of IgE low affinity (CD23) and high affinity receptors (FcεRI) on variety of cell types, B cells, CD4 T cells, DCs, macrophages, mast cells, basophils and structural cells including epithelial and smooth muscle cells, modulates the immune response to allergen[27-29]. IgE dependent and independent mast cell activation and degranulation occurs within minutes

of allergen exposure and results in the release of preformed mediators, such as histamine, serotonin, proteases, and de novo synthesis of others such as prostaglandins and leukotrienes. These mediators result in vasodilation and bronchoconstriction, by targeting airway smooth muscle cells and vascular endothelium, and cause infiltration of inflammatory cells into tissues[25,30,31]. Mast cells produce T_H2 associated cytokines, chemokines and growth factors hours after activation. Basophils also interact with IgE resulting in release of lipid mediators, histamine and T_H2 cytokines. Basophils contribute to CD4 T cell differentiation into T_H2 cells by expressing MHCII and antigen presentation as well as production of TSLP and IL-4[32-35]. Eosinophils augment T_H2 differentiation by presenting antigen to CD4 T cells, providing an early source of IL-4[36]. Animal models for investigating the allergic airway disorders have provided significant understanding about the immuno-physiological mechanisms and have been widely applied with various sensitization approaches. These approaches fall into two categories; systemic sensitization with allergen and adjuvant to prime and expand antigen specific TCR bearing CD4 T cells followed by local challenge with the same allergen to recruit CD4 T cells to the site of inflammation or a more natural repeated sensitization with prevalent allergens administered directly to the respiratory system (intranasal or intratracheal instillation of allergen)[37,38].

1.5 Innate immune system in asthma

1.5.1 Airway epithelial cells (AEC), a "Central player" in asthma

The respiratory tract has a considerable surface area ($70m^2$ in humans) which provides the opportunity for maximal exposure to air-borne pathogens and allergens[39]. As the first line of defense against environmental insults, the airway epithelium plays a dynamic role in immunity. The innate immune functions of airway epithelium are barrier function, mucociliary clearance and production of antimicrobial peptides, reactive oxygen species (ROS), reactive nitrogen species, cytokines and chemokines. This allows the airway epithelium to communicate with immune and structural cells via secreted mediators as well as by direct contact[40-43]. The heterogeneity in epithelial cell types varies among different compartments of the lung from trachea to more distal airways and includes basal cells, ciliated cells, goblet cells, club cells, neuroendocrine cells and submucosal glands. Alteration of the balance in the composition of the epithelium is evident in asthma and includes an increased number of goblet cells, increased mucus production, decreased

expression of E-cadherin, which is a component of the adherens junction, and impaired luminal junctional complex formation. AECs collectively express pattern recognition receptors (PRR) to respond to pathogen associated molecular patterns (PAMP) found in microorganisms and danger associated molecular patterns (DAMP) released by damaged tissues, including toll-like receptors (TLRs), C-type lectin receptors (CLR), RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs)[44]. The result of PRR activation is the production of danger signals such as uric acid, ATP and lysophosphatidic acid (LPA) as well as cytokines and chemokines. The cross talk between AECs and DCs has been extensively studied. IL-12p40, CCL2 and CCL20 attract DC precursor monocytes and immature DCs. GM-CSF, TSLP, IL-25 and IL-33 induce DCs to express OX40L and suppress IL-12 production leading to T_H2 development. Epithelial cells activate and recruit a variety of immune cells. GM-CSF, IL-25 and IL-33 induce the type 2 innate lymphoid cell (ILC2) expansion that is a source of IL-5 and IL-13. TSLP and IL-33 activate mast cells and basophils independently of IgE[45-47]. Release of the chemokine C-X-C motif ligand 8 (CXCL8/IL-8), a major neutrophil chemoattractant, by epithelial cells in response to PPR activation is also capable of inducing neutrophilia[48]. Inhalation of bronchoconstrictors and mechanical stress can increase transforming growth factor beta (TGF-β) production by AECs which have been postulated to cause airway remodeling that is independent of inflammation[49]. The airway epithelium has such a profound role in the onset of asthma and activation of innate and adaptive immune response that it has initiated the ongoing belief for considering asthma as an airway epithelial disease.

1.5.2 Neutrophils

For a long time, asthma was believed to be a T_H2 driven eosinophilic and mast cell inflammatory disease which responds to corticosteroid therapy. However, steroid resistant neutrophilic inflammation has been shown during asthma exacerbations. Despite the identifiable inflammatory phenotypes in asthma, it has not been possible to set a clear-cut binary classification between eosinophilic and neutrophilic asthma as there is potential overlap observed in affected subjects[50]. Neutrophilic asthma seems not to be associated with T_H2 but rather T_H17 and T_H1 cytokines and has an onset later in life, often with less reversible AHR[6,51,52]. Neutrophils are terminally differentiated cells, generated from pluripotent stem cells in bone marrow and enter the circulation in large numbers ($2x10^{11}$ cells per day)[53]. Neutrophils are short lived with 1.5 and 8-hour half-lives in mouse and human, respectively[54]. Airway neutrophilia can be exacerbated by respiratory tract

viral and bacterial infection, LPS, bacterial flagellin, cigarette smoke and oxidants such as ozone and chlorine. Several soluble mediators and adhesion molecules are involved in recruiting neutrophils to the lung. IL-8 a potent chemokine, present in the sputum and nasal secretions of asthmatics, can be secreted by epithelial cells and macrophages as well as neutrophils in response to IgE and IL-17[42,55-59]. IL-17, IFN-γ, tumor necrosis factor alpha (TNF-α) are neutrophil chemo-attractants, increased in severe asthmatics. Neutrophils have dual roles in airway diseases: protection from pathogens and damaging airway tissues and induction of AHR and remodeling. From a protective perspective, neutrophils are one of the first lines of defense pathogens by phagocytosis of pathogens, production of ROS by the NADPH oxidase (NOX) complex, and release of serine proteases such as neutrophil elastase, cathepsin G, proteinase 3, azurocidin, α-defensins, cathelicidins, lactoferrin, myeloperoxidase (MPO) and neutrophil extracellular traps (NET)[60]. Neutrophils can produce an array of cytokines, chemokines and lipid mediators which contribute to the exacerbation of the inflammatory response, including IL-1β, IFN-ϒ, IL-8, IL-6, IL-17, TNF-α, growth related oncogene-α, macrophage inflammatory protein 1α and β, leukotriene B_4 and eosinophil cationic protein. From a pathogenic perspective, the neutrophil NOX complex and MPO generate ROS and hypochlorous acid (HOCl), respectively which can injure surrounding tissues during the inflammatory process. Elastase also induces AHR, while neutrophil derived elastase, TGF-β and matrix metalloproteinase-9 (MMP9) may promote airway remodeling[50,61].

1.5.3 Eosinophils

Eosinophils are generated from multipotent hematopoietic stem cells in bone marrow and reside in the hematopoietic and lymphatic organs. After eosinophils enter the blood they have a short life span of 8-18 hours, however, tissue infiltrating eosinophils can survive for 2-5 days. In allergic asthma, eosinophilia is a hallmark of early onset of disease. However, in late onset non-allergic asthma the production of T_H2 cytokines by other cell types such as ILC2s and mast cells along with survival factors including GM-CSF, TSLP, IL-3, IL-25 and IL-33 can also promote eosinophilia. Eosinophils migrate to the site of inflammation by IL-4 induced expression of vascular cell adhesion molecule-1 (VCAM-1) which interacts with eosinophil very late antigen-4 (VLA-4). Further production of IL-4, IL-3, IL-5 and GM-CSF by activated mast cells synergizes with T_H2 derived eotaxins 1 and 2 (CCL11/CCL24) as well as regulated upon activation, normal T cell expressed and secreted (RANTES) to promote prolonged eosinophil infiltration and

survival[62,63]. The antimicrobial effect of eosinophils is attributable to production of major basic protein, eosinophil cationic protein, eosinophil derived neurotoxin and eosinophil peroxidase which are toxic to airway and increase mucus production and bronchial responsiveness. Cysteinyl leukotrienes released from eosinophils exert a positive feedback on their activation, enhance vascular permeability, cause airway smooth muscle constriction and increase in mucus production. Moreover, eosinophil derived TGF-β, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF)-2 and MMP-9 can potentially contribute to airway remodeling[64]. In addition to the aforementioned factors, eosinophil extracellular trap formation (EETosis) and release of mitochondrial chromatin in a structure combined with eosinophil derived granule proteins is capable of defending against pathogens. A physiological strategy to keep the eosinophil population in balance is through the induction of apoptosis by lipoxin A4, which reduces IL-5 production, a mechanism that is suppressed in exacerbated asthma. Corticosteroid therapy is an effective approach for suppressing the production of IL-3, IL-5 and GM-CSF, hence, inducing apoptosis in eosinophils. Corticosteroid resistant patients may require direct anti-eosinophil pharmacological approaches which are mainly directed towards IL-5 production such as mepolizumab and benralizumab[65,66].

1.5.4 Mast cells & basophils

Mast cells originate from pluripotent stem cells in the bone marrow and circulate in the blood before infiltrating the tissues where they mature under the influence of tissue stem cell factor (SCF also known as KIT ligand) and IL-3. They can survive for months or even years within a tissue. Mast cells are equipped to defend against pathogens and activate/maintain the adaptive immune response. Activation of mast cells occurs with or without IgE ligation to the high affinity IgE receptor, FcεRI, leading to release and production of mediators; preformed and stored products that can be released within minutes after activation (heparin, chymase, tryptase, cathelicidins, defensins, carboxypeptidase and histamine), as well as cytokines and chemokines (TNF-α, IL-1, IL-6, IL-5, IL-3, IL-4, GM-CSF). Lipid mediators (prostaglandins and cysteinyl leukotrienes) are rapidly synthesized de novo. IgE dependent mast cell activation occurs via single FcεRI bound IgE or cross-linking of bound IgE with antigen. IgE independent mast cell activation can occur through TSLP and IL-33 and stimulation of PRRs. Mast cells induce vasodilation and angiogenesis by releasing histamine, VEGF, FGF2b, TGF-β and TNF-α. Mast cells play a role in orchestrating

the adaptive immune response by recruiting dendritic cells to the regional lymph nodes and biasing their function toward induction of T_H2 differentiation by producing IL-1, TNF-α, histamine, PGE_2 and PGD_2. In asthma, mast cells migrate into epithelium and airway smooth muscle[67]. Histamine, proteases, prostaglandins and leukotrienes induce airway smooth muscle contraction and mucus secretion. Mast cells can also prolong inflammation by recruiting immune cells such as mast cells, basophils, neutrophils, eosinophils, CD4 T cells and inducing expansion of ILCs. Modulation of mast cell activation by histamine and leukotriene receptor antagonists and neutralization of IgE are among the promising therapeutic approaches[68-70].

Basophils are the least abundant granulocyte in the blood consisting of around 1% of the leukocytes. Basophils leave the bone marrow when fully mature and have a life span of less than three days. They share the same functional features as mast cells and express the IgE high affinity receptor FcεRI. Basophil activation occurs through two pathways, binding with IgE and TSLP, causing the release and synthesis of histamine and lipid mediators or exposure toT_H2 type cytokines including IL-4 and IL-13[68,71].

1.5.5 Macrophages

Macrophages are sentinel phagocytes at the body-environment interface that effectively link innate and adaptive immune responses by performing antigen presentation. The phenotypic diversity of pulmonary macrophages has been the core reason for challenging approaches to investigate their role in the context of asthma. Pulmonary macrophages are normally among the most abundant leukocytes in the conducting airways and alveoli[59]. Resident pulmonary macrophages consist of two phenotypically and originally different populations, alveolar macrophages (AM) and interstitial macrophages (IM). Conferring both pro- and anti-inflammatory properties, AMs are not generated from bone marrow derived monocytes but the embryonic yolk sac and polarize into M1 and M2 subtypes under the influence of the cytokine and chemokine milieu[72]. Classically activated M1 macrophages express high levels of MHCII and CD86 and have pro-inflammatory functions. So-called anti-allergic M1 cells can efficiently induce a T_H1 biased immune response by producing IFN-γ and has a bigger population in less severe compared to severe asthma induced by HDM in murine models. The inflammatory response caused by M1 cells is induced through production of inducible nitric oxide synthase (iNOS), IL-1 β, and TNF-α[73,74]. IFN-γ and GM-CSF are potent

mediators of M1 polarization. When differentiated through the influence of IL-13, IL-33 and M-CSF, M2 cells express low levels of the surface markers, MHCII, CD86 and CD206, low intracellular expression of iNOS2 and high levels of arginase-1. M2 cells can further polarize into M2a ($_H$2 biasing properties by producing IL-4 and IL-13), M2b and M2c (anti-inflammatory properties by producing IL-10) subtypes[75,76]. Comprising about 30-40% of the lung macrophage population, IMs reside in the lung parenchyma and can be distinguished from AMs by the intensity of surface marker expression, namely high levels of CD11b and low levels of CD11c. IMs are thought be involved in antigen presentation, tissue maintenance and remodeling; besides, they can effectively suppress the T_H2 response. The origin of IMs is unclear and they may be either derived from a common unknown progenitor or the macrophage/monocytic lineage[77-80]. Given the altered sub-populations and dysregulated function of pulmonary macrophages in asthma and the essential immunological roles they play in the lungs, targeting macrophages to modulate asthma may have therapeutic potential[80-82].

1.5.6 Dendritic cells (DC)

DCs have a pivotal role in antigenic tolerance and in promoting both innate and adaptive immune responses by producing inflammatory mediators as well as presenting antigen to both T and B cells and determining the fate of T cell differentiation. In the lungs, DCs reside above and beneath the basement membrane of the epithelium throughout the respiratory tract starting from nose to nasopharynx, trachea, bronchi, bronchioles and alveoli. Pulmonary DCs fall into two sub-types, conventional/myeloid DCs (cDC1 and cDC2) and plasmacytoid DCs (pDC). Under inflammatory conditions, monocytes can differentiate into monocyte-derived DCs (moDC). cDCs can efficiently present antigen via MHCI (cross presentation) and MHCII to CD4 T cells. pDCs have limited ability for the uptake or presentation of antigen and their tolerogenic function is linked to high levels of MHCII and low levels of co-stimulatory molecule expression. Furthermore, they can induce tolerance by inducing regulatory T cell differentiation. In the lung, DC activation occurs by the uptake of antigen, stimulation of PRRs or indirectly by AEC inflammatory mediators[83]. To prime T_H2 differentiation, AECs program DCs by producing innate pro-T_H2 cytokines such as TSLP, IL-33, GM-CSF and IL-1α/β. Additionally, activation of DCs by PRR agonists and ligation of FcεRI and FcγRIII bias T_H2 differentiation. As DCs are not a source of IL-4, it has been suggested that the induction of Th2 polarization happens when DCs fail to produce the T_H1 and

T_H17 differentiating cytokines, IL-12, TGF-β, IL-23 and IL-6[83,84]. DCs migrate to the lymph nodes mainly by responding to the concentration gradient of CCL19 and CCL21 via CCR7. DC interaction with other immune cells is mainly determined by the microenvironment. In the lymph nodes, both naive B and T cells probe DCs. DCs present unprocessed antigens to B cells via the complement system and FcγRIIB[85]. Antigen presentation to T cells occurs at the immune synapse (IS), a multi molecular structure, supported by co-stimulatory molecules and cytokines. This active mutual process requires transmission of different signals and remodeling of plasma membrane architecture and distribution of surface receptors in both T cells and DCs. On the T cell side, the IS structure is highly dynamic and consists of a central supra-molecular activation cluster (cSMAC, rich in TCR), peripheral supra molecular activation cluster (pSMAC, rich in adhesion molecules LFA-1 and talin) and distal supra molecular activation cluster (dSMAC, rich in filamentous actin). Polarization of the T cell's secretory apparatus toward the IS directs delivery of mediators and IS-related molecules. A sustained signaling in T cells (lasting from hours to days depending on the naïve/effector status of the cells and T cell experience for encountering antigen/MHC complex) activates intracellular signaling pathways involving phosphatidylinositol-3-kinase (PI3K), phospholipase C gamma (PLC-γ), mitogen activated protein kinase (MAPK), SH2 domain containing leukocyte protein of 76kDa (SLP-76) and sustained Ca^{2+} signaling and activation of certain transcription factors (Nuclear factor of activated T-cells (NFAT), activator protein 1 (AP1), nuclear factor-κB (NF-κB) necessary for proliferation and expression of cytokines and chemokines[86]. On the DC side of the IS, DCs express neural synaptic molecules (semaphorins, plexins and neuropilins), essential for adhesion and cytoskeletal remodeling. Cytoskeletal remodeling is an active process involving re-shaping of both actin and tubulin structures highly dependent on Rho GTPases, Rac1, Rac2, formin, Wiskott–Aldrich Syndrome protein (WASp) family and positioning of microtubule organizing center adjacent to the synaptic membrane which leads to cytoskeletal polarization, directing the secretory machinery toward the synapse and providing the third signal for T cell activation. MHCII and CD86 are the first molecules clustered in the synapse; while adhesion molecules such as intercellular adhesion molecule (ICAM)-1/3, make the interaction more sustained. Synapse formation activates anti apoptotic signals in DCs by recruitment and activation of protein kinase B (PKB)/Akt and subsequently NF-κB[86-88]. Given the immunogenic and tolerogenic functions of DCs, understanding the biology of these cells and their

interaction with T cells at detailed molecular level may help us to design novel strategies for controlling allergic diseases and asthma.

1.5.7 Innate lymphoid cells (ILCs)

Falling into three sub-groups, ILC1, ILC2 and ILC3, ILCs contribute to tissue homeostasis, repair and inflammation. Generated from multipotent stem cell progenitors residing in the bone marrow, ILCs are considered T lymphocyte counterparts with parallel "innate" programs in the absence of T cell receptors or lineage markers. Group one ILCs includes natural killer cells (NK cells) and ILC1, both of which produce cytokines such as IFN-γ, IL-12 and IL-18. Since they cannot detect MHCI molecules and are not equipped with perforin and granzyme B, ILC1s lack the ability to perform cell-mediated cytotoxicity that makes them close counterpart for T_H1 cells, while MHCI recognition (self/non-self-recognition) by NK cells places them as counterparts of CD8 T cells. Although both cell types are generated in the bone marrow, it is not clear if there is a common progenitor for natural killer (NK) cells and ILC1s. However, T-box transcription factor TBX21 (T-bet) is essential for the development and function of both cell types; the T box transcription factor eomesodermin (Eomes) is expressed by NK cells exclusively. ILC2s (nuocytes) are characterized as T_H2 counterparts, expressing GATA binding protein 3 (GATA3) as the signature transcription factor and producing T_H2 type cytokines (IL-5, IL-13, IL-6 and IL-9) in response to epithelial cell derived IL-33, TSLP and IL-25. ILC2s may contribute to tissue repair and remodeling in asthma by producing amphiregulin[68,89]. ILC3s are counterparts of T_H22 and T_H17 by producing IL-22 and IL-17, respectively. This sub-class of ILCs expresses retinoic acid receptor related orphan receptor gamma (ROR-γt) as its signature transcription factor[90,91]. Over the past few years the discovery of ILCs has changed our perception of immune regulation showing how innate immunity can effectively shape the development of asthma in ways that resemble the role of the adaptive immune system.

1.6 Adaptive immune system in asthma

1.6.1 T cells

The infiltration and residence of T cells in the asthmatic pulmonary tissue have been documented and for a longtime asthma has been known as a T cell disease[92]. While allergic asthma is

predominantly characterized by T_H2 cell effector functions, a range of other T cell subsets are involved in pathogenesis of asthma. The variety and plasticity of T cell subsets have an additive effect on the complexity of the pathophysiology of the disease. T cell biology and functionality is highly dependent on the state of cellular metabolism and mitochondrial function. However, T cells rely on different degrees of metabolic pathways; mitochondrial oxidative phosphorylation is essential in baseline conditions. Naïve T cells mainly use oxidative phosphorylation to remain in their quiescent state, while effector T cells fulfill their increased need for energy consumption by increasing glycolysis rate and switching fatty acid oxidation to fatty acid synthesis[93]. T cells primarily fall into two sub types, αβ and γδ expressing TCR. Expression of either CD4 or CD8 surface markers further increases T cell heterogeneity[94]. Since the identification of T_H1 and T_H2 as the first CD4 T cell subsets, new pro and anti-inflammatory subsets have been defined based on expression of signature cytokines and transcription factors; however, these subsets are highly plastic and flexible in phenotype[95,96]. From circulating in the blood to migration to the secondary lymph nodes and tissues through vascular endothelial cells, T cells have adopted various mechanisms to communicate with both immune and structural cells in contact dependent and independent fashions, by which they exert their physio-immunological functions. Production of cytokines, chemokines and growth factors, trogocytosis, synaptic contacts and membrane nanotube formation are known documented mechanisms of interaction with other cells[97-103].

1.6.1.1 Membrane nanotubes (MNTs): a T cell communication mechanism

Intercellular communication and exchange of biological information is a smart means to maintain the integrity and homeostasis of complex organisms, occurring via soluble mediators, extracellular vesicles, direct cell-cell interaction and plasma membrane connections called MNTs. MNTs are tube-like structures formed as plasma membrane extensions, either one or both communicating cells can participate in their formation. These tubular structures may be open or closed ended, which determines the capability for intercellular transfer of materials and organelles. The diameter of MNTs predicts the cytoskeletal composition. The smaller MNTs (type I MNTs, <700nm) contain only filamentous actin (F-actin) whereas larger MNTs contain both F-actin and microtubules (type II MNTs, >700nm). The composition of type II MNTs provides the potential for transferring organelles through the tubes. Due to lack of specific known markers, it is confusing and difficult to distinguish MNTs from similar structures such as filopodia and cytonemes;

however, they may be interchangeable[104,105]. There have been mechanisms suggested to be involved in MNT formation but whether or not they are cell specific is not known. By using latrunculin and cytochalasin B, it has been proven that actin polymerization is a requirement for MNT formation. Additionally, Rho family GTPase, cell division control protein 42 (CDC42), and Ras family small GTPase, RalA, have been shown to serve as an actin microfilament organizer[106,107]. Anchoring the MNT to the target cell may also require N-cadherin and β-catenin[108]. Environmental stress such as hypoxia and oxidative stress can be a driving factor for MNT formation by activating EGFR mediated PI3K/ mammalian target of rapamycin (mTOR) pathway via P53 activation. Activation of M-sec, reported under hypoxia, can be another mechanism regulating MNT formation by regulating actin polymerization[107,109,110]. Moreover FAS/FAS ligand facilitates the initiation of interaction between CD4 T cells, and therefore MNT formation[111]. Several molecules and organelles have been reported to be transferred through MNTs such as signaling ions (Ca^{2+}), molecules (myeloid cell leukemia sequence 1 (Mcl-1), caspase-3, perforins, mRNAs and miRNAs), fragments of Golgi apparatus and reticulum endoplasmic, mitochondria, lysosomes and endosomes[101,112-120]. Cell types which have been reported to make MNTs include structural cells (neural cells, cancer cells, stem cells) as well as lymphoid and myeloid immune cells (macrophages, T cells, B cells, NK cells, dendritic cells). *In vivo* formation of MNTs has been observed in several studies [121-124]. T cells can interact with other immune (T and B cells) and structural cells such as ASMCs as well as endothelial cells but not the human epithelial cell line BEAS2B cells (data unpublished) through MNTs. [100,101,118,125-131]. CD4 T cell infiltration into the ASM bundles and their proximity to proliferating ASMCs *in vivo* has been shown[132]. In vitro, activated CD4 T cells connect to ASMCs via membrane nanotube-like structures facilitating the transfer of Mcl-1 from ASMCs to CD4 T cells, resulting in reduced apoptosis in T cells. Increased proliferation of rat ASMCs in the presence of CD4 T cells has been shown to be contact dependent[92,101] which strongly raises the possibility of MNT involvement in transfer of mitogenic signals and/or materials.

1.6.1.2 CD4 T cells: heterogeneity and plasticity

An efficient adaptive immune response ensures differentiation of CD4 T cells, a process regulated by transcription factors and genetic programming. The heterogeneity of CD4 T cells has gone beyond the first identified dichotomy, T_H1 and T_H2, since they initially were described[96]. There is

a degree of flexibility in CD4 T cell differentiation and an established fate may be a result of the extent of cell division, epigenetic and chromatin modifications that occur under repeated stimulation and chronic immune response. However, different subsets of CD4 T cells express different arrays of cytokines, with some degree of overlap, and demand different states of cellular metabolism and oxygen consumption rates[133,134]. Allergic asthma immunopathology is strongly associated with T_H2 cytokines and their role in inducing IgE production and eosinophilic bronchitis[135]. Upon differentiation under the influence of highly specific transcription factors, STAT-6 and GATA3, T_H2 cells express cytokines (IL-4, IL-5, IL-13, IL-9, IL-6, IL-31), chemokines and growth factors (amphiregulin) to mediate their immunological functions[102,136]. Molecular heterogeneity in type 2 asthma has led to categorizing asthma into T_H2 high and T_H2 low endotypes. T_H2 high asthmatics can be diagnosed by signs of airway type 2 inflammation and are responsive to corticosteroids; whereas in T_H2 low asthmatics the type 2 inflammation is comparable to non-asthmatic subjects and the affected subjects are not responsive to steroid therapy[137].

T helper 1 cell development requires lineage signature transcription factors T-bet and signal transducer and activator of transcription 4 (STAT4)[95]. T_H1 cells are believed to be inhibitory for T_H2 driven allergic asthma by producing IFN-γ, TGF-β and IL-12 and inhibiting T_H2 development[138]. Spontaneous development of allergic asthma hallmarks is shown in T-bet deficient mice[139]. Moreover, in asthmatic human airways, a decrease in gene expression of T-bet and increase in GATA3 has been detected[140].

T helper 17 cell differentiation depends on induction of transcription factors STAT3 and ROR-γt expression by IL-23, IL-6, TGF-β and IL-1β[135]. T_H17 cells have a certain degree of plasticity under physiological and pathological conditions toward T_H1, T follicular helper cells (T_{FH}) and T regulatory cells[141]. T_H17 cells have been suggested to induce steroid-resistant neutrophilic asthma by producing IL-17A and F cytokines. Increased IL-17 expression has been shown to be correlated with the severity of asthma[142]. In a murine model of chronic asthma T_H17 cells induced airway remodeling by stimulating the production of HB-EGF by airway epithelial cells, a potent mitogen for ASMCs[143].

Regulatory T cells (Treg) contribute to maintaining and balancing the equilibrium of the immune response by regulating the inflammatory functions of T_H1 and 2, ILC2, mast cells, eosinophils, neutrophils and B cells. There are five sub-group of Tregs based on the expression of the transcription factor FoxP3. Thymus derived/natural (tTreg, nTreg) and peripheral Tregs (pTreg) are $FOXP3^+$, whereas Tr1, T_H3 and CD8 Tregs are $FOXP3^-$. pTregs are derived from CD4 T cells by TGF-β and are directed against non-self-antigens. These cells suppress the immune response by producing IL-10, TGF-β and IL-35[135,144]. Tregs are distinguishable in vitro from other effector CD4 T cells by engaging differential use of metabolic pathways; they are more reliant on fatty acid oxidation and oxidative phosphorylation[145]. In allergic asthma attenuation of the T_H2 immune response may be defective. Children with asthma have fewer Tregs with reduced suppressive function in BAL[146].

The heterogeneity of CD4 T cells extends beyond T_H1, T_H2, T_H17 and Tregs. T_H9 cells can be driven from either T_H2 or naïve CD4 T cells in the presence of TGF-β and IL-4 to produce IL-9 which is a mast cell growth factor as well as regulator of their function by promoting expression of mast cell cytokines. Moreover, IL-9 increases eosinophil differentiation and maturation as well as mucus production by AECs[147]. T_H22 cells differentiate in an IL-6 rich milieu and produce IL-22 and TNF-α, but not IL-17, IL-4 or IFN-γ. T_H22 cells express fibroblast growth factors (FGF) and have a protective effect on epithelial cells[148].

1.6.1.3 CD8 T cells

While the predominant focus in the field of asthma has been devoted to CD4 T cells due to their pivotal role in disease, the role of CD8 T cells has been marginalized in the published studies. Based on the cytokine production profile and surface marker expression, CD8 T cells are divided into two groups Tc1 and Tc2 with close cytokine profiles to T_H1 and Th_H2 cells, respectively. Tc1 express mainly IFN-γ and IL-12, attenuating T_H2 responses and asthmatic phenotypes whereas Tc2 express IL-4 as well as less IFN-γ and higher levels of CD40L and CD28, inducing T_H2 polarization. Although CD8 T cells with regulatory phenotype (identified by production of TGF-β and IL-10) have been identified, a physiological role has not been established[149]. A clinical study showed a significant correlation between the CD8 T cell count and annual decrease in FEV_1 in bronchial biopsies from asthmatics[150]. In mouse models of allergic asthma IL-4 and IL-5

producing CD8 T cells in the lung were predominant compared with their IFN-γ producing counterparts, suggesting Tc2 polarization is favored in inflamed lungs in the presence of T_H2 derived inflammatory mediators. Furthermore, CD8 T cell depletion prior to allergen sensitization increased T_H2 response[151]. Adoptive transfer of CD8 cells from OTI mice into naïve animals followed by Ova challenge resulted in a Tc1 phenotype which interestingly inhibited eosinophilia in Ova sensitized mice by increased IL-12 and decreased IL-13 and IL-5 in bronchoalveolar lavage fluid[152]. Given the evidence, CD8 T cells may have a protective role in the initial course of sensitization in asthma but contribute to disease progression after inflammation is established.

1.6.1.4 γδ T cell

γδ T cells are enriched in the mucosal-epithelial compartments and represent a minor population of lymphocytes. γδT cells protect mucosal tissues and the epithelium against environmental stimuli as a first line of defense. γδT cell protect epithelial integrity by producing growth factors (IGF-1, VEGF, PDGF, KGF, EGF and FGF-2 and 9.)[153,154]. γδT cells react to insults independently of MHC molecules and antigen presentation through recognition of unprocessed peptides, small organic phosphate molecules, alkylamines and DAMPs. Their immunoregulatory role in allergic inflammation is mediated by producing T_H1, T_H2 and T_H17 cytokines, hence, bridging innate and adaptive immune responses[155]. Evidence from Ova induced asthma in γδT cell deficient mice indicates a protective role of this cell type in terms of AHR in response to an inhaled bronchoconstrictor [156]. γδCD8 T cells have been shown to be protective in rats; transfer of γδCD8 T cells from naïve to allergen sensitized rats attenuates type 2 inflammation and eosinophilia[157].

1.6.2 B cells

B cells are essential in ensuring both long lasting protection as well as immunopathology and are a fundamental arm of adaptive humoral immunity. B cells go through selection processes after generation in the bone marrow as well as during maturation in the secondary lymph nodes where low affinity B cell receptor bearing cells are selected for survival[158,159]. B cell activation occurs in the germinal center of secondary lymphoid organs with the help of cognate follicular helper T cells (T_{FH}) which leads to immunoglobulin class switching and recombination in the presence of IL-4 and IL-13, and consequent production of IgA, IgG and IgE as well as development of memory B cells and plasma cells[160]. B cell antigen sensitization and IgE production can occur in the nasal

mucosa in patients with atopic and non-atopic asthma as well as lymphoid organs[26]. Local IgE production may occur through long-lived tissue resident plasma cells. Antigen specific B cells function in a process called epitope-spreading via expression of CD23 where they take up specific or non-specific antigen-IgE complexes and present different epitopes to cognate T cells, thereby resulting in an immune response to more epitopes of the same antigen for which they are specific as well as other antigens[161]. Therefore, antigen-spreading may facilitate the "atopic march" where one type of allergy can promote other kinds[25]. Apart from producing antibodies, B cells contribute to the immune response by presenting antigen to T cells, producing cytokines (IFN-**γ**, TNF-**α**, and IL-17) but also may attenuate the immune response via IL-10 production[162].

1.7 Airway smooth muscle (ASM)

Having no well-defined physiological purpose while causing pathological manifestations, ASM has been referred to as the appendix of the lung[163]. The ASM layer surrounds the airway tree from the trachea to the bronchioles and contributes to airway narrowing through contraction of ASM. The helix-antihelix orientation of the ASM bundles in the distal airways ensures the efficiency of ASM contraction in bronchoconstriction[164]. The main neural mechanism for bronchoconstriction is parasympathetic, induced by the release of acetylcholine. The sympathetic system releases the adrenergic neurotransmitter, norepinephrine, but there is no direct innervation of ASM by this system. However, there are abundant β-2 receptors on ASM that mediate relaxation when stimulated by circulating epinephrine or exogenously administered agonists such as salbutamol. Additionally, the autonomic nervous system releases the non-adrenergic non-cholinergic (NANC) contracting (neuropeptide Y, substance P, ATP and neurokinins) or relaxant (nitric oxide and vasoactive intestinal peptide) agonists[165]. Mediators released from structural and inflammatory cells (histamine, cysteinyl leukotrienes, endothelin, IL-5, IL-13, TNF-α, IL-1β and ATP) can either directly or indirectly modulate ASM contraction[166].

ASM is increased in mass and altered in structure and function in asthmatic subjects. The origin of the excess ASM cells is the subject of great debate; in addition to hypertrophy and hyperplasia of ASM cells, several ASM cell progenitors have been suggested such as the myofibroblasts, fibrocytes, epithelial-mesenchymal transition of epithelial cells, as well as mesenchymal stem cells[167,168]. ASM structural and functional changes make the asthmatics more susceptible to a wide

array of bronchoconstrictors. The plausible mechanisms for alterations in ASM mechanical properties contributing to AHR are increased maximal shortening, velocity of shortening and reduced relaxation[169]. ASM cells are equipped with contractile molecular apparatus including actin-myosin machinery, α-actin, desmin, myosin heavy chain (MHC) and myosin light chain (MLC) kinase[166]. Difference in contractile properties in asthmatic ASM may arise at the molecular level. Due to the possible role in ASM mass increase, proliferation has been a topic of great interest. The majority of our knowledge about ASM cell proliferation comes from in vitro experiments. The proximity of inflammatory cells such as CD4 T cells and mast cells to ASM has been shown *in vivo*; these cells could potentially produce growth factors including bFGF2 and amphiregulin; shown to be mitogens for ASM cells[67,132,170,171]. *In vitro* studies show that freshly isolated ASM cells have a contractile phenotype and switch to a synthetic-proliferative phenotype in culture by down regulating the expression of contractile molecules. Whether or not this contractile to proliferative "phenotype switching" occurs in vivo has been subject to debate and remains to be elucidated.

ASM is capable of producing inflammatory cytokines and chemokines such as eotaxin, RANTES, CXCL8, monocyte chemoattractant protein (MCP) 1, 2 and 3, thymus- and activation-regulated chemokine (TARC) and mast cell chemotactic chemokines (CCL11, CXCL10, CX3CL1); thereby modulating inflammation[172]. ASM facilitates T cell adhesion and possibly activation by expressing adhesion molecules (intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and CD44), MHCII and co-stimulatory molecules (CD28, CD80, CD86, CD40 and OX40 ligand)[173]. The fact that ASM contributes to the pathogenesis of asthma by both induction of bronchoconstriction and by taking part in inflammatory response via synthesis of inflammatory cytokines and chemokines, has made it the focus for many current therapeutic approaches. Novel approaches to target ASM are an area of active research for asthma treatment.

1.8 EGFRs & EGFRLs in asthma and airway remodeling

Few studies have focused on the role of growth factors in airway remodeling but have rather addressed the efficacy of anti-inflammatory strategies in animal models. The observation that epidermal growth factor receptor (EGFR) and EGFR ligand (EGFRL) expression is increased in asthmatic human airways, airway epithelium, glands and ASMCs by immune-histochemical

staining suggests a possible role of EGFR and EGFRLs in airway remodeling[174,175]. In animal models as well as in *in vitro* studies, a variety of stimuli and mediators induce EGFR-dependent responses, leading to airway epithelial repair, mucus synthesis, goblet cell metaplasia and sustained inflammatory responses by mediating production of inflammatory substances. TNF-α induces EGFR expression both in vivo and in vitro[176]. Cigarette smoke increases the expression and activation of EGFR both in vitro and in vivo[177]. Human eosinophils, neutrophils and neutrophil elastase induce EGFR activation in vitro[178-180]. Moreover, leukotrienes induce mucus production in vivo in an EGFR-mediated manner[181]. In addition to AECs, other major sources of EGFR ligands such as EGF, TGF-α, HB-EGF and amphiregulin, can be recruited inflammatory cells including macrophages, eosinophils, mast cells and CD4 T cells[102,170,182,183]. EGFR ligand shedding occurs via increase in the expression of metalloproteinases by cigarette smoke, mechanical stress, ROS and increased activation by stimuli such as lipopolysaccharides, TNF- α, G-CSF, IL-8[184,185]. EGFR activation in vitro increases the synthesis and release of IL-8, from primary bronchial epithelial cells, suggesting a mechanism for initiating/prolonging neutrophil recruitment[186]. The convergent outcomes of EGFR activation in AECs by various stimuli renders inhibition of this pathway of great therapeutic interest to prevent/reverse airway remodeling in pulmonary diseases. However, this approach may be limited by the toxicity of such treatments.

1.8.1 Epidermal growth factor receptor ligands (EGFRLs)

Epidermal growth factor receptor ligands (EGFRLs) consist of a single peptide in the worm, a group of four peptides in the fly and eleven peptides in human. This progressive increase in the number of EGFRLs and the complexity of EGFR signaling is phylogenetically in accordance with the higher demand for cell metabolism, growth and development[187]. Known EGFRLs in humans include epidermal growth factor (EGF), epigen (EPI), amphiregulin, heparin binding like-growth factor (HB-EGF), transforming growth factor alpha (TGF-α), epiregulin (EPR), betacellulin (BTC) and neuregulin1-4 [188,189]. EGFRLs are synthesized as type I transmembrane proteins consisting of a carboxy-terminal domain, a hydrophobic transmembrane domain, juxta membrane domain, the EGF domain and N-terminal domain (pro-peptide). Homology between protein sequences of EGFR ligands is low (~25%) and they also differ in the distribution of glycosylation sites, the presence of a heparin-binding domain, and other biochemical properties. The EGF domain is a 40 amino acids sequence containing six conserved cysteine residues with three disulfide bridges

between C1 and C3, C2 and C4, and C5 and C6, forming the A, B, and C loops, respectively. Some poxviruses are capable of encoding EGF-like domains, which stimulate cell proliferation at the primary infection site. Membrane-bound EGFRLs can induce homo and hetero-dimerization of EGF receptors on adjacent cells in a juxtacrine manner. [187]. EGF ligand ectodomains can be shed proteolytically at the juxtamembrane motif by MMPs and ADAMs which allows the soluble domain to act in autocrine, paracrine and endocrine manner[190].

1.8.2 Heparin binding EGF-like growth factor (HB-EGF)

HB-EGF was first discovered by Higashiyama as a secreted factor by a macrophage cell line, U-937[191]. HB-EGF is a mitogen and chemoattractant for many cell types and regulates various cellular processes including growth, differentiation, apoptosis, adhesion, and migration[192]. In humans, HB-EGF gene is located on chromosome 5, (chromosome 18 in mice) and is transcribed as a 20-22 KD transmembrane glycoprotein[193,194]. Human pro-HB-EGF has the longest residency time at the cell surface and is the diphtheria toxin receptor[195,196]. HB-EGF is expressed by a variety of cell types including monocytes/macrophages, CD4 T cells, neutrophils, eosinophils, myeloma cells, vascular smooth muscle cells, endothelial cells and epithelial cells[171,191,197-202]. Upon shedding by integral membrane metalloproteases such as ADAM 9, 10, 12, 17, the soluble domain of HB-EGF exerts autocrine, paracrine or juxtacrine effects. The intracellular c-terminal domain of either HB-EGF or the truncated cytoplasmic domain after cleavage of soluble domain, can translocate into the nucleus and regulate cell proliferation and survival by binding to and repressing nuclear transcriptional repressors such as promyelocytic leukemia zinc finger (PLZF) and B-cell lymphoma 6 (Bcl6). The EGF domain of HB-EGF binds to Erbb1 (EGFR) and ErbB4 (EGFR4), while the heparin-binding domain (HBD) binds to heparan-sulfate proteoglycans (HSPGs) present on the cell surface and in the extracellular matrix. Membrane-bound HB-EGF is associated with membrane tetraspanin proteins such as CD63, CD81 and CD9 which enhance its juxtacrine activity. The role of accessory tetraspanin proteins includes removal of a steric constraints imposed by another structural feature of the ligand, such as the heparin-binding domain. The accessory protein could be required to present the ligand to the receptor by forming part of a multiprotein complex[203-205].

1.8.3 Epidermal growth factor receptors (EGFRs)

The EGFR family members (EGFR/Her1/ErbB1; Her2/ErbB2, also known as the neu oncogene; Her3/ErbB3; and Her4/ErbB4) are among almost 60 receptor tyrosine kinases in human genome regulating cell growth and differentiation and potentiating mitogenic responses. The EGFR family has 40-45% homology, and evolved by a series of gene duplications early in vertebrate evolution[40]. The growth factor ligands bind at the dimer interface, effectively serving to cross-link the two receptors as a dimer. Hetero and homo dimerization between EGFR family members can be either ligand dependent or independent; enhanced expression of the receptors can drive dimerization. Seven ligands bind to and activate EGFR. The high affinity ligands include EGF, TGFα, HB-EGF and BTC; the low affinity ligands are AREG, EREG, and EPGN.

Each of the EGFR ligands binds to EGFR with different affinities and potentials to induce hetero or homo dimerization of the receptor, which suggests a potential heterogeneity in signaling and biological outcomes. For instance, AREG induces 50% fewer dimers of EGFR:ErbB2 and EGFR:EGFR compared to the other ligands. Although EGF and TGFα have a distinct potential to induce EGFR:ErbB2 heterodimers vs. EGFR:EGFR homodimers, BTC and AREG induce equal dimers of both types. Post-translational modifications affect EGFR signaling following ligand binding. For example, methylation at R198 and R200 in the EGFR ectodomain increases the affinity for EGF binding by altering the dissociation constant value three-fold[206,196]. In addition to signaling from the cell membrane, ErbB family members may translocate into the nucleus and regulate gene expression. EGFR dimerization induces its intracellular protein tyrosine kinase activity and autophosphorylation of multiple tyrosine residues in the *C*-terminal domain, leading to downstream activation of signaling pathways[207]. Transactivation of EGFR can occur independent of EGFR ligands via G protein coupled receptors (GPCRs) which mediates activation of intracellular protein tyrosine kinases (PTKs) such as Src family proteins. An increase in PTK activity phosphorylates EGFR on its cytosolic domain[208].

Unlike other members of the EGFR family, ErbB2 does not have ligand binding site but is favored in heterodimerization due to constitutive expression of a dimerization arm[190]. Nuclear translocation of ErbB2 has been shown to regulate the expression of certain genes such as cyclooxygenase-2 and cyclin D1[209,210].

The cytoplasmic domain of ErbB3 has impaired kinase activity with 100-fold reduced function for auto and substrate phosphorylation; thus, it is dependent on heterodimerization with other ErbB family members. Other kinases may phosphorylate and activate ErbB3, such as cyclin dependent kinase 5 (Cdk5) and the breast cancer-associated tyrosine kinase BRK (protein tyrosine kinase 6, PTK6)[211].

ErbB4 ligands include EREG, HBEGF, BTC and NRGs. Similar to other members of the EGFR family, ErbB4 can translocate into the nucleus and regulate gene transcription[212].

1.8.4 EGFR & EGFRL expression by CD4 T cells

In 1994 expression of EGFR ligand, HB-EGF, by human CD4 and CD8 T cells was shown for the first time, when cells were activated.[171] Amphiregulin can be expressed by T_H2 cells which induces intestinal epithelial cell regeneration in vivo in helminth infection; therefore, enhancing elimination of the parasite. The finding led to defining amphiregulin as a T_H2 cytokine[102].

T cell receptor activation induces EGFR expression in mouse T_H2 cells. In intestinal infection with helminth, heterodimerization of EGFR with IL-33 receptor on T_H2 cells activates intracellular MAPK signaling pathway which consequently induces IL-13 expression[213]. Mouse Tregs also express EGFR; amphiregulin induces an increase in suppressive efficacy of this cell type both *in vitro* and *in vivo* by activating ERK/MAPK pathway[183].

1.9 Fibroblast growth factor receptors (FGFR) & ligands

From early stages of development to adult tissues, the 18-member FGF family interacts with four tyrosine FGF receptors (FGFR1-4) to maintain development and organogenesis as well as metabolic function, tissue repair and homeostasis by regulating proliferation, migration, differentiation and survival at a cellular level. Due to the diverse functions of FGFs, tight regulation is provided to the pathway by binding the ligands to heparin sulfate proteoglycans which limits their diffusion via binding to extracellular matrix (ECM). An additional regulatory mechanism is via expression of a fifth decoy non-tyrosine kinase receptor, FGFR5. Moreover, Micro RNAs can also regulate FGF and FGFR expression and signaling[214-216]. The mammalian FGF family consists of 4 intracellular factors (iFGF11-14) with no known interaction with receptors and 18 secreted ligands (FGF1-10 and 15-22). iFGF functions have not been fully

investigated but are implicated in regulating voltage gated sodium channels, microtubule stabilization (FGF13) and participation in intracellular signaling pathways (FGF12)[217-219]. Alternative splicing of FGFR mRNA determines their affinity for ligands. Downstream signaling pathways of FGFRs involve adaptor proteins such as FGFR substrate 2α (FRS2α), growth factor receptor-bound 2 (GRB2) and tyrosine phosphate SH2 as well as signaling molecules including, STAT1, 3 and 5, PLCγ, PI3K, PKB/AKT and RAS-MAPKs[220,221]. The involvement of the FGF/FGFR system in asthma has not been intensively explored; however, elevated FGF2 has been reported in sputum from moderate to severe asthmatic patients[222].

1.10 Aims and Hypothesis

CD4 T cells are described to synthesize growth factors that act on the EGFR. Heparin-binding EGF is reported to cause proliferation of ASM when CD4 cells are placed in direct contact with ASM. However, the role of HB-EGF in the modulation of adaptive immune responses is to date largely unexplored. HB-EGF has the potential to be involved in the interaction of dendritic cells and T cells through juxtacrine actions. Moreover, we aimed to study the role of HB-EGF synthesized by CD4 T cells in airway inflammation and AHR in acute murine model of asthma. Further, we wished to explore the contact-dependent interactions of the CD4 cells with ASM with a particular focus on MNTs. We addressed the signals emanating from ASM that participated in promoting MNT formation by the T cell and the role of the MNTs in transferring materials between ASM and T cell.

Hypothesis 1. HB-EGF synthesized by CD4 T cells facilitates the allergic inflammatory response and airway dysfunction.

Aim 1. To establish a method to knockdown HB-EGF in a CD4 selective manner.

Aim 2. To study the role of membrane-bound HB-EGF in mediating CD4 T cell interaction with DCs.

Aim 3. To explore the role of HB-EGF synthesized by CD4 T cells in pathophysiology of allergic asthma.

Hypothesis 2. Similar morphological features of MNTs formed by CD4 T cells to neural outgrowths suggests the same mechanistic pathway for induction of their formation.

Aim 1. To investigate the molecular pathway inducing the formation of MNT-like structures by CD4 T cells in interaction with ASMCs.

Aim 2. To examine the possibility that MNTs allow the transfer of material between ASMCs and CD4 T cells.

CHAPTER 2

Pathophysiological Role of HB-EGF Synthesized by CD4 T Cells

2.1 Prologue

In this study, we aimed to investigate the pathophysiological and immunological role of CD4 T cell derived HB-EGF in the context of acute allergic asthma in an experimental murine model. CD4 T cells have been shown to express EGFR ligands, HB-EGF as well as amphiregulin. The expression of amphiregulin by T_H2 cells has been attributed to intestinal epithelial repair in helminth infection; however, there is no defined role for HB-EGF synthesized by CD4 T cells. Therefore, we opted to elucidate synthesis and function of HB-EGF by CD4 T cells in AHR and pulmonary inflammation following allergen sensitization and challenge. We hypothesized that HB-EGF of CD4 T cell origin exacerbates inflammation, AHR and lung function in an acute model of asthma.

2.2 Abstract

CD4 T cells express the epidermal growth factor (EGF) receptor ligand, heparin binding EGF (HB-EGF) with no defined immune-pathophysiological function. Therefore, we wished to explore the function of HB-EGF synthesized by CD4 T cells in its interaction with dendritic cells as well as in the context of allergic pulmonary inflammation and the asthma surrogate, airway hyperresponsiveness, in a murine acute model. Here, we show CD4 T cell membrane-bound HB-EGF facilitates interaction with dendritic cells and enhances follicular helper T cell differentiation as well as T_H2 response in the lung in response to allergen sensitization and challenge. Activation stimuli increases expression of HB-EGF by CD4 T cells which regulates T_H2 properties. HB-EGF deficiency in CD4 T cells *in vivo* attenuates interleukin-5 (IL-5) synthesis in the lung that is accompanied by diminished eosinophilic inflammation and airway hyperresponsiveness. HB-EGF co-immunoprecipitates with the transcriptional repressor B cell lymphoma 6 (Bcl-6) in CD4 T cells. Knocking down HB-EGF in CD4 T cells results in increased Bcl-6 binding to the IL-5 gene and decreased IL-5 mRNA expression. Thus, these findings suggest immunoregulatory functions for intrinsic HB-EGF expressed by CD4 T cells in T_H2 inflammation and airway dysfunction by modulating T_H2 driven immune response and IL-5 expression via binding to and inhibiting the repressive function of Bcl-6.

2.3 Introduction

CD4 T cells play crucial roles in inflammatory diseases by differentiating into various subsets, each acquiring particular characteristics in the adaptive responses by secreting varying patterns of cytokines, chemokines and growth factors. Typically, αβ T cell receptor expressing CD4 T cells are subdivided into T_H1, T_H2, T_H17, follicular helper cells and regulatory T cells but more recently the classification has been further expanded to include additional subtypes including T_H3, T_H9, T_H23 and T_H25[223]. In addition to the secretion of pro-inflammatory cytokines, T cells express growth factors, which may be anticipated to affect the physiology of the tissues. For example, CD4 T cells synthesize HB-EGF and basic fibroblast growth factor 2 (FGF2b) in human[171]. T_H2 cells synthesize amphiregulin which contributes to intestinal epithelial proliferation in a murine model of helminth infection, facilitating the clearance of parasite[102].

HB-EGF signals through ErbB1 and ErbB4 directly and indirectly via ErbB2 and ErbB3. HB-EGF is produced as a transmembrane pre-formed protein; while the heparin binding domain of HB-EGF facilitates cell-cell interaction by binding to heparan sulfate proteoglycans (HSPGs) on the cell membrane, the extracellular domain is cleaved by MMPs and members of the ADAM family of sheddases which results in formation of soluble HB-EGF (sHB-EGF) and cytoplasmic HB-EGF [224]. Upon ectodomain shedding, the cytoplasmic domain of HB-EGF binds to and inhibits the function of gene transcriptional repressors, Bcl-6 and promyelocytic leukemia zinc finger (PLZF) [203,225,226]. However, HB-EGF interaction with Bcl-6 in immune cells has not been documented.

CD4 T cells acquire immunoregulatory functions upon interaction with DCs. This interaction takes place in several ways including direct contact which is crucial for formation of the immunological synapse. Antigen presentation to CD4 T cells takes place at the interface of the immunological synapse, the formation of which is facilitated by adhesion molecule LFA-1 on T cells[227]. The strength and duration of the physical contact between DCs and CD4 T cells may determine the differential fate of the CD4 T cells; prolonged interaction has been shown to induce differentiation toward T_{FH}[228-230]. Due to the fact that membrane-bound HB-EGF may potentially enhance cell-cell interaction via the heparin binding domain, we sought the possibility of its involvement in the interaction between CD4 T cells and DCs.

Transcriptional repressor Bcl-6 is a sequence specific multifunctional regulator of lymphocyte differentiation and immune responses for both T and B cells. Bcl-6 is a master regulator of follicular helper T cell differentiation as well as the expression of T_H2 cytokine genes[231]. Bcl-6 has been shown to have a consensus binding site for IL-4 and IL-5 genes[232,233]. Bcl-6 deficient mice develop spontaneous eosinophilic inflammation in the lung and in vitro activated CD4 T cells from Bcl-6 deficient mice have exaggerated mRNA expression for IL-4, IL-5 and IL-13[234]. A T_H1 polarizing milieu induces higher levels of Bcl-6 protein expression by CD4 T cells compared to T_H2 skewing conditions at the early stages of polarization[235].

T_H2 cytokines and chemokines such as, IL-3, IL-4, IL-5, IL-13, GM-CSF, eotaxin and RANTES have a pivotal role in eosinophilic inflammation in asthma[236]. IL-5 is crucial for the development of eosinophils from bone marrow progenitors as well as promoting their migration, activation and survival[237]. Increased IL-5 mRNA expression in the bronchoalveolar lavage fluid correlates with eosinophilic inflammation in asthmatic patients; whereas neutralization of IL-5 has been effective in reducing asthma exacerbations[65,238].

The aim of this study was to investigate the immunological and pathophysiological properties of HB-EGF synthesized by CD4 T cells. In the current study, we found that in interaction with DCs membrane-bound HB-EGF in CD4 T cells co-localizes with the marker of the immunological synapse, CD3, and facilitates the physical interaction which translates into enhanced T_{FH} differentiation and synthesis of T_H2 cytokines in the lung. Ovalbumin sensitized and challenged HB-EGF $^{lox/lox}$CD4CreER^{T2} mice with HB-EGF-deficient CD4 T cells display reduced AHR, lung levels of IL-5 and eosinophilia. Our results further suggest that decreased IL-5 expression by HB-EGF deficient lung resident T_H2 cells may be attributed to diminished interaction of HB-EGF and Bcl-6, resulting in increased binding of Bcl-6 to IL-5 gene. We also confirmed in human a higher frequency of HB-EGF expression by peripheral IL-5 producing T_H2 cells from asthmatic subjects compared to non-asthmatics.

2.4 Methods

Animals

CD4CreER^{T2} mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). HB-EGF lox knock-in mice were purchased from the RIKEN Institute (Wako, Japan). Bcl-6$^{lox/lox}$CD4cre and DO.11.10 mice were kindly provided by Dr. Irah King and Dr. Elizabeth Fixman (McGill University, Montreal, Canada), respectively. Male BALB/c and C57BL/6J mice, 8-10 weeks of age, were purchased from Charles River Laboratories. Animals were bred in a conventional animal care facility at the McGill University Health Center Research Institute. Protocols and procedures were approved by an institutional animal care committee and were in conformity with the standards of the Canadian Council of Animal Care.

Antibodies and chemicals

Anti-mouse CD16/32, FITC, PerCP and APC-conjugated anti-mouse CD4, V500-conjugated anti human CD4, PE and PerCP-conjugated anti-mouse CD11c, PE-Cy7-conjugated anti-mouse MHCII, brilliant violet 421-conjugated anti-mouse CD69, PE-conjugated anti-mouse CD86, Alexa Fluor 647-conjugated anti-mouse GL7, brilliant violet 510-conjugated anti-mouse IgD, PE-Cy7-conjugated anti-mouse CD19, APC and brilliant violet 421-conjugated anti-mouse CXCR5, brilliant violet 510-conjugated anti-mouse CD11b, APC-conjugated anti-mouse IL-5 and brilliant violet 421-conjugated donkey anti rabbit IgG were purchased from BioLegend. efluor 710-conjugated anti mouse Siglec-F was purchased from Thermo Fisher Scientific. Anti-CD3, anti-CD28 and anti-IL-4 antibodies were purchased from Becton Dickinson. Rabbit anti-HB-EGF polyclonal antibody was purchased from Bioss Antibodies. Ovalbumin (Ova), bovine serum albumin (BSA), phorbol myristate acetate ^{239}, ionomycin, GM6001, tamoxifen free base, 4-hydroxy tamoxifen (4HT) and peanut oil were purchased from Sigma Aldrich. Antibody against C-terminal domain of HB-EGF and an anti-Bcl-6 antibody were purchased from Santa Cruz Biotechnologies. Rabbit and mouse IgG were obtained from Cell Signaling Technology.

Allergic sensitization and challenge

BALB/c, C57/BL6 and Bcl-6$^{lox/lox}$CD4cre were sensitized with Ova (100 μg) and aluminum hydroxide (1 mg), Al (OH)3 (Fisher Scientific) in 100 μl of phosphate-buffered saline (PBS) (Invitrogen) by intraperitoneal injection (i.p) on days 1 and 7. One week following the second sensitization, under light isoflurane anesthesia mice were either challenged on three consecutive days with Ova (50 μg) via intranasal instillation or their spleens were collected without challenge. Control mice were sensitized intraperitoneally with Ova and challenged with PBS. Forty-eight hours following the third challenge, mice were euthanized and lungs were harvested. In order to study the *in vivo* effect of HB-EGF synthesized by CD4 T cells on interaction with DCs, either tamoxifen or peanut oil was administered to HB-EGF$^{lox/lox}$CD4CreER^{T2} mice for 5 consecutive days. 5 days following the last dose of tamoxifen animals were sensitized with Ova on days 10 and 17. On day 24 mice were euthanized and mesenteric lymph nodes and blood were collected. T_H2 cytokines in the BAL were measured after challenging animals with Ova intranasally. To examine the role of CD4 T cell derived HB-EGF on allergic airway response, HB-EGF$^{lox/lox}$CD4CreER^{T2} and C57BL/6 mice were sensitized with Ova on day 1 and 7. On day 13 mice were treated with tamoxifen free base (Sigma Aldrich) administered in peanut oil and 10% ethanol (5 mg/animal) by gavage for 5 consecutive days. Vehicle was administered to control mice. Five days after the last tamoxifen dose animals were challenged with Ova intranasally for 3 consecutive days. All experiments were conducted 48 hours following the last challenge.

Tissue collection and cell culture

Spleens and mesenteric lymph nodes from naive DO.11.10 mice, naïve and Ova sensitized BALB/c mice as well as HB-EGF$^{lox/lox}$CD4CreER^{T2} mice were harvested. Suspensions of splenocytes were prepared by mechanical disruption of the spleen, followed by passaging through 40 μm cell strainers. Red blood cells were lysed with ammonium chloride-potassium lysis buffer (ACK; 0.15 M NH4Cl, 10 mM KHCO3, 0.1 mM EDTA). Cells were cultured in 24 well tissue culture plates in a concentration of 5x10^6 cells/ml in RPMI 1640 supplemented with FBS (10%) and penicillin-streptomycin-amphotericin (PSA) (Life Technologies Inc) and incubated at 37°C with 5% CO_2. DO.11.10 CD4 T cells were stimulated by culturing splenocytes with Ova (200 μg/ml) for 3 days. In order to examine the effect of HB-EGF on DC and T cell interaction, cells were co-cultured for 2 hours at a ratio of 1:2 DCs to T cells with either anti HB-EGF antibody or isotype control at the density of 2x10^6 cell/100 μl in sterile FACS tubes and were spun down in

order to enhance the interaction. Cells were fixed immediately and stained for CD4, CD11c and TCR-β markers. In order to inhibiting IL-4 signaling pathway, anti-IL-4 antibody (10 μg/ml) was administered and cells were cultured for 7 days. Every 3 days ½ of medium was replaced with fresh medium and anti-IL-4 antibody. Splenocytes harvested from Ova sensitized BALB/c mice were incubated with Ova (200 μg/ml) *in vitro* for 4, 6 and 8 days. CD4 T cells were purified by magnetic activated cell sorting (MACS), positive selection, following the manufacturer's instructions (Miltenyi Biotec). As an alternative mechanism of CD4 T cell stimulation, CD4 T cells were isolated from naïve BALB/c and HB-EGF$^{lox/lox}$CD4CreER^{T2} splenocytes by MACS, cultured in anti-CD3 antibody (3 μg/ml) pre-coated plates, and were treated with anti-CD28 antibody (1 μg /ml) for 3 days. In order to knock down HB-EGF, 18 hours following stimulation, 4HT (1.5 μg/ml) prepared in methanol was added to the culture medium of HB-EGF$^{lox/lox}$CD4CreER^{T2} CD4 T cells. Control cells were treated with vehicle. Cells were harvested after 48 hours. Lungs from Ova sensitized and challenged animals were digested in collagenase (*clostridium histolyticum*, Type IA, Sigma; 150 CDU/mL) for 1 hour at 37°C. Cells were passed through 40 μm cell strainers, Red blood cells were lysed with ACK Buffer. Cells were resuspended in RPMI-1640, supplemented with FBS (10%) and PSA.

Bone Marrow Dendritic cell (BMDC) differentiation and T cell co-culture

Bone marrow cells harvested from BALB/c femur and tibia were cultured at the concentration of $2.5x10^6$ cells/ml in 6 well plates for 10 days in RPMI-1640 supplemented with non-essential amino acids (0.1 mM), sodium pyruvate (1 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), FBS (10%), L-glutamine (2 mM) and β- mercaptoethanol (50 μM, Sigma Aldrich) in the presence of GM-CSF (20 ng/ml). On day 3, an additional 3 mL of differentiation cocktail was added to each well. On days 6 and 9 half of the volume from each well was replaced with fresh differentiation cocktail. Non-adherent cells were collected, washed, and then cultured at $5x10^5$ cells/ml in a 6 well plate for 24 hours in the presence of 200 μg/ml Ova. DO.11.10 CD4 T cells were purified from splenocytes and co-cultured with DCs at a ratio of 1:4 DC to T cells for 24 hours, with or without Transwell™, in the presence of polyclonal rabbit anti-HB-EGF (1 μg/ml), isotype control or afatinib (5nM) or vehicle. DCs were incubated with mouse recombinant HB-EGF (100 ng/ml) for 24 hours.

Immunogold staining and transmission electron microscopy

Cells were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer and were post fixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide. Samples were dehydrated in a graded series of concentrations of ethanol and infiltrated with Epon 812 and ethanol mixture, 1:1 and 3:1, followed by 100% Epon 812. Samples were embedded in low viscosity, thermally curing Epon resin. Ultrathin sections (70–100 nm) were cut from the resin blocks by a Reichert-Jung Ultra cut E ultramicrotome with a Diatome (Biel, Switzerland) diamond knife. Sections were incubated with Rabbit anti HB-EGF polyclonal antibody (Bioss) for 1 h (1:10 dilution), washed six times in PBS, incubated with the goat anti-rabbit antibody conjugated with 10-nm gold particles (Jackson ImmunoResearch) and washed six times with PBS. Sections were transferred into 200-mesh Cu transmission electron microscopy (TEM) grids with Formvar support film. Imaging was carried out on a FEI Tecnai 12 TEM equipped with an AMT XR80C CCD camera at an accelerating voltage of 120 kV.

Immunofluorescent staining and confocal microscopy

DCs and CD4 T cells were cultured on coverslips and were fixed with 4% paraformaldehyde. Samples were permeabilized with Triton X-100, 0.3% in PBS, and stained with Armenian hamster anti CD3 [240], PE-conjugated anti TCR-β (Biolegend) and rabbit anti HB-EGF (Bioss) antibodies. Samples then were stained with PE-conjugated goat anti rabbit IgG and brilliant violet 405-conjugated donkey anti Armenian hamster IgG. Coverslips were placed on slides using Fluoroshield mounting medium (Sigma Aldrich). Samples were visualized using a confocal microscope (LSM 700; Zeiss) with a 63X immersion oil objective.

Human cell culture

Healthy and asthmatic subjects were recruited under protocol approved by the Ethics Review Board of the McGill University Health Centre. Blood was collected following informed consent. Peripheral blood mononuclear cells (PBMCs) were collected using Ficoll-Paque PLUS (GE Healthcare). CD4 T cells were purified using MACS, positive selection, and stimulated with anti-CD3 (2 μg/ml) and anti-CD28 (1 μg/ml) in RPMI 1640 supplemented with 10 % FBS and 1% PSA for 72 hours.

Intracellular cytokine assessment by flow cytometry

To assess intracellular cytokines, cells were stimulated with PMA (75 ng/ml) and ionomycin (1 μM) in the presence of GolgiPlug for 4 hours. Cells were stained with viability dye, eFluor780. Following blocking with anti-mouse CD16/32, cells were stained for surface markers, CD4, CD11b, Siglec-F and CD11c in FACS buffer (3% FBS in PBS). After fixation and permeabilization using Cytofix/Cytoperm^{TM} and Perm/Wash buffer (BD Biosciences), cells were stained for intracellular markers: IL-5 and HB-EGF. Stained cells were analyzed by LSRFortessa cell analyzer (BD Biosciences) and Flowjo software (Tree Star Inc.).

Quantitative PCR (qPCR)

Total RNA from purified CD4 T cells was extracted, using RNeasy mini kit (Qiagen) according to manufacturer's instructions. Reverse transcription was performed using Affinity Script qPCR cDNA synthesis kit and using oligo dT primers according to manufacturer's instructions (Agilent Technologies). qPCR was performed using iTaq SYBR green supermix containing ROX (Bio-Rad). cDNA was amplified using Step-One-Plus machine (Applied Biosystems). Relative mRNA expression was calculated using the "ΔΔCt" method. Data were normalized to the housekeeping gene, S9. Results were expressed as a fold change over the control.

Protein extraction and ELISA

In order to measure HB-EGF produced by CD4 T cells, following incubation of the cells with Ova, cells were purified by MACS and cultured at the concentration of $5x10^6$ cells/ml in ultra-low binding plates with PMA (75 ng), ionomycin (1 μM) and GM6001 (25 μM). After 4 hours, cells were harvested and culture medium was collected and stored at -80°C for further analysis by ELISA. Cell lysate was prepared using lysis buffer (Nacl (250 mM), EDTA (5 mM), NaF (50 mM), Na3VO4 (1mM), NaN3 (0.02%), sodium deoxycholate (0.5%), Tris-Cl (50 mM), NP-40 (1%), PMSF (1mM) and proteinase inhibitor cocktail (Sigma-Aldrich). HB-EGF was quantified using a mouse HB-EGF ELISA kit (USCN Life Science Inc.)

Assessment of respiratory system mechanics and airway responsiveness to methacholine

Mice were sedated with xylazine hydrochloride (8mg/kg, i.p.) and anesthetized with sodium pentobarbital (50mg/kg i.p.). A tracheostomy was performed using an 18-gauge cannula which was subsequently used to connect the mouse to a small animal ventilator (flexiVent FX1, Scireq, Montreal, QC, Canada). Mice were ventilated with a tidal volume of 10 mL/kg, maximum inflation pressure of 30 cmH20, a positive end expiratory pressure (PEEP) of 3 cmH2O and a frequency of 150/min. Rocuronium pentabromide (0.2mg/kg, i.p.) was administered to induce muscle paralysis and prevent spontaneous breathing. Airway responsiveness was measured by exposing mice to increasing concentrations of methacholine in PBS (0, 6.25, 12.5, 25 and 50 mg/mL), using an inline nebulizer. The software took 6 measurements per dose and used the constant phase model to compute changes in respiratory system mechanics.

Bronchoalveolar lavage fluid (BALF) and serum preparation

Following the assessment of respiratory system mechanics, 1 mL of sterile PBS was injected into the lungs through the tracheal cannula and the recovered fluid was kept on ice. BALF was centrifuged at 1500 rpm for 5 min at 4°C and the supernatant was collected for analysis at -80°C. The cell pellet was re-suspended in 500 µl sterile PBS. Cells were counted using a hemocytometer. Cells were mounted on slides using a cytocentrifuge (Shandon) and stained with Diff-Quik (Medical Diagnostics). Differential cell counts were evaluated by identifying 300 cells per slide based on morphology. Blood was collected via cardiac puncture and transferred to a 1.5mL Eppendorf tube. After centrifugation at 3000 rpm for 10 min at room temperature with slow deceleration, the serum was removed and stored at -80C.

Cytokine/chemokine multiplex bead assay of BAL

Cytokine levels in BAL were measured using a Milliplex map mouse cytokine/chemokine magnetic bead-based kit and data were acquired on a Luminex FLEXMAP 3D system (Millipore).

Histological analysis

Paraffin-embedded lungs sections were stained with periodic acid–Schiff. The staining was performed with a PAS staining kit (Merck, Darmstadt, Germany). Quantification of goblet cell

hyperplasia was performed by counting the number of cells and standardizing for airway size by dividing the count by the perimeter of the basement membrane ($\mathbf{P}_{BM}$). $\mathbf{P}_{BM}$ was measured with the Image J software. Lung inflammation was scored for cellular infiltration around the airways: 0, no infiltrates; 1 layer of inflammatory cells; 2, 2 layers of inflammatory cells; 3, 3 layers of inflammatory cells and 4, 4 and more layers of inflammatory cells.

Immunoblotting and immunoprecipitation

CD4 T cell were purified by MACS and stimulated with anti-CD3 and anti-CD28 for 3 days. Cells were lysed in RIPA lysis buffer (1% NP-40, 150mM NaCl, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 50 mM Tris pH 8 and 1mM EDTA) supplemented with anti-phosphatase and protease inhibitor cocktail (Sigma-Aldrich). Protein A/G beads (Life Technologies) and either anti-HB-EGF antibody or IgG2a isotype control were added to 500µg of protein extract. Samples were incubated overnight at $4^{\circ C}$ with gentle agitation. Immunoprecipitates were washed 3 times with lysis buffer and boiled in 2x Laemmli buffer. Samples were resolved by SDS-PAGE and electro-transferred onto polyvinylidene difluoride membrane (Bio-Rad). The membrane was blocked with BSA (5%) and incubated overnight at $4^{\circ C}$ with either anti-HB-EGF or anti-Bcl-6 primary antibodies. Membranes were incubated with HRP-conjugated secondary antibodies for 1 hour at room temperature. Signal was detected using clarity ECL kit (Bio-Rad) and visualized by Chemidoc MP system (Bio-Rad).

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using an EZ-Magna ChIP A/G chromatin immunoprecipitation kit (EMD Millipore), following the manufacturer's instruction. CD4 T cells were purified by MACS from spleens of HB-EGF$^{lox/lox}$CD4CreER^{T2} mice and stimulated with anti CD3 (3 µg/ml) and anti CD28 (1 µg/ml) for 24 hours. CD4 T cells were treated with either 4HT (Sigma-Aldrich) dissolved in methanol (1.5 µg/ml) or the vehicle for 48 hours. 1.5 x10^6 live cells were fixed with 1% formaldehyde and lysed with lysis buffer. Cell lysates were sonicated using a 150T digital sonic dismembrator (Fisher Scientific). Immunoprecipitation was performed with either rabbit anti mouse Bcl-6 antibody, 5 µg, (Santa Cruz) or normal rabbit IgG (Cell Signaling Technology) as control. Purified DNA was subjected to qPCR for IL-5 binding site for Bcl-6 using following

primer sequence: 5′-TGGGCCTTACTTCTCCGTGTAACT-3′ (forward), 5′-CTCCAGTGACCCTGATACCTGAAT-3′ (reverse)[233]. The fold-enrichment was quantified by normalizing the specific antibody enriched chromatins against the nonspecific IgG enriched chromatins.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad). All data are expressed as means plus or minus one standard error of the mean with at least 2 independent experiments. To test for statistical differences among more than 2 groups, one-way ANOVA with Tukey's post hoc test and two-way ANOVA with Bonferroni post-test were applied, as appropriate. To compare 2 groups, Student's t-tests were performed. P values <0.05 were considered significant.

2.5 Results

Stimulation of CD4 T cells upregulates HB-EGF expression

The Ova transgenic DO.11.10 CD4 T cells expressed mRNA for EGF, HB-EGF, amphiregulin, epiregulin, β-cellulin, TGF-α and neuregulins 1-4, in the absence of stimulation. Following stimulation with Ova, the mRNA for HB-EGF increased whereas those for the other tested EGFR ligands either did not change or were significantly lower than in unstimulated cells (Fig. 1a). To confirm that HB-EGF mRNA expression could be induced in non-transgenic mice, purified CD4 T cells from the spleen of BALB/c mice were stimulated with anti-CD3 and anti-CD28 antibodies (Fig. 1b). Additionally, splenocytes from *in vivo* Ova sensitized BALB/c mice were challenged with Ova for 4, 6 and 8 days *in vitro* and subsequently CD4 T cells were purified (Fig. 1c). In both instances HB-EGF mRNA expression was significantly increased compared to control.

We next quantified HB-EGF production at the protein level in both cell lysates and supernatant of DO.11.10 CD4 T cells after Ova stimulation. HB-EGF release occurs following activation of matrix metalloproteinases (MMPs)[241-244]; we also examined if release was affected by GM6001, a broad spectrum MMP inhibitor. We additionally used PMA and ionomycin stimulation as a control to measure soluble HB-EGF since the stimuli induces shedding of its N-terminal domain from cell membrane, possibly by activating MMPs[245-247]. The HB-EGF concentration in cell lysates from Ova stimulated CD4 cells was significantly higher than in control unstimulated cells; PMA and ionomycin did not affect intracellular HB-EGF synthesis (Fig. 1d)c. The medium from CD4 T cell cultures after 4 hours demonstrated that HB-EGF release was induced by PMA and ionomycin, while inhibited by GM600l (Fig. 1e). The biased differentiation of T_H2 was confirmed by increased GATA3 expression in the Ova stimulated condition (Supplementary fig. 1a). We next examined the effect of inhibition of T_H2 differentiation on HB-EGF synthesis. Ova stimulated CD4 T cells synthesized less HB-EGF when treated with anti-IL-4 neutralizing antibody (Fig. 1f). To safely conclude that increased HB-EGF synthesis is induced by Ova protein and not its LPS contamination, we used Ova peptide instead which similarly resulted in increased HB-EGF protein synthesis in the cell lysate (Supplementary figure 1b).

HB-EGF co-localizes with CD3 on CD4 T cell interacting with DCs.

HB-EGF presence on the CD4 T cell membrane was visualized by immunogold labeling of N-terminal domain of protein (Fig. 2a). HB-EGF has the potential to serve as an EGFR ligand or adhesion molecule by binding to negatively charged heparan sulfate proteoglycans[248]. To visualize the localization of membrane bound HB-EGF in CD4 T cells interacting with DCs we performed confocal microscopy. Images showed that HB-EGF was present in the contact site, co-localized with CD3 (Fig. 2b).

HB-EGF facilitates the interaction between CD4 T cells with DCs and enhances activation of both cell types

We next investigated the immunological role of HB-EGF in the interaction between CD4 T cell and DC. Anti-HB-EGF neutralizing antibody, against the N-terminal domain of protein covering heparin binding domain, reduced the aggregate formation of CD4 T cells and DCs *in vitro* (Fig. 3a), Compared to the physically separated condition by Transwell, direct contact of Ova loaded DCs with Ova specific TCR bearing CD4 T cells induced expression of the early activation marker, CD69, (Fig. 3b) in T cells and augmented MHCII and CD86 expression in DCs (Fig. 3d and f); these outcomes were inhibited by an anti HB-EGF neutralizing antibody in the first 24 hours. Treating cells with EGFR tyrosine kinase inhibitor, afatinib, confirmed that HB-EGF mediated activation of CD4 T cells and DCs was EGFR independent (Fig. 3c, e and g).

In vivo, interfering of CD4 T cell and DC interaction diminishes differentiation of T_{FH} cells and T_H2 cytokines in Ova induced acute model of asthma

The kinetics and strength of interaction between CD4 T cells and dendritic cells are key factors for T_{FH} cell differentiation. *In vivo* knocking out of HB-EGF in CD4 T cells prior to sensitization reduced the T_{FH} frequency in the mesenteric lymph nodes measured as $CD4^+$, $CXCR5^+$, and $Bcl\text{-}6^+$ population (Fig 4b). Additionally, the population of germinal center (GC) B cells ($CD19^+$, $GL7^+$, IgD^-) and the level of IgG1 in the serum were reduced (Fig. 4c and d); however, serum IgE was unchanged (Fig. 4e). following intranasal challenge of mice with Ova, IL-4 and IL-5 in BAL were diminished in knockout mice (Fig. 4f and g).

Mice with CD4 T cells deficient in HB-EGF mount an attenuated airway inflammation and hyperresponsiveness

To investigate the immune-physiological role of HB-EGF synthesized by CD4 T cells, a novel strain of mice, HB-EGF$^{lox/lox}$CD4CreER^{T2}, was generated and the efficiency of HB-EGF knockout in CD4 T cells purified from spleen was assessed by measuring mRNA expression and qPCR (Supplementary fig. 2). Mice were sensitized with Ova, HB-EGF knockout in CD4 T cells was performed by administration of tamoxifen followed by intranasal challenge of mice with Ova (Fig. 5a). Goblet cell hyperplasia and lung inflammation were lower in HB-EGF$^{lox/lox}$CD4CreER^{T2} mice (Fig 5b, c and d). Reduced eosinophils in the BAL and lung tissue from HB-EGF knockout animals were confirmed by differential cell count and flow cytometry (CD11c^{-}, CD11b^{+}, Siglec-F^{+}), respectively (Fig. 5e and f).

Eosinophilia has been associated with effector functions of T_H2 immune response through synthesis and secretion of IL-5, essential for eosinophil differentiation and trafficking[239]. To examine the role of HB-EGF by CD4 T cells in IL-5 synthesis, HB-EGF$^{lox/lox}$CD4CreER^{T2} mice were sensitized and challenged with Ova and IL-5 protein in the lung was measured by flow cytometry and by a multiplex assay. The frequency and total cell number of IL-5 expressing CD4 T cells were reduced in the lung of mice with HB-EGF deficient CD4 T cells (Fig. 5g and h). In addition, IL-5 cytokine in BAL was present in lower levels (Fig. 5i).

A reduced immune response to allergen and inflammation may attenuate AHR in the acute model of asthma [249,250]. Hence, we examined respiratory mechanical properties in response to increasing doses of inhaled methacholine which demonstrated that knocking down HB-EGF in CD4 T cells attenuated AHR in the peripheral lung, shown as tissue elastance (H) (Fig. 5j). To exclude the possible effect of tamoxifen on airway inflammation and hyperresponsiveness wild type C57BL/6J mice were treated with the same protocol. AHR and eosinophilic inflammation, IL-5 in BAL and AHR were unchanged by tamoxifen administration (Supplementary fig. 3a, b and c)

IL-5 mRNA expression by T_H2 cells is modulated via HB-EGF interaction with Bcl-6

The HB-EGF C-terminal domain of HB-EGF is shown to bind to and repress Bcl-6 function in non-immune cells[203,225]. Bcl-6 is a transcriptional repressor of IL-5 gene in T_H2 cells[232]. We

aimed to investigate whether HB-EGF in CD4 T cells could induce transcription of the IL-5 gene by antagonizing the effects of Bcl-6. Consistent with the hypothesis *in vitro* HB-EGF knockdown in anti-CD3 and anti-CD28 stimulated CD4 T cells resulted in reduced IL-5 mRNA expression (Fig. 6a and b). Co-immunoprecipitation of HB-EGF and Bcl-6 confirmed their interaction in CD4 T cells stimulated with anti-CD3 and anti-CD28 antibodies (Fig. 6c). Deficiency of HB-EGF in CD4 T cells enhanced Bcl-6 binding to the IL-5 gene assessed by CHIP assay (Fig. 6d).

To determine whether Bcl-6 is involved in allergic airway inflammation and disfunction via repressing IL-5 synthesis, Ova sensitized and challenged Bcl-$6^{lox/lox}$CD4Cre mice were shown to develop greater eosinophilia (Fig. 6e), IL-5 level in the serum (Fig. 6f) as well as AHR (Fig. 6g).

HB-EGF expression by T_H2 cells from asthmatic patients is greater than healthy subjects

To determine the association between IL-5 synthesis and HB-EGF expression in human CD4 T cells, we examined CD4 T cells from asthmatics which showed a higher level of HB-EGF expression by IL-5 expressing T_H2 cells compared to healthy subjects after *in vitro* stimulation (Fig. 7).

Figure 1. Stimulation induces increased expression of HB-EGF by CD4 T cells. (a) splenocytes from DO.11.10 mice were stimulated with Ova *in vitro* for 3 days and purified CD4 T cells were examined for expression of EGFR ligands by RT-qPCR (n=3-4). (b) purified CD4 T cells from BALB/c mice were stimulated with anti-CD3 and anti-CD28 antibodies for 3 days. HB-EGF mRNA expression was examined by RT-qPCR (n=3). (c) splenocytes from Ova sensitized BALB/c mice were stimulated with Ova *in vitro* for 3 days. CD4 T cells were purified and mRNA expression was measured by RT-qPCR (n=4-5). Data are means ± 1 SE. Student's t-test; *P<0.05, **P<0.01. DO.11.10 splenocytes were stimulated with Ova *in vitro* for 3 days. CD4 T cells were purified and treated with PMA, ionomycin and GM6001 for 4 hours. HB-EGF protein was measured (d) in cell lysate and (e) supernatant (n=5-6). Data are mean± 1SE. One-way ANOVA with Tukey posttest; *P<0.05, ***P<0.001. (f) splenocytes from DO.11.10 mice were stimulated with Ova for 7 days with either anti IL-4 antibody or isotype control. Purified CD4 T cells were tested for HB-EGF protein expression by ELISA (n=4). Data are means ± 1 SE. Student's t-test; *P<0.05.

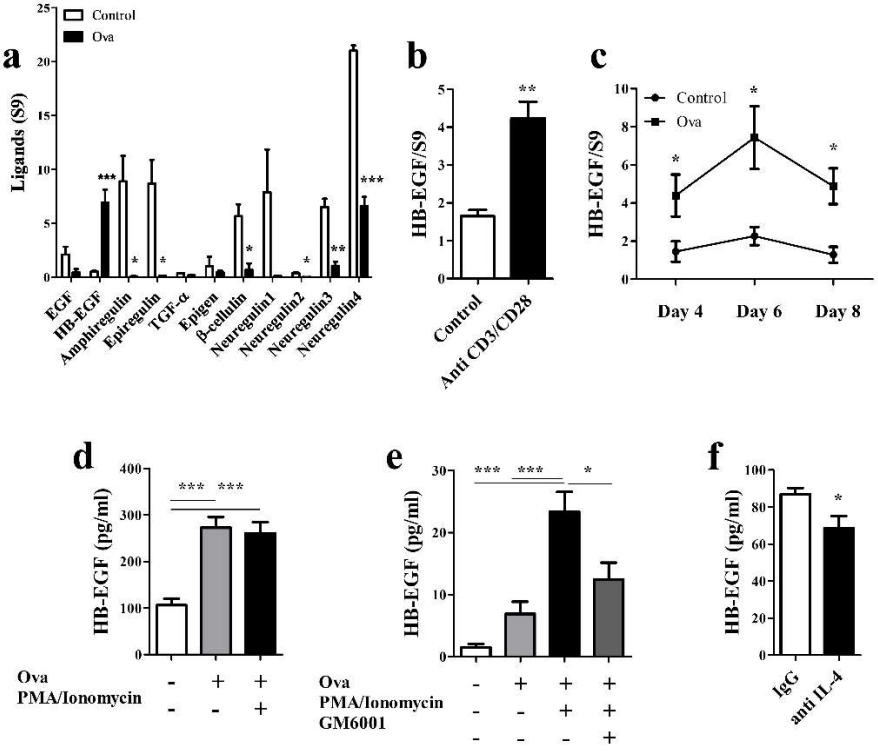


Figure 2. HB-EGF co-localizes with CD3 on CD4 T cells interacting with DCs. (a) the presence of membrane bound HB-EGF on CD4 T cells was confirmed by immunogold labeling and transmission electron microscopy. The arrows indicate the location of 10 nm gold particles. (b) Ova loaded BMDC from BALB/c mice were co-cultured with CD4 T cells purified from DO.11.10 spleens for 12 hours. Following fixation and permeabilization, cells were stained for CD3 (blue) and HB-EGF (red) and visualized by a confocal microscope.

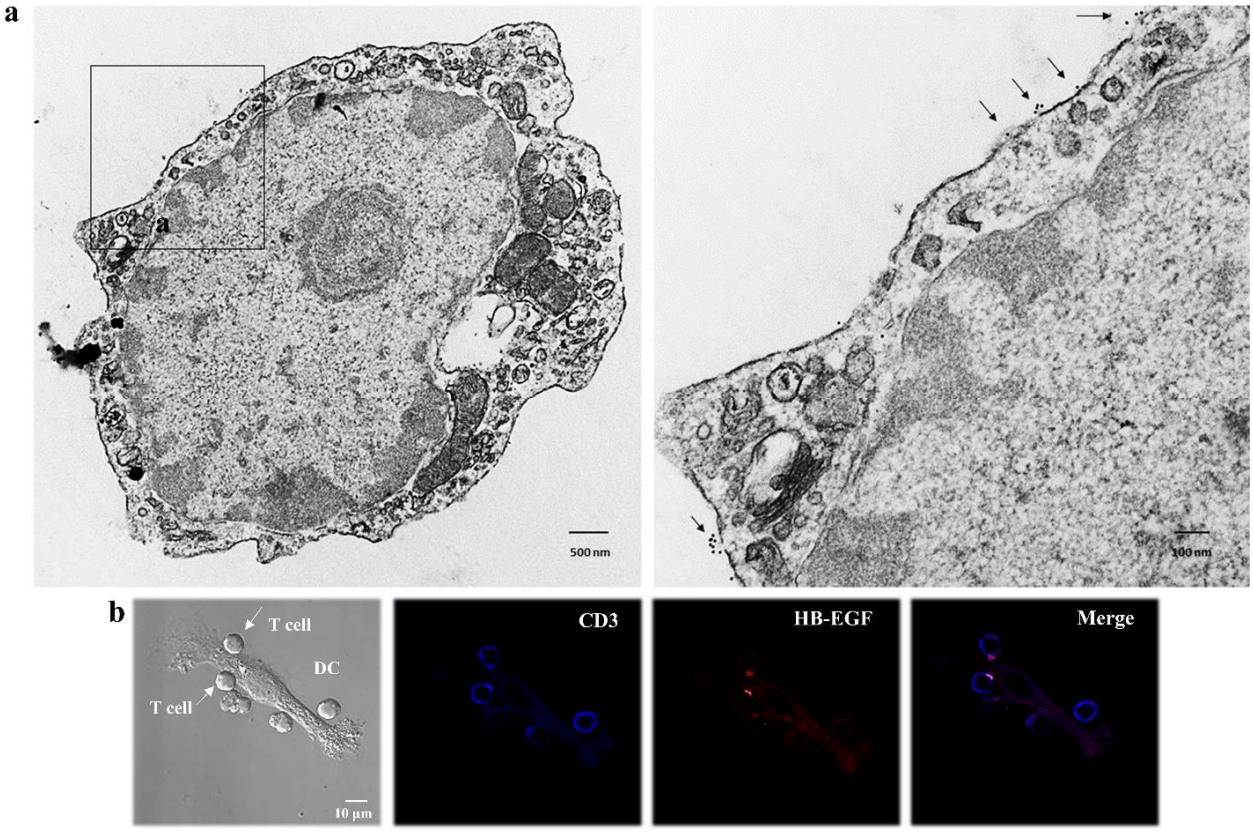


Figure 3. Membrane-bound HB-EGF facilitates the interaction between CD4 T cells and DCs. (a) aggregate formation between CD4 T cells and DCs in *in vitro* co-culture in the presence of either anti HB-EGF neutralizing antibody or isotype control was measured based on the expression of CD4, CD11c and TCRβ by flow cytometry (n=6). (b and c) Ova loaded BMDC from BALB/c mice were co-cultured with CD4 T cells purified from DO.11.10 spleens for 24 hours either in Transwell or directly (CC) with either HB-EGF neutralizing antibody or isotype control (1 µg/ml). In order to examine the involvement of EGFR in HB-EGF mediated CD4 T cell and DC interaction, cells were treated with either afatinib or vehicle. The activation status of CD4 T cells as well as DCs was examined by flow cytometry. The frequency (%) of early activated CD4 T cells was measured by gating viable cells; eFluor780^{-}, followed by CD4^{+}, CD11c^{-} and CD69^{+}; n=8. (d-g) The intensity (MFI) of DC activation markers, MHCII and CD86, was assessed in CD11c^{+} population; n=8, data represent 1 dataset of 2 independent experiments. Data are means±SE. Student's t-test; *P<0.05, **P<0.01, ***P<0.001.

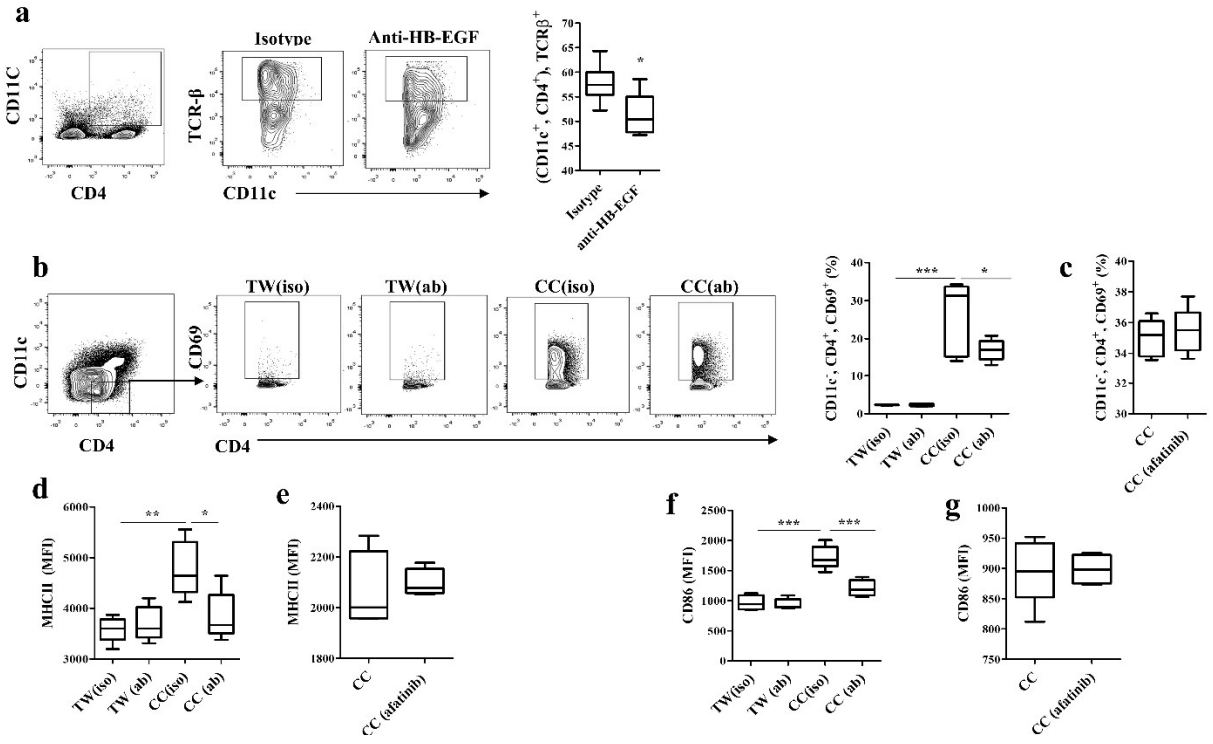


Figure 4. Interfering with CD4 T cells interaction with DCs *in vivo* by knocking out HB-EGF in CD4 T cells prior to sensitization reduces T_{FH} cell population in the mesenteric lymph nodes and TH2 cytokines in the airways in an acute model of allergic asthma. (a) schematic presentation of tamoxifen administration and acute asthma induction in HB-EGF $^{lox/lox}$CD4Cre^{ERT2} mice. (b) T_{FH} cell frequency in the mesenteric lymph nodes was measured by flow cytometry. eFluor780^{-} cells were gated for CD4, CXCR5 and Bcl-6 (n=5). (c) GC B cell frequency was assessed by gating on CD19^{+}, GL7^{+} and IgD^{-} cell (n=5). (d and e) serum IgG1 and IgE were measured by ELISA (n=7-10). (f and g) IL-4 and IL-5 cytokines were measured in BAL following intranasal challenge of mice with Ova (n=8). Data are means±SE. Student's t-test; *P<0.05.

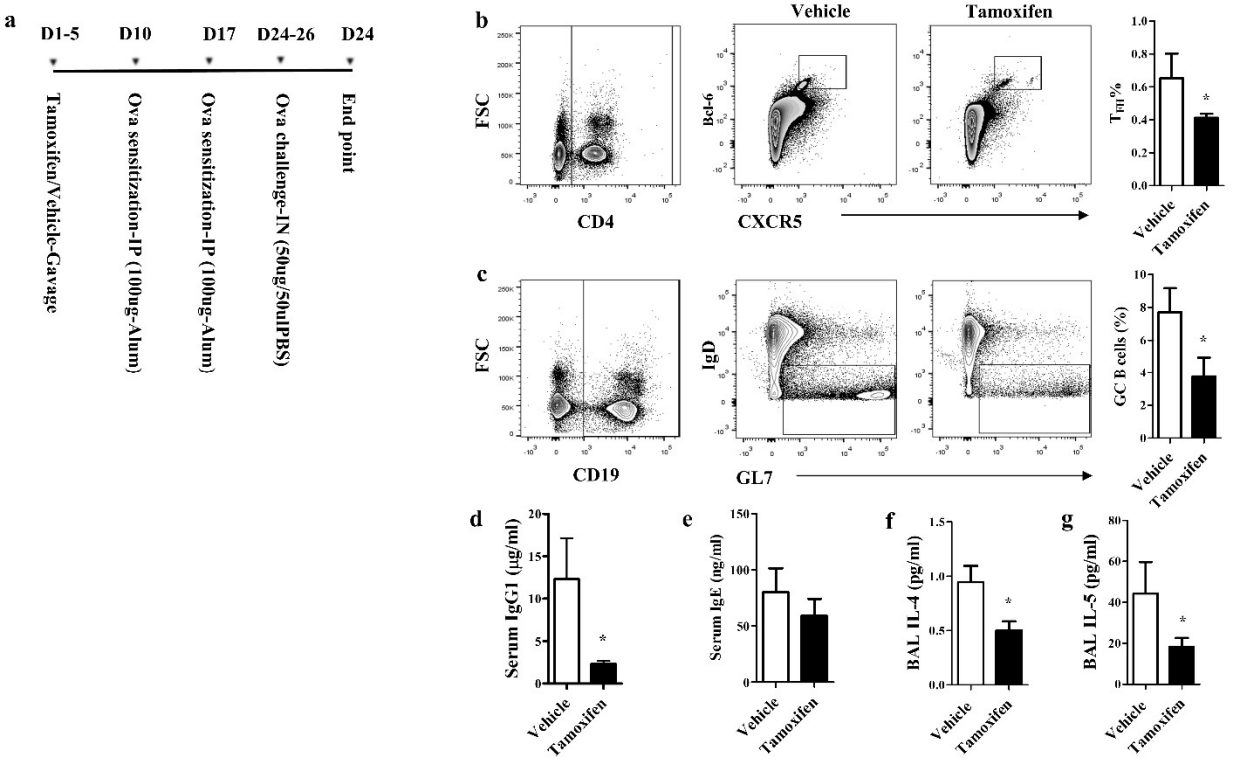


Figure 5. HB-EGF synthesized by CD4 T cells positively contributes to eosinophilic inflammation and AHR in an acute model of asthma. (a) The schematic representation of Ova sensitization and challenge using HB-EGF $^{lox/lox}$CD4Cre^{ERT2} mice. (b) representative PAS stained lung sections from Ova sensitized and challenged HB-EGF $^{lox/lox}$CD4Cre^{ERT2} mice. **(**c) goblet cell hyperplasia and (d) inflammation (n=5) were quantified. (e) Differential cell count was performed for inflammatory cells in BAL (n=7). (f) eosinophil population in the lung tissue was assessed by flow cytometry (n=6). Gating strategy represents CD11b^{+}, Siglec-F^{+} population gated on viable CD11c^{-} cells. Flow cytometric analysis of IL-5 expressing CD4 T cells presented as (g) frequency (%) and (h) total cell number in the lung of Ova sensitized and challenged HB-EGF $^{lox/lox}$CD4Cre^{ERT2} (n=6). eFluor780^{-} viable cells were gated for CD11c^{-}, CD4^{+} population. IL-5^{+} cells were gated within the CD4 T cell population. (i) IL-5 protein was quantified in BAL from Ova sensitized and challenged HB-EGF $^{lox/lox}$CD4Cre^{ERT2} mice (n=7). (j) Ova sensitized and challenged HB-EGF $^{lox/lox}$CD4Cre^{ERT2} mice were examined for lung tissue elastance (H) in response to methacholine 48 hours after the last challenge (n=6-7). Data are shown as the mean ± SE. Student's t-test and two-way ANOVA with Bonferroni multiple comparison; *P<0.05, **P<0.01, ***P<0.001.

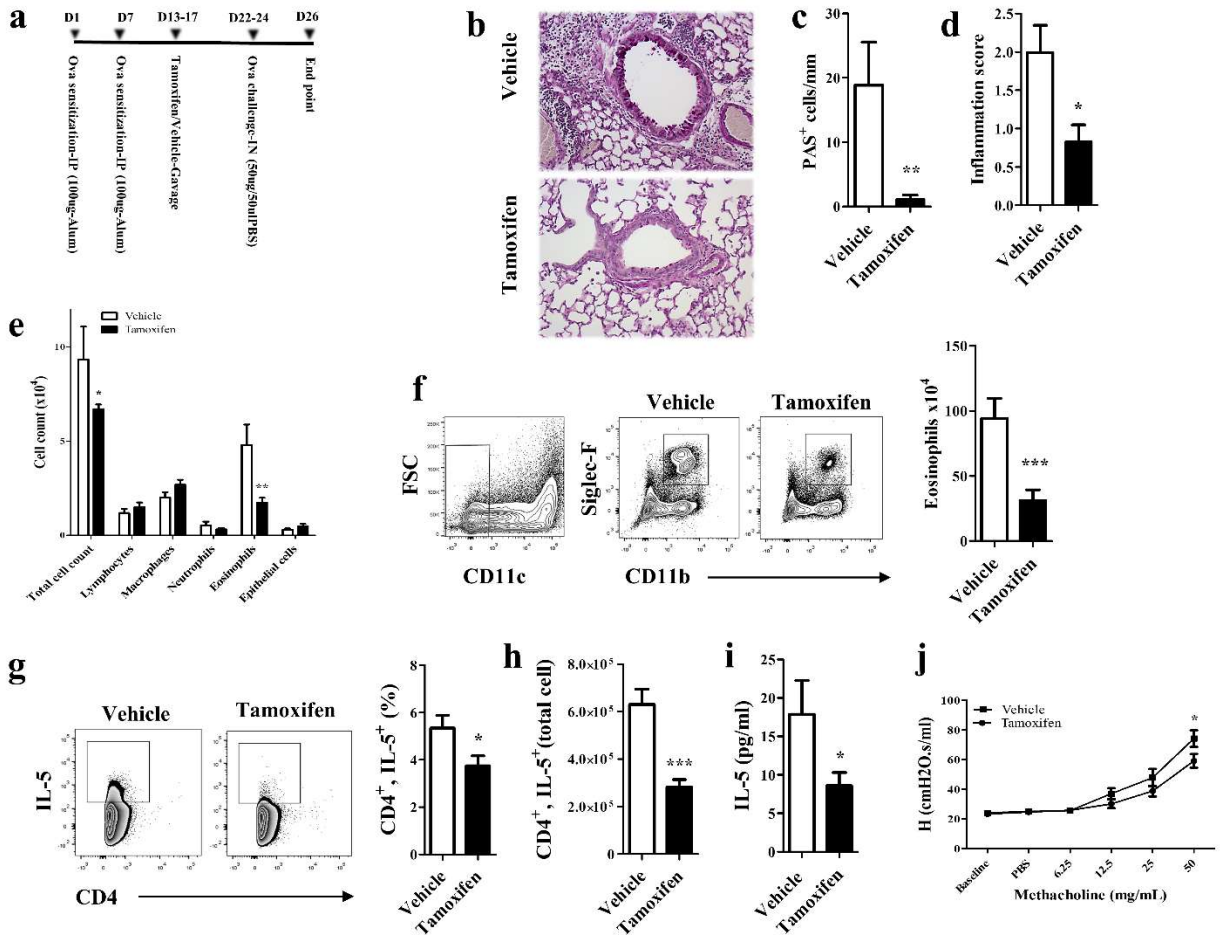

a
D1
D7
D13-17
D22-24
D26
Ova sensitization-IP (100μg-Alum)
Ova sensitization-IP (100μg-Alum)
Tamoxifen/Vehicle-Gavage
Ova challenge-IN (50ug/50ulPBS)
End point
b
Vehicle
Tamoxifen
c
PAS+ cells/mm
Vehicle
Tamoxifen
d
Inflammation score
Vehicle
Tamoxifen
e
Vehicle
Tamoxifen
Cell count (x10⁴)
Total cell count
Lymphocytes
Macrophages
Neutrophils
Eosinophils
Epithelial cells
f
Vehicle
Tamoxifen
FSC
CD11c
Siglec-F
CD11b
Eosinophils x10⁴
Vehicle
Tamoxifen
g
Vehicle
Tamoxifen
IL-5
CD4
CD4+, IL-5+ (%)
Vehicle
Tamoxifen
h
CD4+, IL-5+(total cell)
Vehicle
Tamoxifen
i
IL-5 (pg/ml)
Vehicle
Tamoxifen
j
Vehicle
Tamoxifen
H (cmH2O.s/ml)
Baseline
PBS
6.25
12.5
25
50
Methacholine (mg/mL)

Figure 6. Intracellular HB-EGF regulates IL-5 expression by CD4 T cells via interacting with Bcl-6. (a) CD4 T cells from HB-EGF $^{lox/lox}$CD4Cre^{ERT2} mice spleens were stimulated with anti-CD3 and anti-CD28 antibodies in the presence of either 4-OHT or vehicle (n=8). (b) Knock down of CD4 T cell HB-EGF *in vitro* reduced IL-5 mRNA expression (n=8). (c) CD4 T cells isolated from spleen of wild type mice were stimulated with anti-CD3 and anti-CD28 for 3 days. Immunoprecipitation was performed on the cell lysate using antibody against the C-terminal domain of HB-EGF. Samples were analyzed by immunoblotting for HB-EGF and Bcl-6 (n=3). (d) Analysis of Bcl-6 binding to IL-5 gene by ChIP assay followed by qPCR. To prepare samples CD4 T cells, purified from spleen of HB-EGF $^{lox/lox}$CD4Cre^{ERT2} mice were stimulated with anti-CD3 and anti-CD28 in the presence of either 4-OHT or vehicle. Data are presented as fold enrichment of the IL-5 gene immunoprecipitated by anti-Bcl-6 antibody against IgG enriched chromatin (n=3-4). In an acute model of asthma induced by Ova (e) eosinophilic inflammation in BAL and (f) IL-5 level was measured in HB-EGF $^{lox/lox}$CD4Cre^{ERT2} (n=3-5). (g) AHR was measured as tissue elastance (H) (n=4) (n=3-4). Data are shown as the mean ± 1SE. Student's t-test and two-way ANOVA with Bonferroni multiple comparison; *P<0.05, **P<0.01. UD: undetectable.

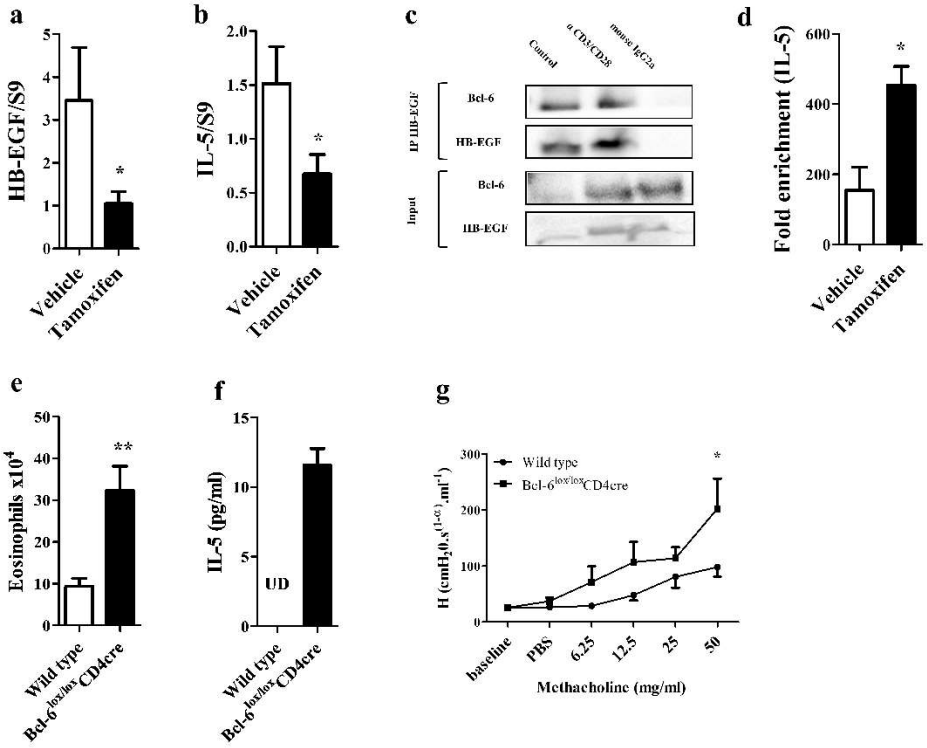
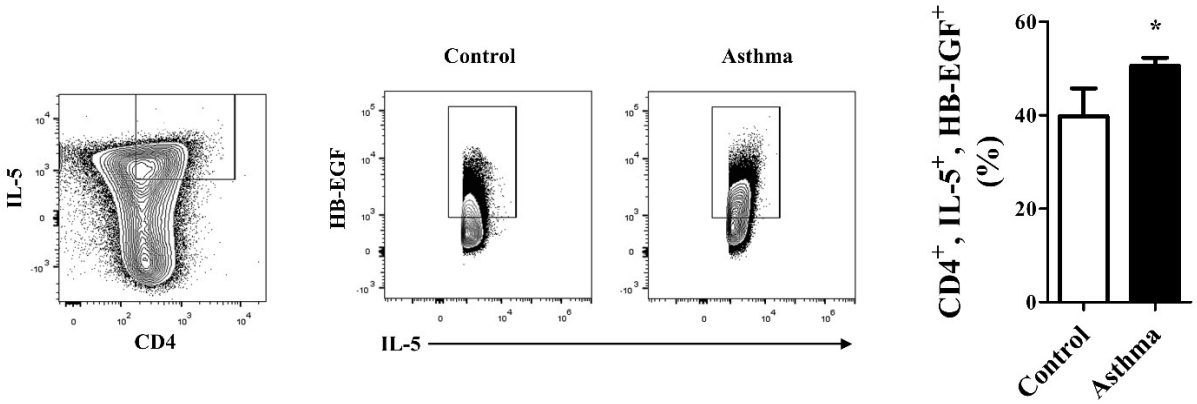
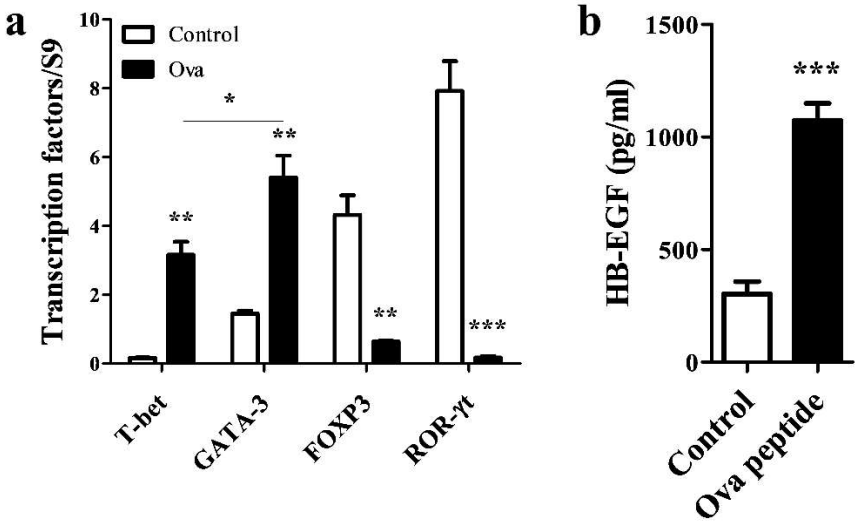


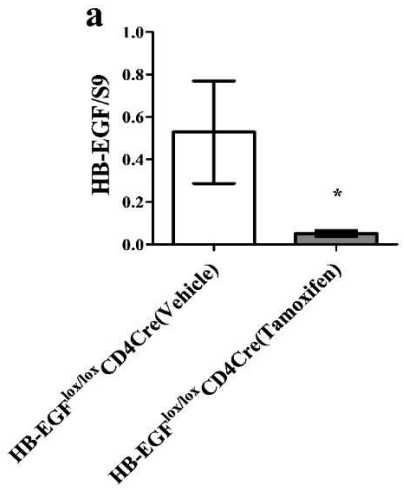
Figure 7. T_H2 cells from asthmatic patients express more HB-EGF. CD4 T cells from PBMC from healthy and asthmatic subjects were stimulated with anti-CD3 and anti-CD28 for 3 days. HB-EGF expression by IL-5 expressing $CD4^+$ T cells population was measured by flow cytometry. eFluor780$^-$ cells were gated for CD4 and IL-5 and HB-EGF (n=4-6). Data are shown as the mean ± 1SE. Student's t-test and two-way ANOVA with Bonferroni multiple comparison; *P<0.05.



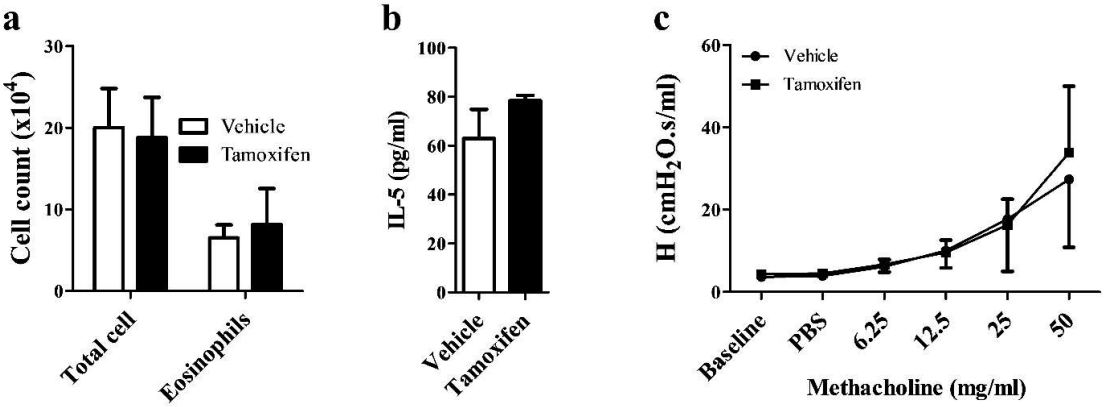
Supplementary figure 1. (a) The differentiation pattern of CD4 T cells from DO.11.10 mice stimulated with Ova *in vitro* based on mRNA expression for signature transcription factor of T_H1 (T-bet), T_H2 (GATA-3), T_H17 (ROR-γt) and T regulatory cells (FOXP3) measured by qPCR(n=3). (b) HB-EGF synthesis by CD4 T cells from DO.11.10 mice stimulated with Ova peptide, 10 µg/ml, in vitro. Splenocytes were stimulated with Ova peptide for 3 days. CD4 T cells were purified and HB-EGF protein in the cell lysate was measured by ELISA (n= 3-4). Data are shown as the mean ± 1SE. One-way ANOVA with Tukey posttest; *P<0.05, **P<0.01, ***P<0.001.



Supplementary figure 2. HB-EGF knockdown in HB-EGF $^{lox/lox}$CD4Cre^{ERT2}. (a) HB-EGF lox/loxCD4Cre mice were treated with either tamoxifen or vehicle by gavage for 5 consecutive days. Data are mean ± 1SE. Student's t-test; *P<0.05.



Supplementary figure 3. The effect of tamoxifen on acute allergic response in wild type mice. C57BL/6J mice were sensitized and challenged with Ova while either tamoxifen or vehicle was administered between sensitization and challenge phase similar to HB-EGF $^{lox/lox}$CD4Cre^{ERT2}mice. (a) total cell count and eosinophilic inflammation were assessed in BAL (n=5). (b) IL-5 was measured in BAL by ELISA (n=3). (c) AHR was measured as tissue elastance in response to increasing doses of methacholine (n=5-6). Data are mean ± 1SE. Student's t-test.



2.6 Discussion

This study has elucidated novel immunological functions for CD4 T cell derived HB-EGF. CD4 T cell interaction with dendritic cells is modulated through membrane-bound HB-EGF which determines the differentiation of CD4 T cells toward T_{FH} cells. Interfering with CD4 T cell interaction with DCs *in vivo* by knocking out HB-EGF prior to sensitization of mice with allergen leads to a decreased population of T_{FH} cells and, as a result, a decreased population of germinal center B cells, IgG1 production and reduced T_H2 cytokines in the BAL. Further, knocking out HB-EGF following the sensitization process abrogates the T_H2 response in a model of allergic asthma. HB-EGF synthesis by CD4 T cells increases in response to various activation stimuli. HB-EGF synthesized by CD4 T cells enhances IL-5 gene expression, contributing to eosinophilia and possibly AHR in a model of acute allergic asthma. Our data suggest that the HB-EGF immunoregulatory mechanism occurs via two mechanisms: facilitating the interaction of CD4 T cells with DCs in the sensitization phase as well as interaction with and suppression of Bcl-6 transcriptional repressive function for IL-5 gene expression in T_H2 cells.

Expression of epidermal growth factors by CD4 T cells has long been documented although their precise functions have received little attention. Amphiregulin synthesis by CD4 T cells has been attributed the function of tissue repair when produced by T_H2 cells and the induction of pulmonary fibrosis when produced by memory T_H2 cells[102,251]. Although HB-EGF synthesis by human CD4 T cells has been reported[171] there is a lack of studies investigating its immune-physiological function. We demonstrated DO.11.10 CD4 T cells, bearing Ova specific TCR, increased both mRNA and protein expression of HB-EGF *in vitro* after stimulation with Ova, while, mRNA expression for other EGFR ligands that we examined did not follow the same pattern. Using GM6001 as a broad spectrum MMP inhibitor, we confirmed the involvement of MMPs in the cleavage and release of HB-EGF from CD4 T cells. PMA and ionomycin, shown to induce HB-EGF shedding from the cell membrane[245-247], was used as a control for enhanced HB-EGF release without affecting the synthesis. mRNA expression for the signature transcription factors of T_H1, T_H2, T_H17 and T_{reg} suggested increased expression of HB-EGF mRNA correlated with a dominant expression of GATA3 as an indicator of T_H2 differentiation. Antibody neutralization of IL-4 during CD4 T cell stimulation reduced HB-EGF synthesis which suggests a mechanistic role for

increased HB-EGF expression by T_H2 cells. HB-EGF mRNA expression by stimulated CD4 T cells was further confirmed in non-transgenic wild type mice.

Membrane-bound HB-EGF has a function as adhesion molecule by binding to heparan sulfate proteoglycans present in cell membrane and extra-cellular matrix[224]. The presence of HSPGs has been shown on DCs[252], supporting a possible role of HB-EGF in the interaction of CD4 cells and DCs. We visualized the availability of membrane-bound HB-EGF on CD4 T cells freshly purified from spleen by immunogold labeling and transmission electron microscopy using an antibody against the N-terminal domain of protein comprising the heparin binding domain. Using confocal microscope, HB-EGF was detected co-localized with CD3 in the contact site with DCs which suggests it may function as an adhesion molecule to strengthen and prolong the interaction. Neutralizing HB-EGF, where CD4 T cells and DCs were co-cultured reduced the aggregates of the two cell types as well as the early activation of both CD4 T cells and DCs in a direct contact dependent manner, confirmed by physically separating cells by Transwells. Using EGFR tyrosine kinase inhibitor, afatinib, suggested the reduced activation of both cell types was EGFR independent. Strong and prolonged interaction of CD4 T cells with DCs enhances T_{FH} cell differentiation[228]. *In vivo* knocking out HB-EGF in CD4 T cells prior to sensitization with Ova reduced frequency of T_{FH} cells in mesenteric lymph nodes and as a consequence reduced population of GC B cells and a diminished IgG1 level in the serum. Intranasal challenge of the mice with Ova following sensitization phase resulted in diminished T_H2 cytokines, IL-4 and IL-5 in the BAL which may be a consequence of reduced IgG1, aberrant antigen uptakes by antigen presenting cells and reduced antigen presentation to CD4 T cells.

To explore the pathophysiological role of HB-EGF by CD4 T cells in allergen induced airway inflammation and AHR we examined the responses of the conditional inducible HB-EGF knockout mice, HB-EGF$^{lox/lox}$CD4CreER^{T2} to sensitization and challenge with Ova. Knocking out HB-EGF in CD4 T cells by tamoxifen following sensitization resulted in modest effects on responses to inhaled methacholine, reflected in reduced dynamic elastance (H) or AHR in the peripheral airways. We observed reduced eosinophilic inflammation and goblet cell hyperplasia in the lung which was associated with a reduced level of IL-5 synthesis by CD4 T cells deficient in HB-EGF, both *in vitro* and i*n vivo*. To exclude an effect of tamoxifen itself on responses to allergen challenge we used wild type mice treated similarly to HB-EGF$^{lox/lox}$CD4CreER^{T2} animals.

Tamoxifen did not affect allergen induced eosinophilic inflammation, IL-5 and AHR. The reduction of eosinophilia may be anticipated to have a number of favorable effects on asthma. Eosinophils may adversely affect airway mechanics by producing lipid mediators such as cysteinyl-leukotrienes, MBP, EPO and IL-13. Furthermore, in long term and chronic sensitization, eosinophils may contribute to the severity of decreased lung function by inducing airway remodeling, hence, increasing the dynamic stiffness of pulmonary tissues[253]. However, the relationship between eosinophilic airway inflammation and AHR is not straightforward. IL-5 deficient mice do not develop eosinophilia and AHR in the pulmonary allergic reaction to Ova[254]. Two different transgenic mice deficient in eosinophils have resulted in contradictory conclusions showing either no role or inducing role in allergic AHR[255,256]. Eosinophils are important for effective Th2 responses so that prolonged deficiency impairs responses and inhibits AHR in response to allergen challenge[36]. The acute neutralization of IL-5 in mice and also a single intravenous administration of anti-IL-5 monoclonal antibody prior to allergen challenge in human although reducing allergen-induced eosinophilia, does not prevent AHR[257,258]. The duration or magnitude of the inhibition of eosinophilia in the current experiment may have been insufficient to have a major impact on AHR. Eosinophil depletion by humanized monoclonal antibodies against IL-5 have been successful in reducing exacerbation rates and allowing a reduction in corticosteroid use in eosinophilic asthma[65,259,260].

Our results suggest that HB-EGF deficiency may be mediated by an unopposed action of Bcl-6 on IL-5 synthesis. Bcl-6 has a binding site on the IL-5 gene and CD4 T cells deficient in Bcl-6 synthesize higher levels IL-5[232,235]. The repressive function of Bcl-6 can be reversed by its binding to the cytoplasmic domain of HB-EGF in non-immune cells[203,225]. In this study we attempted to confirm the interaction between HB-EGF and Bcl-6 in CD4 T cells. Co-immunoprecipitation of HB-EGF with Bcl-6 in wild type CD4 T cells together with increased Bcl-6 binding to the IL-5 gene when CD4 T cells are deficient in HB-EGF suggests a mechanism by which HB-EGF regulates IL-5 synthesis and therefore, eosinophilia. In agreement with the inhibitory interaction of HB-EGF with Bcl-6, Bcl-6$^{lox/lox}$CD4Cre mice sensitized and challenged with Ova developed higher eosinophilia, IL-5 production and AHR.

To determine if IL-5 expression had association with HB-EGF synthesis, a comparison of asthmatics with healthy subjects revealed higher frequency of the T_H2 cells in the peripheral blood

expressing IL-5 that also synthesized higher level of HB-EGF. It would be of interest to investigate whether this increased expression of HB-EGF is due to aberrant genetic or epigenetic properties of CD4 T cells in asthma.

Our data indicate that HB-EGF synthesized by CD4 T cells has an important contributory role in launching adaptive immune response by mediating CD4 T cell interaction with DCs. Besides, in T_H2 cells HB-EGF mediates allergen-induced pulmonary eosinophilia and AHR, potentially by augmenting IL-5 synthesis. The evidence suggests a mechanism of increased IL-5 production is through the interaction of HB-EGF with Bcl-6, thereby inhibiting its repressive action on IL-5 gene expression. The novel immune-regulatory role of HB-EGF synthesized by CD4 T cells provides a more complete understanding of the regulation of CD4 T cell immunological functions which may provide new insight into therapeutic modulation of allergic immune responses.

CHAPTER 3

Basic Fibroblast Growth Factor 2 is a Determinant of CD4 T Cell-Airway Smooth Muscle Cell Communication through Membrane Conduits

3.1 Prologue

Previous studies have shown that contact between activated CD4 T cells triggers ASMC proliferation. Subsequently so-called nanotubes were identified running between T cells and the transfer of an anti-apoptotic factor MCl-1 was identified within the tubes. T cells are present within smooth muscle bundles in the airways of asthmatic subjects, strategically placed to affect ASM structure and function. The current study was designed to investigate structural and functional properties of lymphocyte-derived membrane conduits (LMC) generated by activated CD4 T cells as well as delineating mechanism(s) inducing their formation. Given the importance of ASM role in asthma and existing evidence of their interaction with CD4 T cells, we were interested in further studying the possible implications of these cell to cell connections. For this purpose, an *in vitro* co-culture of primary human ASMCs and CD4 T cells was employed and LMC-mediated cell-cell communication was explored.

3.2 Abstract

Activated CD4 T cells connect to airway smooth muscle cells (ASMCs) *in vitro* via lymphocyte derived membrane conduits (LMCs) structurally similar to membrane nanotubes with unknown intercellular signals triggering their formation. We examined the structure and function of CD4 T cell derived LMCs and we established a role for ASMC derived basic fibroblast growth factor 2 (FGF2b) as well as fibroblast growth factor receptor 1 (FGFR1) in LMC formation. Blocking FGF2b's synthesis and FGFR1 function reduced LMC formation. Mitochondrial flux from ASMCs to T cells was partially FGF2b and FGFR1-dependent. LMC formation by CD4 T cells and mitochondrial transfer from ASMCs was increased in the presence of asthmatic ASMCs that expressed more mRNA for FGF2b compared to normal ASMCs. These observations identify ASMC-derived FGF2b as a factor needed for LMC formation by CD4 T cells, affecting intercellular communication.

3.3 Introduction

Once activated, CD4 T cells acquire the ability to migrate to the site of inflammation, a process that enables them to communicate with a variety of immune and structural cell types. A range of molecular mechanisms ensures optimal communication of CD4 T cells with their cellular environment via cytokines and growth factors, exosomes, direct cell to cell contact, membrane nanotubes (MNTs) and trogocytosis [101,102,111,261-265]. CD4 T cells have been shown in close proximity to ASMCs *in vivo*, indicating the plausibility that contact-dependent communication of CD4 T cells and ASMCs may be responsible for altered functionality of these cells in airway diseases with increased lymphocyte infiltration [92,266]. In several airway pathologies, including asthma, increased thickness of ASM bundles as a result of hyperplasia and hypertrophy may account for excessive airway narrowing in response to various stimuli [267]. The tubular connections that have been usually referred to as MNTs have been shown previously to connect CD4 T cells to ASMCs, T cells and B cells [100,101,264]. *In vitro*, upon activation, primary human CD4 T cells interact with ASMCs via MNTs, allowing myeloid cell leukemia protein-1 (Mcl-1) transfer from ASMCs to CD4 T cells and resulting in suppression of CD4 T cell apoptosis [101]. Hence, characterization of the structure of CD4 T cell derived LMCs communicating with ASMCs, as well as the mediator(s) inducing their formation is essential for understanding how intercellular communication between ASMCs and CD4 T cells alters their functionality in airway diseases.

MNTs are a continuum of cell membrane extensions forming bridges between cells. MNTs can be formed by a variety of cell types including neural, cancer, stem and stromal cells as well as lymphoid and myeloid immune cells [100,101,127,128,264,268-272]. MNT functionality is determined by their size and cytoskeletal composition, whether containing either actin alone or also tubulin [273]. Aside from the transfer of surface proteins due to membrane fluidity, MNTs can transfer calcium ions, micro RNAs, vesicles, organelles and pathogens [100,120,127,268,274-277]. Although there is very limited information concerning the cause and molecular mechanisms involved in the formation of this mode of intercellular communication, a number of conditions including cellular stress, availability of cytokines and specific ligands such as Fas are known to initiate MNT formation [110,111,118,120,268]. However, as a process highly dependent on cell types and micro environments, mechanisms controlling MNT formation are likely not shared among all cell types.

The fibroblast growth factor (FGF) family, consisting of 22 structurally related polypeptides in human, is involved in physiological processes by controlling cell proliferation, migration, survival and differentiation as well as embryonic development. FGFs exert their activity by binding to surface receptor tyrosine kinases, FGFR1-FGFR4. FGF2b induces chemotaxis, cell adhesion, migration and neuronal proliferation. Furthermore, FGF2b induces formation of neurite outgrowths and branching mediated by FGFR1 [278-282]; suggesting their possible role in MNT formation that share the same morphological features.

In this study, we hypothesized that the formation of neurite outgrowths may share the same mechanisms and triggering factors by T cells for the formation of MNT-like structures. We have opted to use the term lymphocyte-derived membrane conduits (LMCs) throughout because the range of sizes of LMCs often exceeds the nanometer scale. We have demonstrated that the formation of LMCs by CD4 T cells, rich in filamentous actin (F-actin) and tubulin, induces marked changes in the CD4 T cell membrane structure topologically. Importantly, as a factor inducing actin polymerization in CD4 T cells, FGF2b was expressed by ASMCs which induced LMC formation by the T cells. FGFR1 was established as a receptor on the T cell surface with the potential to mediate the effect of FGF2b. LMCs appear to transfer mitochondria from ASMCs to CD4 T cells, a process sensitive to LMC frequency. Asthmatic ASMCs were found to express more FGF2b compared to control ASMCs, inducing more LMC formation and mitochondrial transfer.

3.4 Methods

Reagents

DMEM, RPMI 1640, fetal bovine serum (FBS), penicillin, streptomycin and amphotericin B (PSA), phosphate buffered saline (PBS), Cell light Mitochondria-RFP, BacMan 2.0, MitoTracker Red CM-H2Xros, calcein AM, FITC conjugated phalloidin and recombinant human FGF2b were purchased from Invitrogen. Anti-human CD4 antibodies, conjugated with PerCP, Alexa Fluor 647 and Brilliant Violet 421, were purchased from Biolegend. Rabbit anti human FGF2b neutralizing antibody, rabbit anti human FGFR1, rabbit anti human α/β-tubulin, Alexa Fluor 647 conjugated donkey anti rabbit IgG and Fluoroshield mounting medium with DAPI were purchased from Abcam. Viability dye, eFluor 780, was purchased from eBiosience. Phorbol 12-myrsistate 13-acetate [239] and ionomycin were purchased from Sigma Aldrich. Magnetic activated cell sorting kit, negative selection, (MACS) was obtained from Miltenyi Biotec. PD 173074 and PD 161570 were purchased from Cayman Chemical.

Cell culture

Primary human airway smooth muscle cells were obtained from transplant grade lungs procured by the International Institute for the Advancement of Medicine. Protocols were approved by the McGill University Health Center Ethics Review Board and all methods were performed in accordance with the relevant guidelines and regulations. Tissues were treated with elastase and collagenase for 20 minutes at 37 °C with gentle agitation to digest the tissues. Cells were plated in 6-well plates in DMEM supplemented with 10 % fetal bovine serum and 1 % PSA in the density of $5x10^4$ cell/well.

Following informed consent, human peripheral blood was collected from healthy volunteers in heparin-coated tubes and was diluted with PBS in at 1:2 ratio (blood: PBS). Diluted blood was deposited on Ficoll-Paque PLUS (GE Healthcare) and centrifuged at 1400 rpm for 35 minutes. The peripheral blood mononuclear cell (PBMC) layer was isolated from the centrifuged blood. PBMCs were cultured in RPMI 1640 supplemented with 10 % FBS and 1% PSA, 20 ng/ml of PMA and 250 nM ionomycin for 48 hours. CD4 T cells were isolated from PBMCs by MACS kit.

Jurkat cells (ATCC, TIB-152™) were activated and co-cultured in conditions similar to CD4 T cells.

Co-culture

ASMCs were seeded 24 hours before co-culture. Following isolation with an average purity of 80%, CD4 T cells were co-cultured either unstained or stained with calcein AM, 7 nM in PBS incubated in CO_2 chamber at 37 °C for 30 minutes. Following three washes with serum-free RPMI, stained CD4 T cells were co-cultured with airway smooth muscle cells at $5x10^5$cells/well for 24 hours in a mixture of RPMI 1640 and DMEM (1:1) supplemented with either 10% or 0.1% FBS and 1% PSA. Where appropriate, cells were treated with the FGFR inhibitors, PD 173074; 10 nM and 30 nM, PD 161570; 100 nM and 200 nM, anti-FGF2b neutralizing antibody; 1µg/ml or human recombinant FGF2b; 50 ng/ml; at the time of co-culture in the presence of starvation medium (0.1% FBS).

Real time- qPCR reaction

FGF2b, BDNF, NGF, NT3, IGF-1 and NT-4/5 were measured by RT-qPCR in human airway smooth muscle cells and T cells. Total RNA was extracted, using RNeasy mini kit (Qiagen) according to manufacturer's instructions. Reverse transcription was performed with an AffinityScript qPCR cDNA synthesis kit and using oligo dT primers (Agilent Technologies) qPCR was performed using iTaq SYBR green supermix (Bio-Rad). cDNA was amplified in the StepOnePlus realtime PCR system (Applied Biosystems). Relative mRNA expression was calculated using the "ΔΔCt".

siRNA transfection

ASMCs were treated with FGF2b and control siRNA as well as siRNA transfection reagent according to the manufacturer's protocol (Santa Cruz). The highest efficiency of FGF2b knock down was measured based on FGF2b mRNA expression by qPCR 72 hours after siRNA transfection (not shown). After 48 hours of transfection, ASMCs were collected and seeded in 6 well tissue culture plates and co-cultured with calcein AM stained CD4 T cells the following day.

Staining and microscopy

ASMCs were cultured on cover slips and co-cultured with calcein AM pre-stained CD4 T cells. Co-cultured cells were either fixed in 4% paraformaldehyde or fixed and permeabilized with triton x-100, 0.3% in PBS, and stained with phalloidin, rabbit anti-FGFR1 primary antibody and rabbit anti α/β-tubulin primary antibody. Anti-rabbit IgG-Alexa Fluor 647 was used as secondary antibody. Cover slips were placed on slides using Fluoroshield mounting medium with DAPI. Cells were visualized using either a fluorescence microscope (Olympus) with 40X immersion oil objective or confocal microscope (Zeiss LSM 700) with a 63X immersion oil objective. LMCs were quantified in a blinded manner with 40X magnification. The proportion of CD4 T cells bearing LMCs/total CD4 T cells was determined.

Actin polymerization measurement

Total PBMCs were incubated with PMA and ionomycin for 48 hours. Cells were collected and incubated with or without recombinant human FGF2b for 15, 20 and 30 minutes. After each time point, cells were fixed and permeabilized with fixation/permeabilization buffer (BD Biosciences) and stained with anti-CD4 antibody and phalloidin. Samples were acquired by FACS Canto ^{240} and analyzed by FlowJo software.

Scanning electron microscopy

Co-cultured cells were fixed with 4% paraformaldehyde and were post-fixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide. Samples were dehydrated in a graded series of ethanol, and critical point dried with 100% ethanol. Samples were coated by platinum using Leica EM ACE600 Sputter Coater and imaging was performed with a FEI Inspect F50 FE-Scanning electron microscope at an accelerating voltage of 5 kV.

Transmission electron microscopy

Co-cultured cells were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer and were post-fixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide. Samples were stained with tannic acid, 1% in 0.1M sodium cacodylate and dehydrated in a graded series of concentrations of ethanol. Samples were infiltrated with Epon 812 and ethanol mixture, 1:1 and 3:1, followed by 100% Epon 812. Samples were embedded in low-viscosity, thermally-curing Epon resin. Ultrathin sections (70–100 nm) were cut from the resin blocks by a Reichert-Jung

Ultracut E ultramicrotome with a Diatome (Biel, Switzerland) diamond knife. The sections were transferred onto 200-mesh Cu transmission electron microscopy (TEM) grids with Formvar support film. Imaging was carried out on a FEI Tecnai 12 TEM equipped with an AMT XR80C CCD camera at an accelerating voltage of 120 kV.

Mitochondrial transfer assay

ASMCs were stained with either Cell light Mitochondria-RFP, BacMan 2.0 or MitoTracker Red CM-H2Xros following manufacturer's instruction prior to co-culture with CD4 T cells and were co-cultured with either un-stained CD4 T cells or CD4 T cells stained with calcein AM for 18 hours in RPMI 1640 and DMEM [168] supplemented with 10% FBS and 1% PSA. Cells were co-cultured either directly or were separated by Transwell (Costar). Transfer of mitochondria was either visualized and imaged after fixation of cells with paraformaldehyde (4%) by confocal microscopy under a 63X oil immersion objective or quantified by flow cytometer (either LSRII or FACS Canto, BD) following staining with the viability dye eFluor 780, and anti-human CD4 antibody. Data were analyzed by FlowJo software.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 software. Data are presented as the mean +/- one standard error of the mean, unless otherwise specified. Either Student's t-test or one-way Analysis of Variance followed by Tukey's post-test was used to compare either the means of two or more groups, respectively. P-values <0.05 considered as significant.

3.5 Results

CD4 T cells undergo marked changes in cell membrane topology in making LMCs

It has been shown that MNTs can be formed by either one or both communicating cells [283]. We determined to examine the distribution of length and diameter as well as cytoskeletal composition of LMCs. Scanning electron microscopy [284] was used to measure the range of diameters, 0.6-2 μm, and lengths, 2.9-60.5 μm of LMCs. The length and width were related as a power function (Fig. 1A-D). Confocal microscopy and selective staining of CD4 T cells established that LMCs connecting CD4 T cells to ASMCs were of CD4 T cell origin (Fig. 1E).

Jurkat cells, an immortalized line of human CD4-expressing T lymphocytes, also generated LMCs with the same morphological features as primary CD4 T cells connecting to ASMCs (Fig. 1F).

To respond to stimuli which induce changes in the cell shape and morphology, it has been shown that cells use their surface membrane reservoir, in the form of membrane microvilli [285-287]. Consistent with this mechanism of membrane recruitment, the SEM images indicated that CD4 T cells have a distinct cell membrane morphology and density of membrane microvilli when forming no LMC (Fig. 1A), medium-sized LMC (Fig. 1B) or long LMC (Fig. 1C). Longer LMCs were associated with a smoother cell membrane.

There is reported variability in the content of cytoskeletal components, F-actin and tubulin, among LMCs originating in different cell types [283]. Confocal micrographs confirmed the presence of both F-actin and α/β tubulin in all LMCs from CD4 T cells selectively stained with calcein AM (Fig 2A and B).

FGF2b synthesized by ASMCs induces LMC formation by CD4 T cells

Next, we explored the mechanism(s) by which ASMCs induce LMC formation by CD4 T cells. Given the morphological similarity of the LMCs observed in this study to neurite outgrowths, we examined mRNA expression by ASMCs of a number of factors, well known to induce neurite outgrowth formation including, FGF2b, nerve growth factor (NGF), insulin-like growth factor 1 (IGF1), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3) and neurotrophin 4/5

(NT4/5) [278,288-291]. Although all of the above factors tested were expressed by ASMCs, only FGF2b expression was increased in ASMCs when co-cultured with CD4 T cells (Fig 3A).

To examine the contribution of FGF2b to LMC formation, we administered a rabbit polyclonal neutralizing antibody against FGF2b in co-culture conditions. There was a significant reduction in the ratio of CD4 T cells with LMCs to total CD4 T cells, enumerated using fluorescence microscopy (Fig 3B). Rabbit IgG isotype control did not affect the LMC formation by CD4 T cells compared to non-antibody treated cells (Supplementary figure 1). Neutralizing FGF2b antibody did not affect the viability of CD4 T cells assessed by flow cytometry and eFluor780 (Supplemental Figure. 2B).

To determine if FGF2b synthesized from ASMCs was the inducer of LMC formation, we knocked down FGF2b mRNA expression in ASMCs, using siRNA, and co-cultured them with CD4 T cells, stained with calcein AM. The ratio of CD4 T cells with LMCs to total CD4 T cells was reduced by the suppression of FGF2b mRNA expression by ASMCs and LMC formation was restored by the addition of exogenous FGF2b (Fig 3C). Compared to control, control siRNA as well as FGF2b siRNA did not affect the total number of T cells (Supplemental Figure. 2A).

Based on our observation showing F-actin contained within LMCs and the requirement of actin polymerization for LMC formation, we wished to confirm the effect of FGF2b on MNT formation by quantifying actin polymerization. Exogenous FGF2b increased mean fluorescence intensity (MFI) of F-actin in the CD4 T cell population at the time points, 15, 20 and 30 min post exposure (Fig. 3D).

FGFR1 mediates LMC formation by CD4 T cells

Having confirmed the effect of ASMC-derived FGF2b on LMC formation and based on the prior demonstration of the involvement of the FGF2b/FGFR1 axis in the formation of neurite outgrowths [278], we studied the role of FGFR1 in LMC formation. We performed immunofluorescent staining of co-cultured cells with an anti FGFR1 antibody and visualized the cells by confocal microscopy. Our observations confirmed the expression of FGFR1 protein by CD4 T cells, present in the cell body as well as along the length of the LMCs (Fig. 4A). Additionally, we measured mRNA expression of FGFR1 by CD4 T cells, co-cultured with

ASMCs. Compared to CD4 T cells alone, co-culture increased mRNA expression of FGFR1 in CD4 T cells (Fig 4B).

To further investigate the role of FGFR1 in LMC formation, we used two chemical compounds known to selectively inhibit the tyrosine kinase activity of FGFR1, PD161570 and PD173074. Co-cultured cells treated with either of the inhibitors had a decreased ratio of CD4 T cells with LMCs to total CD4 T cells, again demonstrating the involvement of FGFR1 in LMC formation (Fig. 4C and D). Compared to control PD17307 and PD161570 did not affect the viability of CD4 T cells measured by flow cytometry stained by viability dye eFluor780 (Supplemental Figure. 2B).

LMCs permit mitochondrial transfer from ASMCs to CD4 T cells

Since LMCs formed between T cells and ASMCs contain microtubules which are potentially necessary for organelle movement we used transmission electron microscopy (TEM) on co-cultured cells to visualize the organelles present in these structures. We observed mitochondria in LMC structures (Fig. 5A); hence, we examined the possibility of mitochondrial transfer from ASMCs to CD4 T cells. We transduced ASMCs with baculoviral particles loaded with a fusion construct of the leader sequence of E1 alpha pyruvate dehydrogenase tagged with red fluorescent protein (Cell light Mitochondria-RFP, BacMan 2.0). Co-culture of transduced ASMCs with calcein AM stained CD4 T cells was performed and cells were visualized by confocal microscopy (Fig. 5B). After visualization, transferred mitochondria were quantified by flow cytometry following staining of the cells with a viability dye and an anti-CD4 antibody (Fig. 5C). In order to confirm mitochondrial transfer and the necessity of cell contact for this process, we stained ASMCs with MitoTracker Red CM-H2Xros. Mitochondrial transfer to co-cultured CD4 T cells, either physically separated by Transwell® or in direct contact, was quantified by flow cytometry. There was no detectable transfer of mitochondria when CD4 T cells were physically separated but shared the same medium with ASMCs; however, mitochondrial transfer was detected in a substantial fraction of T cells (15.3±2.2%) when cells were co-cultured directly (Fig. 5D).

To investigate the dependence of mitochondrial transfer on LMCs and to confirm that the process was sensitive to FGF2b and FGFR1, we demonstrated decreased mitochondrial transfer from ASMCs stained with Mito Tracker to CD4 T cells in the presence of either anti-FGF2b neutralizing antibody or PD161570 (Fig. 5E). Transfer of mitochondria from CD4 T cells to ASMCs was not

detected, using confocal microscopy (images not shown), suggesting a possible polarity to the mitochondrial transfer.

For the study of mitochondrial transfer, CD4 T cells and ASMCs were co-cultured in medium supplemented with 10% FBS rather than starvation medium, which induces a significant increase of FGF2b mRNA expression relative to S9 by ASMCs compared to medium supplemented with 0.1% FBS, 2.23±0.16 and 1.4±0.1; respectively (n=8; $p<0.001$). The enriched medium also resulted in LMC formation by an increased proportion of CD4 T cells, 0.21±0.03 and 0.44±0.01; respectively. (n=5-12; $P<0.001$).

Asthmatic ASMCs augment communication via LMCs when in contact with CD4 T cells

In order to study the communication of CD4 T cells with asthmatic ASMCs in the context of a disease in which increased infiltration of CD4 T cells in the area of ASM is seen [92], we explored first the expression of FGF2b mRNA by asthmatic ASMCs compared to healthy ASMCs and found that it was increased (Fig. 6A). We quantified the ability of CD4 T cells to make LMCs in contact with asthmatic ASMCs by fluorescence microscopy and found an elevated ratio of CD4 T cells with LMCs to total CD4 T cells (Fig. 6B).

To examine the significance of increased LMC formation induced by asthmatic ASMCs, we assessed the frequency of viable CD4 T cells containing mitochondria from asthmatic ASMCs, in comparison with healthy ASMCs. ASMCs from asthmatic subjects transferred mitochondria to a greater fraction of CD4 T cells. Mitochondrial transfer from both healthy and asthmatic ASMCs was diminished by neutralizing FGF2b (Fig. 6C).

Figure 1. Visualization and measurement of LMCs. SEM images of CD4 T cells with (A) no LMC, (B) medium length LMC and (C) long LMC showing the progressive smoothening of the surface membrane and loss of membrane microvilli. Scale bars represent 10 µm. (D) SEM images of CD4 T cells co-cultured with ASMCs was used for analyzing the distribution of LMC diameter and length. 51 cells were analyzed (R^2=0.36, P<0.0001). (E) Confocal image of activated CD4 T cells labeled with calcein AM (green) and co-cultured with ASMCs, nucleus stained with DAPI (red), showing LMCs formed by a CD4 T cell. (F) Jurkat cells, stained with calcein AM (green), were co-cultured with unstained ASMCs form LMCs. Nuclei were stained with DAPI (red). Scale bars represent 10 µm.

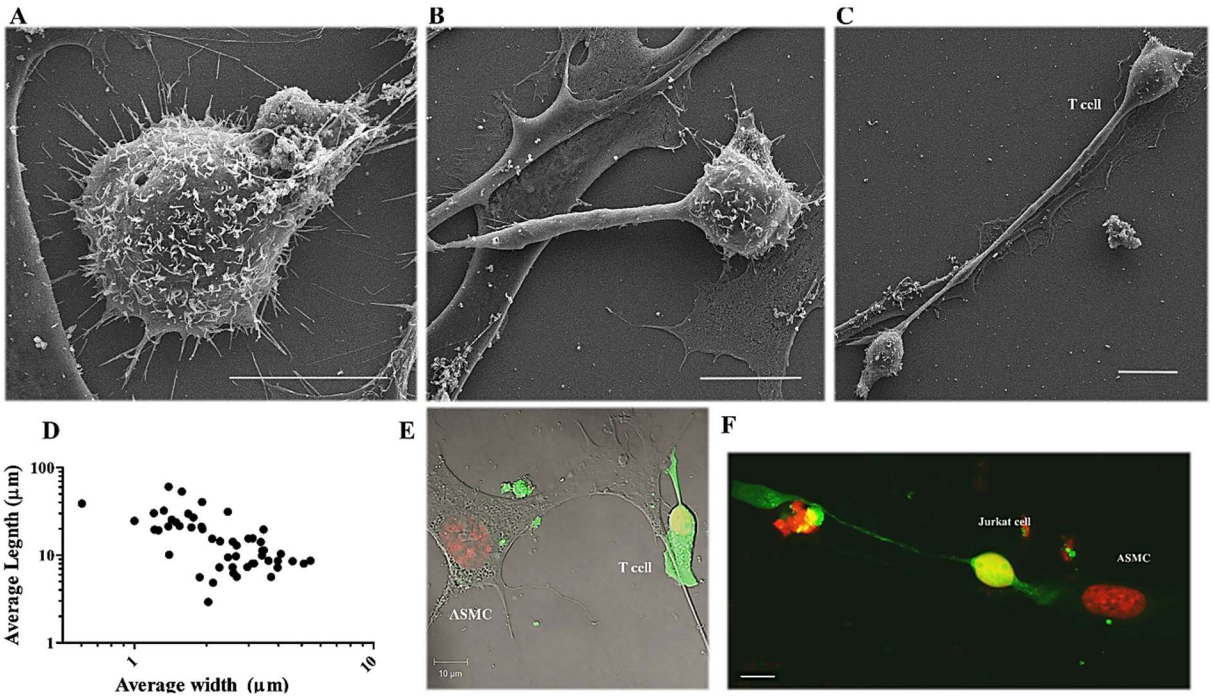


Figure 2. LMCs composed of both cytoskeletal components, F-actin and α/β-tubulin. (A) CD4 T cell were stained with Calcein red-orange AM before co-culture with ASMCs. Co-cultured cells were stained with phalloidin labeled F-actin (green). (B) CD4 T cells were stained with Calcein AM green before co-culture with ASMCs. Co-cultured cells were stained with anti α/β-tubulin antibody (red). Scale bars represent 10 μm.

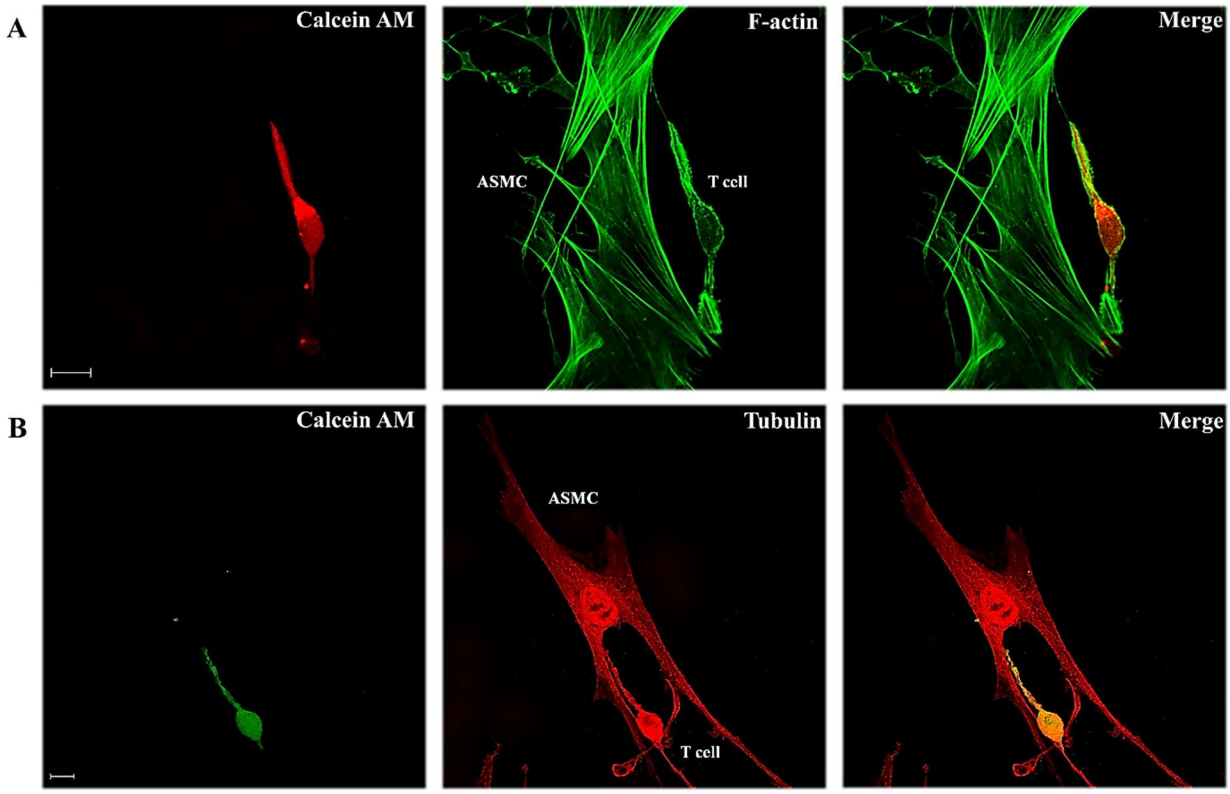


Figure 3. ASMC derived FGF2b induces LMC formation. (A) mRNA expression of the factors known to induce neurite outgrowth formation by ASMCs alone and co-cultured with CD4 T cells was measured by qPCR (ASMC: FGF2b, n=7; NGF, n=8; IGF1A, n=8; BDNF, n=4; NT3, n=4; NT4/5, n=4. ASMC C-C: FGF2b, n=8; NGF, n=10; IGF1A, n=11; BDNF, n=4; NT3, n=4; NT4/5, n=4). (B) CD4 T cells were stained with Calcein AM and co-cultured with ASMCs with (n=4) and without (n=5) anti FGF2b neutralizing antibody. CD4 T cells with LMCs and total CD4 T cells were counted by fluorescence microscopy (n≥190 cells per replicate). (C) ASMCs were transfected with control (n=9) and FGF2b siRNA and co-cultured with Calcein AM stained CD4 T cells with (n=3) and without (n=12) recombinant FGF2b. CD4 T cells with LMCs and total CD4 T cells were counted by fluorescence microscopy (n≥200 cells per replicate). (D) PMA/ionomycin treated PBMCs were incubated with (n=8) and without (n=4) recombinant FGF2b and stained with anti CD4 antibody and phalloidin. Mean fluorescence intensity of polymerized actin was measured by flow cytometry. Gating strategy is shown for the 30 minutes time point post exposure to FGF2b. Data are mean±SEM; *P<0.05, **P<0.01, ***P<0.001, Student's t-test or one-way ANOVA with Tukey's post-test multiple comparisons.

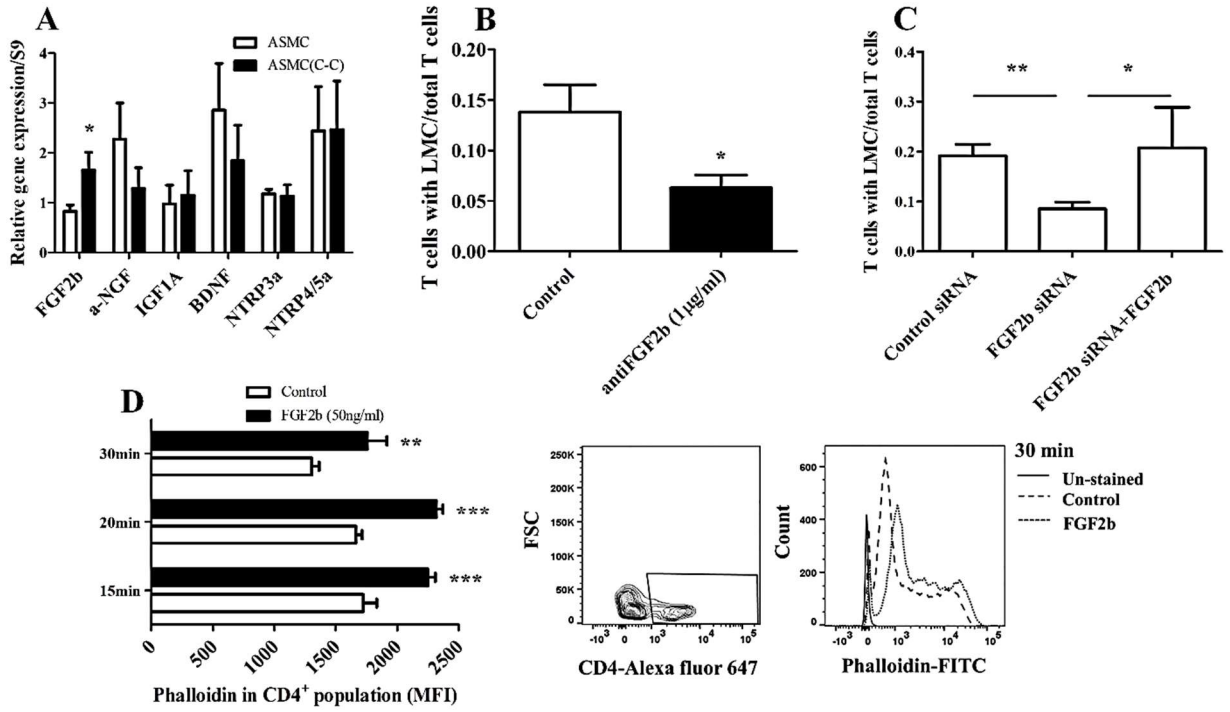


Figure 4. FGFR1 mediates LMC formation. (A) CD4 T cells were stained with Calcein AM (green) and co-cultured with ASMCs. Cells were stained with anti FGFR1 antibody (red) and nuclei were stained with DAPI (blue). Scale bars represent 10 μm. (B) mRNA expression of FGFR1 in CD4 T cells, alone and co-cultured with ASMCs,was measured by qPCR (n=5). (C and D) CD4 T cells were stained with Calcein AM and co-cultured with ASMCs with or without either PD161570 (control, n=7; 100nM, n=6; 200nM, n=3) or PD173074 (n=7). CD4 T cells with LMCs and total CD4 T cells were counted by fluorescence microscopy (n≥200 cells per replicate). Data are mean±SEM; *P<0.05, **P<0.01, Student's t-test.

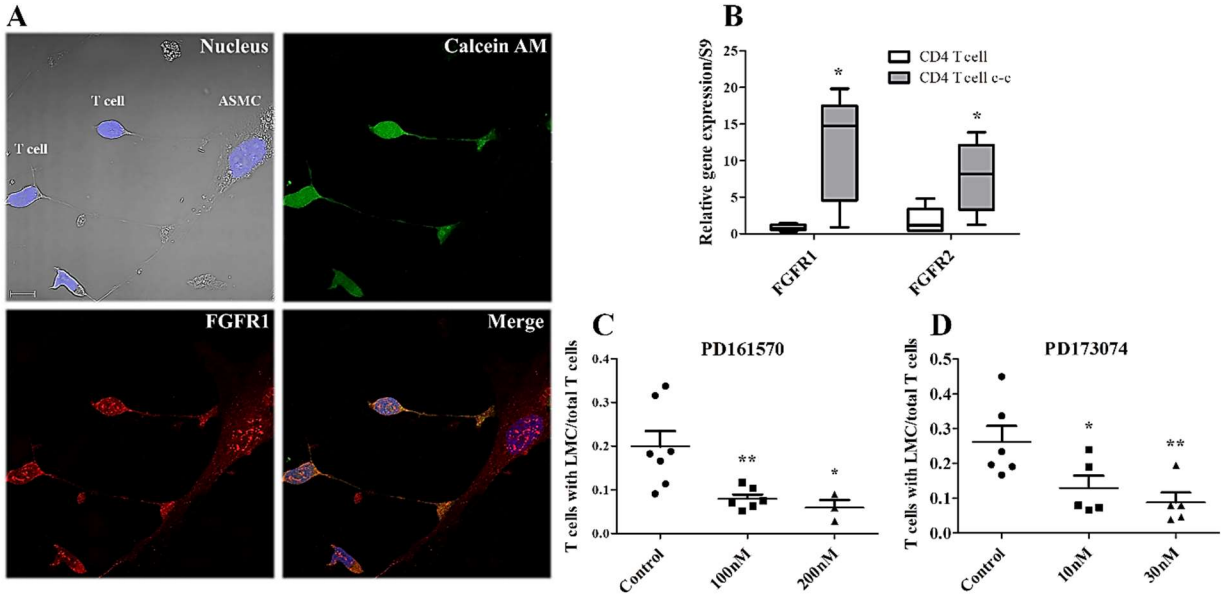


Figure 5. Mitochondrial transfer from ASMCs to CD4 T cells. (A) TEM showed the presence of mitochondria in the LMC structure of a CD4 T cells. (B) Before co-culture, ASMCs were transduced by Cell light BacMan2.0 (red) and CD4 T cells were stained with Calcein AM (green). Nuclei of co-cultured cells were stained with DAPI (blue). Scale bars represent 10 µm. (C) ASMCs transduced with Cell Light Mitochondria-RFP, BacMan 2.0 and co-cultured with CD4 T cells. Cells were stained with viability dye and anti-CD4 antibody and analyzed by flow cytometry (n=5). (D) ASMCs were stained with MitoTracker Red CM-H2Xros and co-cultured with CD4 T cells in Transwells® (T.W, n=14) or direct contact (C-C, n=12). After staining with viability dye and anti-CD4 antibody, mitochondrial transfer was assessed by flow cytometry. (E) ASMCs stained with MitoTracker Red CM-H2Xros and co-cultured with CD4 T cells. Co-cultured cells were treated with either PD161750 or anti-FGF2b neutralizing antibody. following staining with viability dye and anti-CD4 antibody, MFI of Mito Tracker in CD4 T cell population was measured by flow cytometry (control, n=4; PD161570, n=5; anti FGF2b, n=5). Data are mean±SEM; *P<0.05, **P<0.01, ***P<0.001, Student's t-test.

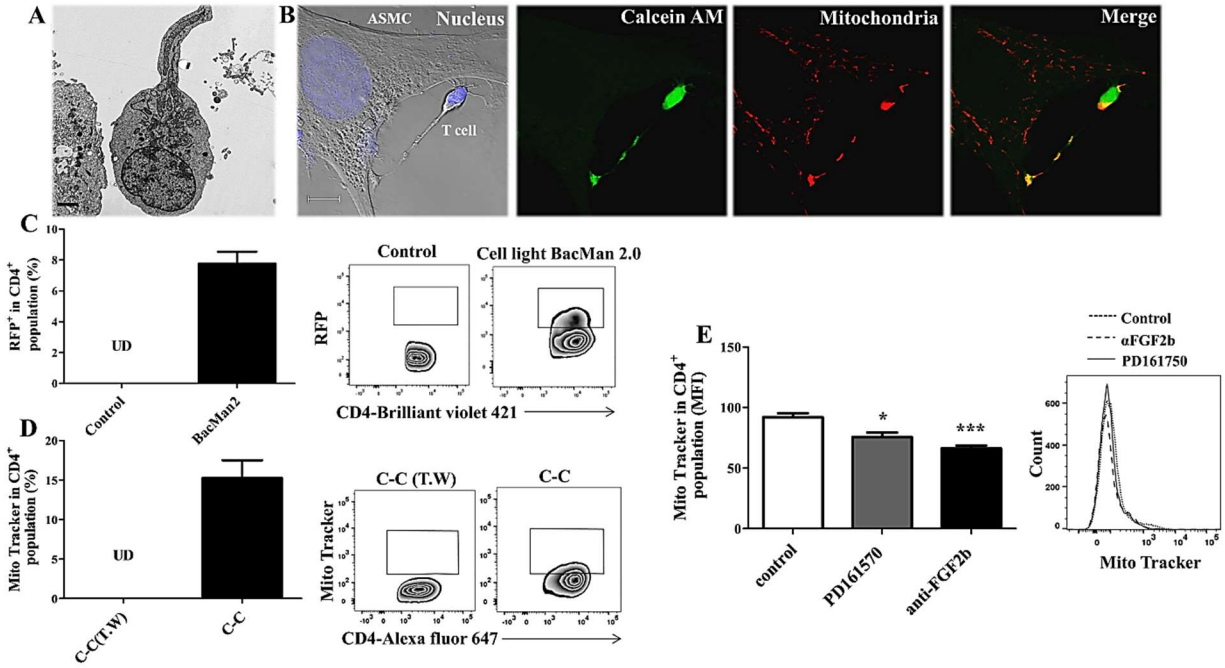
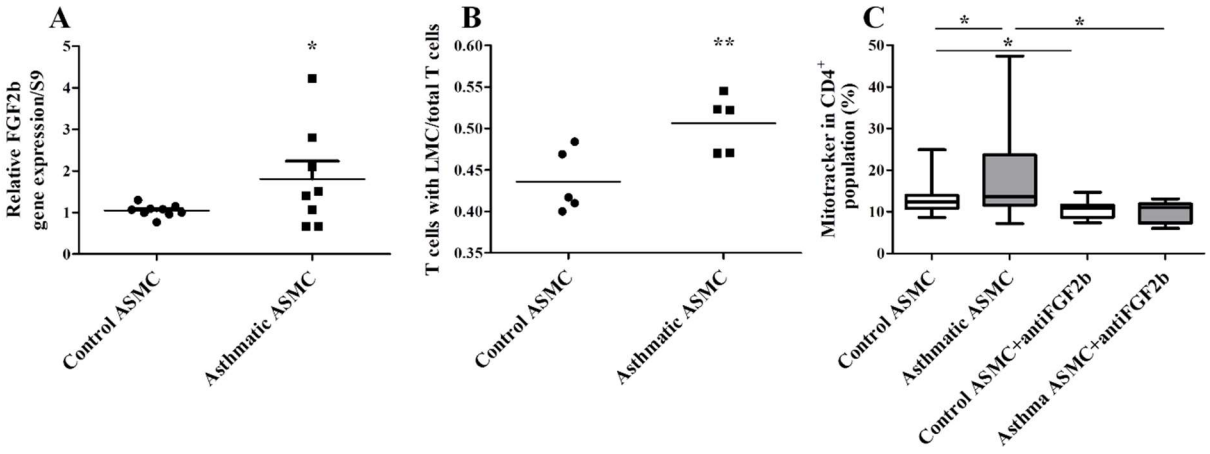
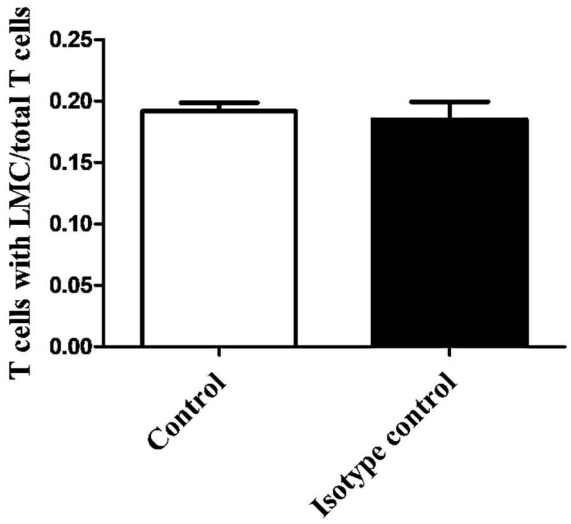


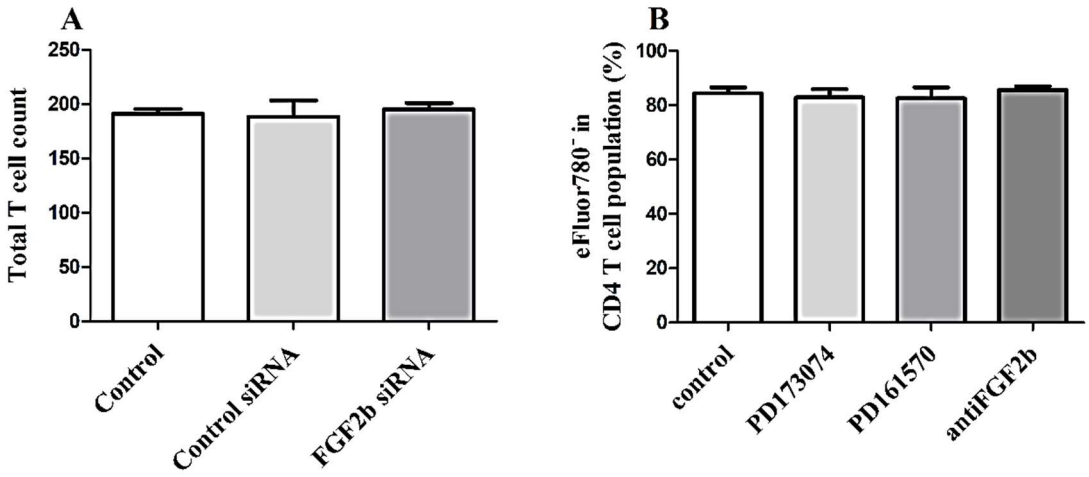
Figure 6. Enhanced communication between asthmatic ASMCs and CD4 T cells supports FGF2b significance in LMC formation. (A) FGF2b mRNA expression by ASMCs harvested from healthy (control) (n=9) and asthmatic (n=8) subjects was measured by qPCR. (B) CD4 T cells were stained with Calcein AM and co-cultured with either healthy or asthmatic ASMCs (n=5). CD4 T cells with LMCs and total CD4 T cells were counted by fluorescence microscopy (n≥200 cells per replicate). (C) ASMCs from healthy subjects and asthmatic subjects were stained with Mito Tracker and co-cultured with CD4 T cells with or without FGF2b neutralizing antibody (n=9-20). Cells were stained with a viability dye as well as anti-CD4 antibody and analyzed by flow cytometry to measure the frequency of CD4 T cell population containing Mito Tracker. Data are mean±SEM; *P<0.05, **P<0.01, Student's t-test.



Supplementary figure 1. Rabbit IgG isotype control did not affect LMC formation by CD4 T cells. CD4 T cells were stained with calcein AM and co-cultured with ASMCs with or without rabbit IgG isotype control for 24h. After fixation, LMC formation was visualized and quantified by fluorescence microscopy (n=6). Data are mean±1SEM, P>0.5, Student's t-test.



Supplementary figure 2. Effects of FGF2b siRNA, anti FGF2b antibody, PD173074 and PD161570 on CD4 T cells. (A) ASMCs were transfected with either control siRNA or anti FGF2b siRNA and co-cultured with calcein AM stained CD4 T cells. Total CD4 T cells were counted by fluorescence microscopy (n≥190 cells per replicate) (n=9-12). (B) After 24 hours co-culture with ASMCs in the presence of either PD173074, PD161570 or anti FGF2b, CD4 T cells were stained with a viability dye and anti CD4 antibody. The frequency of viable CD4 T cells was assessed by flow cytometry (n=9-15). Data are mean±1SEM; P>0.5, one-way ANOVA.



3.6 Discussion

In the present study, we have demonstrated an increase in FGF2b mRNA expression by ASMCs when in co-culture with CD4 T cells. There was a concomitant increase in the expression of the FGFR1 in CD4 T cells. Our findings suggest a substantive role for ASMC derived FGF2b and FGFR1 in the induction of LMC formation by CD4 T cells. The formation of LMCs was associated with topological changes in the CD4 T cell plasma membrane and the LMCs were structurally equipped to transfer mitochondria. The movement of mitochondria occurred from ASMCs to CD4 T cells. ASMCs harvested from asthmatic subjects expressed a higher level of FGF2b mRNA, induced more CD4 T cells to form LMCs and increased the fraction of CD4 T cells with transferred mitochondria.

CD4 T cells have been reported to connect to other structural and immune cells by LMCs [100,101,264]. Due to the fact that the formation of LMC is highly influenced by the micro environment and they do not share the same structure and function among different cell types [283], we further characterized these intercellular connections. The surface of lymphocytes is covered with short microvilli, dynamic structures that become flat during cell morphogenesis [285-287]. We also noted on SEM images a loss of surface villi in association with the formation of long LMCs. This observation suggests that the formation of LMCs, ranging in length from 2.9-60.5 μm and therefore in some instances extending to several times the diameter of the cell body, requires the recruitment of cell membrane from the plasma membrane microvilli.

The dimensions of LMCs as well as their cytoskeletal components predict the ability of LMCs to transfer materials, including ions, vesicles and organelles [283]. Hence, in our study, the diameter of LMCs and their complement of both F-actin and tubulin, confirmed by immunostaining and confocal microscopy, suggested that these tubular structures might also transfer organelles. Besides, we have previously reported that Mcl-1, a mitochondrial anti apoptotic protein was present in LMCs and when expressed in ASMCs appeared in LMC connected CD4 T cells [101]. We speculate that this protein may have been transported in conjunction with mitochondria. Using two different tools to track ASMC mitochondrial movement, by expression of a mitochondrial associated peptide tagged with RFP and Mito Tracker Red, we found a population of CD4 T cells containing ASMC mitochondria. Physically separating ASMCs and CD4 T cells by Transwells®

(pore size 400 nm) inhibited transfer of mitochondria (500nm-10μm in diameter). However, whether other mechanisms of intercellular exchange such as trogocytosis and microvesicles might also be involved in mitochondrial transfer was not excluded by our study.

There appeared to be a unidirectional transfer of mitochondria from ASMCs to CD4 cells although since T cells contain relatively few mitochondria compared to ASMCs our detection technique may have missed the transfer of a small number of mitochondria from T cells to ASMCs. The rich mitochondrial network in ASMCs is likely to have favoured the direction of mitochondrial flow from these cells to T cells.

Although there is a dearth of information concerning the mechanisms of LMC formation, a few mechanisms have been proposed [110,111,118,120,268]. The morphological similarity of T cell- derived LMCs to neurite outgrowths prompted us to examine ASMCs for factors known to induce neurite outgrowth formation. Of those tested, only the mRNA expression of FGF2b was increased in ASMCs in the presence of CD4 T cells. Confirmed by different approaches, FGF2b from ASMCs induced LMC formation by CD4 cells, possibly by increasing actin polymerization. Although there are four known receptors for FGF2b, FGFR1-4, in our search for the receptor on CD4 T cells mediating LMC formation we focused our exploration on FGFR1; given its role in neurite outgrowth formation in response to FGF2b [278]. Consistent with this idea, pharmacological inhibition of the FGFR1 with two selective tyrosine kinase inhibitors, PD173074 and PD161570 suppressed LMC formation. Reduced mitochondrial transfer in the presence of either neutralizing FGF2b antibody or PD161750 which reduced LMC formation supports the idea that LMCs are involved in organelle transfer. However, the inhibition of theFGF2b/FGFR1 pathway caused only a partial inhibition of both LMCformation and of mitochondrial transfer, suggesting other important residual molecular mechanisms.

ASMCs harvested from asthmatic subjects have been reported to have different characteristics that distinguish them from cells harvested from healthy subjects including enhanced proliferation as well as increased mitochondrial biogenesis [292,293]. We explored the possibility that asthmatic ASMCs might therefore have differences in their propensity to form intercellular connections via LMCs with CD4 T cells. Consistent with the data on ASMCs harvested from non-asthmatic airway tissues, FGF2b also appeared to be important in the interaction between ASMCs and CD4 T cells

via LMCs. Asthmatic ASMCs expressed more FGF2b mRNA and induced more CD4 T cells to make LMCs leading to a greater frequency of CD4 T cells containing mitochondria of ASMC origin. The magnitude of the difference in mRNA expression for FGF2b between healthy and asthmatic ASMCs was greater than the difference in frequency of CD4 T cells bearing LMCs. There are several possible explanations. Firstly, we did not confirm the level of FGF2b protein in the medium of the two sets of cells and secondly there may not be a linear relationship between FGF2b and the number of LMCs formed. Indeed, detection of FGF2b in medium was problematic (data not shown), in all probability because of the high isoelectric point of FGF2b and its avid binding to negatively charged tissue culture plates. The increased transfer of mitochondria from asthmatic ASMCs to T cells compared to non-asthmatic ASM may relate to the reported increased quantity of mitochondria in asthmatic ASMCs [292]. However, we did not assess the mitochondrial mass in the cells we studied.

In conclusion, ASMC-derived FGF2b as well as CD4 T cell-derived FGFR1 regulate the communication between these two cell types by promoting LMC formation by T cells and mitochondrial transfer. The biological significance of LMC-mediated mitochondrial transfer to T cells awaits further elucidation.

CHAPTER 4

Discussion & Future Directions

4.1 Discussion & future directions

Type 2 inflammation has been predominantly associated with allergic asthma. In response to allergen, CD4 T cells are one of the major key players in the disease and have been shown repeatedly to mediate the immune response to aeroallergens and drive allergic airway inflammation. From an abundance of evidence based on *in vitro* and *in vivo* studies, the immunological aspects of CD4 T cell function have been well established. However, the possible modulatory role of CD4 T cells in ASM physiology and function is more controversial and mostly based on *in vitro* observations. For example, the autocrine effect of IL-5 and IL-1β synthesized by ASMC from rabbits induced by activated CD4 T cells increases ASM constriction in response to acetylcholine[294]. Moreover, Ova specific CD4 T cells enhance rat ASM contraction by inducing the expression of contractile proteins, myosin heavy and light chain, as well as increased phosphorylation of myosin light chain kinase in the first 24 hours of co-culture[295]. Besides, although epidermal growth factor expression by CD4 T cells has been reported and some functions have been attributed to amphiregulin, there are not sufficient studies to elucidate the role of growth factor synthesis by CD4 T cells in their immunological and physiological functions. The aim of this dissertation was to reveal mechanisms regulating T cell communication with ASMCs and how growth factor synthesis by CD4 T cells regulates their immune-physiological properties in the context of an acute model of allergic asthma.

As presented in chapter 2, we first focused on EGFR ligand expression by CD4 T cells; considering the prior evidence showing the expression of HB-EGF by CD4 T cells and amphiregulin by T_H2 and Tregs[102,171,296]. We examined the effect of different stimuli to induce activation in CD4 T cells and we evaluated the expression of EGFR ligand expression in comparison with unstimulated cells. Aiming to stimulate CD4 T cells in a physiologically relevant manner with allergen, we used DO.11.10 CD4 T cells which are genetically modified to express the Ova specific TCR. Upon stimulation in the presence of antigen presenting cells in total splenocytes, when the milieu efficiently induces mRNA expression of T_H2 signature transcription factor GATA3, CD4 T cells were found to upregulate the expression of HB-EGF exclusively among the other EGFR ligands examined. In order to show that the finding is not particular to DO.11.10 mice due to their transgenic status, we confirmed HB-EGF mRNA expression in non-transgenic mice with the same

genetic background by both *in vivo* Ova sensitization as well as *in vitro* anti-CD3 and anti-CD28 stimulation.

First, we focused on the heparin-binding domain of HB-EGF and due to the fact that it can mediate adhesive function of the protein to heparan sulfate proteoglycans on the cell membrane and extracellular matrix, we hypothesized that membrane-bound HB-EGF synthesized by CD4 T cells facilitates interaction with DCs. As the first step, we visualized the presence of HB-EGF on the CD4 T cell membrane by immunogold labeling. Then we confirmed HB-EGF localization with CD3 on CD4 T cells in the contact site with DCs. Attenuated aggregate formation of CD4 T cells and DCs in the presence of anti HB-EGF neutralizing antibody resulted in reduced activation of both CD4 T cells and DCs. The lack of effect of the EGFR inhibitor, afatinib, on both cell types suggests that the observed CD4 T cell membrane bound HB-EGF function is independent of the EGFR. Therefore, we postulate that HB-EGF may serve as an adhesion molecule.

Based on evidence showing the strong and long interaction between DCs and CD4 T cells are required for T_{FH} cell differentiation, we hypothesized that *in vivo* knock out of HB-EGF in CD4 T cells prior to sensitization results in an inefficient differentiation. By intraperitoneal sensitization of HB-EGF$^{lox/lox}$CD4CreER^{T2} following HB-EGF knockout, we showed the involvement of HB-EGF in T_{FH} cell differentiation in the lymph nodes which resulted in reduced humoral immune response based on reduced germinal center B cell population in the lymph nodes and IgG1 in the serum. IgG1 receptor FcγRIII mediates antigen uptake by antigen presenting cells which in turn stimulates the initiation of T_H2 immune response[297]. Hence, we showed intranasal challenge of mice with allergen following the sensitization phase in the same mice knockout for HB-EGF in CD4 T cells resulted in reduced T_H2 cytokines, IL-4 and IL-5, in the airways.

After establishing a role for CD4 T cell membrane-bound HB-EGF, in the second part of the study we aimed to examine the effect of increased HB-EGF expression under activation stimuli. Using HB-EGF$^{lox/lox}$CD4CreER^{T2} mice which enable tamoxifen induced knock out of HB-EGF specifically in CD4 T cells, we studied the role of CD4 T cell derived HB-EGF in acute model of allergic asthma induced by Ova. Knocking out HB-EGF in CD4 T cells after sensitization and before challenge phase revealed that CD4 T cell production of HB-EGF exacerbates T_H2 response and production of IL-5 cytokine, eosinophilic inflammation as well as bronchial

hyperresponsiveness in peripheral compartments of the lung. The selective effect of HB-EGF knockout in the peripheral lung is not explained but suggests different mechanisms are responsible for central airway and peripheral airway hyperresponsiveness. CD4 T cell depletion in mice repeatedly exposed to cigarette smoke attenuates alveolar destruction[298], and suggests therefore that the peripheral airways can be the main site of action for CD4 T cells.

The reduced AHR in the absence of HB-EGF in CD4 T cells may be attributed to a reduced eosinophil population. Eosinophilic products can directly or indirectly enhance AHR by inducing ASM contraction[253,299]. T_H2 cytokines and chemokines are pivotal for eosinophil differentiation from bone marrow progenitors, survival and migration. IL-5 is among the most well-known factors crucial for initiating and maintaining eosinophilic inflammation[236,237]. IL-5 deficient mice do not develop eosinophilic inflammation and AHR following Ova sensitization and challenge[254]. Decreased IL-5 synthesis upon knocking out HB-EGF in CD4 T cells may account for the lower eosinophilia. HB-EGF is a ligand functioning in three ways: from the cell membrane as an integral protein, a soluble form upon ectodomain shedding by MMPs and ADAMs as well as an intracellular cytoplasmic form which is shown to inhibit the function of gene repressor factors, including Bcl-6. Since the IL-5 gene contains a silencer element that binds to Bcl-6, we hypothesized that HB-EGF enhances IL-5 production by repressing Bcl-6[232]. Therefore, we assessed the interaction between HB-EGF with Bcl-6 in CD4 T cells by immunoprecipitation. We thereafter showed the increased binding of Bcl-6 to IL-5 gene upon knocking out HB-EGF in CD4 T cells *in vitro*. Using Bcl-$6^{lox/lox}$CD4Cre, we confirmed the protective effect of Bcl-6 expression by CD4 T cells in airway eosinophilic inflammation and AHR which was accompanied by decreased IL-5 in the serum.

Interestingly, in asthmatic patients HB-EGF synthesis by IL-5 expressing CD4 T cells was higher than healthy subjects which further confirms the association between HB-EGF expression and IL-5 production. It also suggests that there might be a genetic or epigenetic diversity for HB-EGF expression by CD4 T cells which may predispose individuals to T_H2 driven allergic response.

Chapter 3 of this dissertation is devoted to investigating the mechanisms modulating CD4 T cell interaction with ASMCs *in vitro*. CD4 T cell infiltration into the airways and their close proximity to the ASM area has been shown *ex vivo* in human asthmatic airways and in airway tissues from

allergen challenged rats[92,132]. Direct co-culture of CD4 T cells with ASMCs enhances proliferation in both cell types compared to physically separated co-cultured cells in rats[92], a process that we have reproduced for human cells (data not shown). Previous work in our lab has shown the connection of primary human CD4 T cells to ASMCs via MNT-like structures which facilitate the transfer of MCL-1, an anti-apoptotic factor, from ASMC to CD4 T cells, resulting in decreased apoptosis of T cells[101]. MNTs are extensions of plasma membrane with the capability of transferring a range of materials and organelles, pathogens[277,300], ions, membrane proteins, vesicles from organelles and mitochondria, depending on size and cytoskeletal composition and may have an origin from one or both interacting cells. Their formation has been observed between immune, stem, neural and cancer cells and a few mechanisms have been proposed for their generation.[301]. Molecular mechanisms involved in MNT formation include, P53 in astrocytes[110], M-Sec in human macrophages[277] as well as adhesion molecules including N-cadherin and β-catenin in an urothelial cell line[108]. Morphologically, MNT-like structures, stemming from CD4 T cells, are similar to neural outgrowths, dendrites and axons. Taken together, we hypothesized that ASMCs may synthesize and release factors that promote neurite outgrowth formation which trigger MNT-like structure generation from CD4 T cells. We confirmed the generation of MNT-like structures exclusively by CD4 T cells by using differential staining of ASMCs and CD4 T cells. Plasma membrane of lymphocytes has been shown to be folded into short microvilli, structures that are flattened in the process of cell morphogenesis[286,287]. Scanning electron micrographs revealed the association of microvilli density with the length of MNT-like structures; the longer the tubes were, the smoother was the plasma membrane. Since the tubes often had diameters larger than the nanometer scale, 0.6-2 μm, we called the tube structures lymphocyte-derived membrane conduits (LMC). In search for a mechanism inducing LMC formation, we detected FGF2b and FGFR1 mRNA upregulation in ASMCs and CD4 T cells, respectively, a pathway involved in dendrite formation in neurons. Inhibiting the FGF2b/FGFR1 pathway either by blocking FGF2b synthesis/function by the ASMCs or inhibiting FGFR1 with selective tyrosine kinase inhibitors, PD161570 and PD173074, resulted in decreased LMC formation.

The cytoskeletal composition of MNTs can be used to predict the type of cargo they can transfer[283]. MNTs may contain actin filaments only or contain microtubules; the latter component allows them to transfer organelles. Indeed, a key molecule in the transport of mitochondria has been recently described. Mitochondrial Rho GTPase 1 (Miro1) mediates mitochondrial transfer from

mesenchymal stem cells to epithelial cells via MNTs and rescues them from stress and injury[302]. We visualized the presence of both actin filaments and microtubules in the LMCs. Therefore, as the necessary machinery for the transfer of organelles was present as well as the previously observed transfer of mitochondrial protein, MCL-1, from ASMCs to CD4 T cells[101], we were prompted to speculate that transfer of whole mitochondria may occur. By both expression of a mitochondrial associated peptide tagged with red fluorescent protein and Mito Tracker Red, we detected mitochondria from ASMC in the CD4 T cells, a process that was inhibited by physical separation of the cells using Transwells™. FGF2b neutralization as well as pharmacological inhibition of FGFR1 by PD161570 reduced mitochondrial transfer which further confirmed the involvement of LMCs in the process. To determine a pathological significance for the involvement of FGF2b/FGFR1 in LMC formation and communication between ASMCs and CD4 T cells in asthma, we found that ASMCs from asthmatic patients synthesized higher level of FGF2b mRNA compared to healthy subjects *in vitro* which resulted in increased generation of LMCs in CD4 T cells. Furthermore, a greater mitochondrial transfer from asthmatic ASMCs and the sensitivity of the process to FGF2b neutralization was observed.

Future directions

Regarding the work presented in chapter 2, generating HB-EGF$^{lox/lox}$CD4CreER^{T2} mice as an efficient tool to study the immunological and physiological role of HB-EGF expressed by CD4 T cells raises various opportunities for future experimentation. HB-EGF has been implicated in increased ASMC proliferation as well as excessive mucus production and airway remodeling in a chronic model of allergic asthma induced by Ova[143]. Moreover, EGFR inhibition suppresses the increased epithelial and goblet cell proliferation as well as ASM hyperplasia in response to OVA sensitization and challenge in rats[303]. Accordingly, it is of interest to study how CD4 T cell expression of HB-EGF contributes to epithelial regeneration, mucus production and airway remodeling in chronic models of asthma.

Moreover, investigating the role of HB-EGF in T_{FH} cell differentiation and function is of great interest. The fact that HB-EGF inhibits Bcl-6, which is a master transcription factor for T_{FH} cells, raises the question whether this specific subset of CD4 T cells regulates, possibly suppresses, HB-EGF expression.

The future research opportunities stemming from the work presented in chapter 3 are rich. The first proposed work would be to devise an experimental plan to observe and confirm the presence of interaction between ASMCs and CD4 T cells via LMC *in vivo,* for which a chronic murine model of asthma to induce increased ASM mass in transgenic mice with fluorescently labeled CD4 T cells may be of great help. Next, clarifying whether a specific subtype of CD4 T cells can potentially generate LMCs and if subsets differentially respond to FGF2b by expressing varying levels of FGFR1. This can be achieved by *in vitro* differentiation of CD4 T cells subsets or using transgenic animals fluorescently tagged for signature cytokine or transcription factors for subsets of CD4 T cells. Mitochondrial transfer can occur in a biased manner to dictate the fate of recipient cells; transfer of a fewer number of aged mitochondria from human mammary stem-like cells to daughter cells during division is essential for recipients to restore their stem cell traits[304]. Hence, given that CD4 T cell function is fundamental in lung inflammation in allergic asthma, it is worthwhile to investigate the characteristics of CD4 T cells dictated by receiving mitochondria from ASMCs. CD4 T cell dependency on mitochondria for the purpose of cell metabolism differs among different subsets, greater in naïve CD4 T cells and Tregs while less significant in T_H1, T_H2 and T_H17 cells[93,305]; therefore, it is of interest to study the effect of mitochondrial transfer in skewing CD4 T cell differentiation toward specific subsets. Additionally, examining the possibility of transfer for other organelles besides mitochondria and probing whether it happens unidirectionally or bidirectionally is of interest. Based on evidence indicating that the induction of ASMC proliferation by T cells occurs in a contact-dependent manner, it is of importance to unravel the possible role of LMCs in the process. It would also be also of interest to test the effect of FGFR inhibition in models of ASM remodeling in mouse or rat. Such models have been developed in our laboratory[303]. Last but not least, we wish to examine the role of FGF2b/FGFR1 axis in MNT formation by other cell types to show whether the mechanism is or is not exclusive for LMC formation by T cells since P53 and M-sec mediated MNT formation are not universal mechanisms for all cell types[301].

4.2 Final conclusion

In conclusion, the work in this dissertation was dedicated to providing evidence for novel mechanisms by which CD4 T cells mediate pathophysiological hallmarks of allergic asthma. We have proposed a role for HB-EGF synthesized by CD4 T cells in exacerbation of disease. In

addition, we elucidated a mechanism for communication of CD4 T cells with ASMCs via LMCs that provides a new venue of study to apply the knowledge in *in vivo* experimentation and manipulating intracellular interaction in order to investigate relevant physiological impact of the finding. Moreover, mitochondrial transfer from ASMCs to CD4 T cells suggests a new mechanism by which ASMCs can regulate function and biological properties of CD4 T cells. In a broader perspective, the findings bring us one step closer to a more comprehensive understanding of cell-cell communications in general and regulatory mechanisms in pulmonary inflammation, although explored currently in allergic asthma in particular.

References

1 Hornung, V. *et al.* Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *Journal of immunology (Baltimore, Md. : 1950)* **168**, 4531-4537 (2002).

2 Holgate, S. T. Pathogenesis of asthma. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* **38**, 872-897, doi:10.1111/j.1365-2222.2008.02971.x (2008).

3 Kudo, M., Ishigatsubo, Y. & Aoki, I. Pathology of asthma. *Frontiers in microbiology* **4**, 263, doi:10.3389/fmicb.2013.00263 (2013).

4 Nunes, C., Pereira, A. M. & Morais-Almeida, M. Asthma costs and social impact. *Asthma research and practice* **3**, 1, doi:10.1186/s40733-016-0029-3 (2017).

5 Burgess, A. W. *et al.* An open-and-shut case? Recent insights into the activation of EGF/ErbB receptors. *Molecular cell* **12**, 541-552 (2003).

6 Lambrecht, B. N. & Hammad, H. The immunology of asthma. *Nature immunology* **16**, 45-56, doi:10.1038/ni.3049 (2015).

7 Guerra, S. & Martinez, F. D. Asthma genetics: from linear to multifactorial approaches. *Annual review of medicine* **59**, 327-341, doi:10.1146/annurev.med.59.060406.213232 (2008).

8 Ober, C. Asthma Genetics in the Post-GWAS Era. *Annals of the American Thoracic Society* **13 Suppl 1**, S85-90, doi:10.1513/AnnalsATS.201507-459MG (2016).

9 Martinez, F. D. *et al.* Asthma and wheezing in the first six years of life. The Group Health Medical Associates. *The New England journal of medicine* **332**, 133-138, doi:10.1056/nejm199501193320301 (1995).

10 Melgert, B. N., Ray, A., Hylkema, M. N., Timens, W. & Postma, D. S. Are there reasons why adult asthma is more common in females? *Current allergy and asthma reports* **7**, 143-150 (2007).

11 Wills-Karp, M. Allergen-specific pattern recognition receptor pathways. *Current opinion in immunology* **22**, 777-782, doi:10.1016/j.coi.2010.10.011 (2010).

12 Holt, P. G. & Strickland, D. H. Interactions between innate and adaptive immunity in asthma pathogenesis: new perspectives from studies on acute exacerbations. *The Journal of allergy and clinical immunology* **125**, 963-972; quiz 973-964, doi:10.1016/j.jaci.2010.02.011 (2010).

13 Borish, L. The immunology of asthma: Asthma phenotypes and their implications for personalized treatment. *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology* **117**, 108-114, doi:10.1016/j.anai.2016.04.022 (2016).

14 Brehm, J. M. *et al.* Serum vitamin D levels and markers of severity of childhood asthma in Costa Rica. *American journal of respiratory and critical care medicine* **179**, 765-771, doi:10.1164/rccm.200808-1361OC (2009).

15 Farchi, S. *et al.* Dietary factors associated with wheezing and allergic rhinitis in children. *The European respiratory journal* **22**, 772-780 (2003).

16 Mohanan, S., Tapp, H., McWilliams, A. & Dulin, M. Obesity and asthma: pathophysiology and implications for diagnosis and management in primary care. *Experimental biology and medicine (Maywood, N.J.)* **239**, 1531-1540, doi:10.1177/1535370214525302 (2014).

17 Tarlo, S. M. & Lemiere, C. Occupational asthma. *The New England journal of medicine* **370**, 640-649, doi:10.1056/NEJMra1301758 (2014).

18 Quirce, S. & Bernstein, J. A. Old and new causes of occupational asthma. *Immunology and allergy clinics of North America* **31**, 677-698, v, doi:10.1016/j.iac.2011.07.001 (2011).

19 Halwani, R., Al-Muhsen, S. & Hamid, Q. Airway remodeling in asthma. *Current opinion in pharmacology* **10**, 236-245, doi:10.1016/j.coph.2010.06.004 (2010).

20 Ranu, H., Wilde, M. & Madden, B. Pulmonary function tests. *The Ulster medical journal* **80**, 84-90 (2011).
21 Walker, J. K., Kraft, M. & Fisher, J. T. Assessment of murine lung mechanics outcome measures: alignment with those made in asthmatics. *Frontiers in physiology* **3**, 491, doi:10.3389/fphys.2012.00491 (2012).
22 Bates, J. H., Rincon, M. & Irvin, C. G. Animal models of asthma. *American journal of physiology. Lung cellular and molecular physiology* **297**, L401-410, doi:10.1152/ajplung.00027.2009 (2009).
23 Creticos, P. S. *et al.* Immunotherapy with a ragweed-toll-like receptor 9 agonist vaccine for allergic rhinitis. *The New England journal of medicine* **355**, 1445-1455, doi:10.1056/NEJMoa052916 (2006).
24 Lambrecht, B. N. & Hammad, H. Dendritic cell and epithelial cell interactions at the origin of murine asthma. *Annals of the American Thoracic Society* **11 Suppl 5**, S236-243, doi:10.1513/AnnalsATS.201405-218AW (2014).
25 Galli, S. J. & Tsai, M. IgE and mast cells in allergic disease. *Nature medicine* **18**, 693-704, doi:10.1038/nm.2755 (2012).
26 Takhar, P. *et al.* Class switch recombination to IgE in the bronchial mucosa of atopic and nonatopic patients with asthma. *The Journal of allergy and clinical immunology* **119**, 213-218, doi:10.1016/j.jaci.2006.09.045 (2007).
27 Wu, L. C. Immunoglobulin E receptor signaling and asthma. *The Journal of biological chemistry* **286**, 32891-32897, doi:10.1074/jbc.R110.205104 (2011).
28 Gounni, A. S. *et al.* Human airway smooth muscle cells express the high affinity receptor for IgE (Fc epsilon RI): a critical role of Fc epsilon RI in human airway smooth muscle cell function. *Journal of immunology (Baltimore, Md. : 1950)* **175**, 2613-2621 (2005).
29 Wang, J. *et al.* IgE actions on CD4+ T cells, mast cells, and macrophages participate in the pathogenesis of experimental abdominal aortic aneurysms. *EMBO molecular medicine* **6**, 952-969, doi:10.15252/emmm.201303811 (2014).
30 Kraneveld, A. D., Sagar, S., Garssen, J. & Folkerts, G. The two faces of mast cells in food allergy and allergic asthma: the possible concept of Yin Yang. *Biochimica et biophysica acta* **1822**, 93-99, doi:10.1016/j.bbadis.2011.06.013 (2012).
31 Robinson, D. S. The role of the mast cell in asthma: induction of airway hyperresponsiveness by interaction with smooth muscle? *The Journal of allergy and clinical immunology* **114**, 58-65, doi:10.1016/j.jaci.2004.03.034 (2004).
32 Yoshimoto, T. *et al.* Basophils contribute to T(H)2-IgE responses in vivo via IL-4 production and presentation of peptide-MHC class II complexes to CD4+ T cells. *Nature immunology* **10**, 706-712, doi:10.1038/ni.1737 (2009).
33 Perrigoue, J. G. *et al.* MHC class II-dependent basophil-CD4+ T cell interactions promote T(H)2 cytokine-dependent immunity. *Nature immunology* **10**, 697-705, doi:10.1038/ni.1740 (2009).
34 Sokol, C. L. *et al.* Basophils function as antigen-presenting cells for an allergen-induced T helper type 2 response. *Nature immunology* **10**, 713-720, doi:10.1038/ni.1738 (2009).
35 Sokol, C. L., Barton, G. M., Farr, A. G. & Medzhitov, R. A mechanism for the initiation of allergen-induced T helper type 2 responses. *Nature immunology* **9**, 310-318, doi:10.1038/ni1558 (2008).
36 Spencer, L. A. & Weller, P. F. Eosinophils and Th2 immunity: contemporary insights. *Immunology and cell biology* **88**, 250-256, doi:10.1038/icb.2009.115 (2010).
37 Eisenbarth, S. C. Use and limitations of alum-based models of allergy. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* **38**, 1572-1575, doi:10.1111/j.1365-2222.2008.03069.x (2008).
38 Gregory, L. G. & Lloyd, C. M. Orchestrating house dust mite-associated allergy in the lung. *Trends in immunology* **32**, 402-411, doi:10.1016/j.it.2011.06.006 (2011).

39 Salvi, S. & Holgate, S. T. Could the airway epithelium play an important role in mucosal immunoglobulin A production? *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* **29**, 1597-1605 (1999).
40 Stein, R. A. & Staros, J. V. Evolutionary analysis of the ErbB receptor and ligand families. *Journal of molecular evolution* **50**, 397-412 (2000).
41 Weitnauer, M., Mijosek, V. & Dalpke, A. H. Control of local immunity by airway epithelial cells. *Mucosal immunology* **9**, 287-298, doi:10.1038/mi.2015.126 (2016).
42 Wang, Y., Bai, C., Li, K., Adler, K. B. & Wang, X. Role of airway epithelial cells in development of asthma and allergic rhinitis. *Respiratory medicine* **102**, 949-955, doi:10.1016/j.rmed.2008.01.017 (2008).
43 Bove, P. F. & van der Vliet, A. Nitric oxide and reactive nitrogen species in airway epithelial signaling and inflammation. *Free radical biology & medicine* **41**, 515-527, doi:10.1016/j.freeradbiomed.2006.05.011 (2006).
44 Hiemstra, P. S., McCray, P. B., Jr. & Bals, R. The innate immune function of airway epithelial cells in inflammatory lung disease. *The European respiratory journal* **45**, 1150-1162, doi:10.1183/09031936.00141514 (2015).
45 Lambrecht, B. N. & Hammad, H. The airway epithelium in asthma. *Nature medicine* **18**, 684-692, doi:10.1038/nm.2737 (2012).
46 Salter, B. M. *et al.* IL-25 and IL-33 induce Type 2 inflammation in basophils from subjects with allergic asthma. *Respiratory research* **17**, 5, doi:10.1186/s12931-016-0321-z (2016).
47 de Boer, W. I. *et al.* Altered expression of epithelial junctional proteins in atopic asthma: possible role in inflammation. *Canadian journal of physiology and pharmacology* **86**, 105-112, doi:10.1139/y08-004 (2008).
48 Sorrentino, R. *et al.* Pattern recognition receptors and interleukin-8 mediate effects of Gram-positive and Gram-negative bacteria on lung epithelial cell function. *British journal of pharmacology* **154**, 864-871, doi:10.1038/bjp.2008.139 (2008).
49 Grainge, C. L. *et al.* Effect of bronchoconstriction on airway remodeling in asthma. *The New England journal of medicine* **364**, 2006-2015, doi:10.1056/NEJMoa1014350 (2011).
50 Ray, A. & Kolls, J. K. Neutrophilic Inflammation in Asthma and Association with Disease Severity. *Trends in immunology*, doi:10.1016/j.it.2017.07.003 (2017).
51 Pelaia, G. *et al.* Cellular mechanisms underlying eosinophilic and neutrophilic airway inflammation in asthma. *Mediators of inflammation* **2015**, 879783, doi:10.1155/2015/879783 (2015).
52 Durrant, D. M. & Metzger, D. W. Emerging roles of T helper subsets in the pathogenesis of asthma. *Immunological investigations* **39**, 526-549, doi:10.3109/08820131003615498 (2010).
53 Soehnlein, O., Steffens, S., Hidalgo, A. & Weber, C. Neutrophils as protagonists and targets in chronic inflammation. *Nature reviews. Immunology* **17**, 248-261, doi:10.1038/nri.2017.10 (2017).
54 Kolaczkowska, E. & Kubes, P. Neutrophil recruitment and function in health and inflammation. *Nature reviews. Immunology* **13**, 159-175, doi:10.1038/nri3399 (2013).
55 Marshall, L. J., Perks, B., Ferkol, T. & Shute, J. K. IL-8 released constitutively by primary bronchial epithelial cells in culture forms an inactive complex with secretory component. *Journal of immunology (Baltimore, Md. : 1950)* **167**, 2816-2823 (2001).
56 Monteseirin, J. *et al.* Human neutrophils synthesize IL-8 in an IgE-mediated activation. *Journal of leukocyte biology* **76**, 692-700, doi:10.1189/jlb.0903441 (2004).
57 Murcia, R. Y., Vargas, A. & Lavoie, J. P. The Interleukin-17 Induced Activation and Increased Survival of Equine Neutrophils Is Insensitive to Glucocorticoids. *PloS one* **11**, e0154755, doi:10.1371/journal.pone.0154755 (2016).
58 Laan, M. & Linden, A. The IL-17 family of cytokines--applications in respiratory medicine and allergology. *Recent patents on inflammation & allergy drug discovery* **2**, 82-91 (2008).

59 Balhara, J. & Gounni, A. S. The alveolar macrophages in asthma: a double-edged sword. *Mucosal immunology* **5**, 605-609, doi:10.1038/mi.2012.74 (2012).
60 Kruger, P. *et al.* Neutrophils: Between host defence, immune modulation, and tissue injury. *PLoS pathogens* **11**, e1004651, doi:10.1371/journal.ppat.1004651 (2015).
61 Monteseirin, J. Neutrophils and asthma. *Journal of investigational allergology & clinical immunology* **19**, 340-354 (2009).
62 Park, Y. M. & Bochner, B. S. Eosinophil survival and apoptosis in health and disease. *Allergy, asthma & immunology research* **2**, 87-101, doi:10.4168/aair.2010.2.2.87 (2010).
63 Felton, J. M., Lucas, C. D., Rossi, A. G. & Dransfield, I. Eosinophils in the lung - modulating apoptosis and efferocytosis in airway inflammation. *Frontiers in immunology* **5**, 302, doi:10.3389/fimmu.2014.00302 (2014).
64 Kay, A. B., Phipps, S. & Robinson, D. S. A role for eosinophils in airway remodelling in asthma. *Trends in immunology* **25**, 477-482, doi:10.1016/j.it.2004.07.006 (2004).
65 Haldar, P. *et al.* Mepolizumab and exacerbations of refractory eosinophilic asthma. *The New England journal of medicine* **360**, 973-984, doi:10.1056/NEJMoa0808991 (2009).
66 Tian, B. P., Zhang, G. S., Lou, J., Zhou, H. B. & Cui, W. Efficacy and safety of benralizumab for eosinophilic asthma: A systematic review and meta-analysis of randomized controlled trials. *The Journal of asthma : official journal of the Association for the Care of Asthma*, 1-10, doi:10.1080/02770903.2017.1379534 (2017).
67 Brightling, C. E. *et al.* Mast-cell infiltration of airway smooth muscle in asthma. *The New England journal of medicine* **346**, 1699-1705, doi:10.1056/NEJMoa012705 (2002).
68 Deckers, J., Branco Madeira, F. & Hammad, H. Innate immune cells in asthma. *Trends in immunology* **34**, 540-547, doi:10.1016/j.it.2013.08.004 (2013).
69 Reuter, S., Stassen, M. & Taube, C. Mast cells in allergic asthma and beyond. *Yonsei medical journal* **51**, 797-807, doi:10.3349/ymj.2010.51.6.797 (2010).
70 Krystel-Whittemore, M., Dileepan, K. N. & Wood, J. G. Mast Cell: A Multi-Functional Master Cell. *Frontiers in immunology* **6**, 620, doi:10.3389/fimmu.2015.00620 (2015).
71 Siracusa, M. C., Kim, B. S., Spergel, J. M. & Artis, D. Basophils and allergic inflammation. *The Journal of allergy and clinical immunology* **132**, 789-801; quiz 788, doi:10.1016/j.jaci.2013.07.046 (2013).
72 Epelman, S., Lavine, K. J. & Randolph, G. J. Origin and functions of tissue macrophages. *Immunity* **41**, 21-35, doi:10.1016/j.immuni.2014.06.013 (2014).
73 Tarique, A. A. *et al.* Phenotypic, functional, and plasticity features of classical and alternatively activated human macrophages. *American journal of respiratory cell and molecular biology* **53**, 676-688, doi:10.1165/rcmb.2015-0012OC (2015).
74 Draijer, C., Robbe, P., Boorsma, C. E., Hylkema, M. N. & Melgert, B. N. Characterization of macrophage phenotypes in three murine models of house-dust-mite-induced asthma. *Mediators of inflammation* **2013**, 632049, doi:10.1155/2013/632049 (2013).
75 Roszer, T. Understanding the Mysterious M2 Macrophage through Activation Markers and Effector Mechanisms. *Mediators of inflammation* **2015**, 816460, doi:10.1155/2015/816460 (2015).
76 Jiang, Z. & Zhu, L. Update on the role of alternatively activated macrophages in asthma. *Journal of asthma and allergy* **9**, 101-107, doi:10.2147/jaa.s104508 (2016).
77 Byrne, A. J., Maher, T. M. & Lloyd, C. M. Pulmonary Macrophages: A New Therapeutic Pathway in Fibrosing Lung Disease? *Trends in molecular medicine* **22**, 303-316, doi:10.1016/j.molmed.2016.02.004 (2016).
78 Tan, S. Y. & Krasnow, M. A. Developmental origin of lung macrophage diversity. *Development (Cambridge, England)* **143**, 1318-1327, doi:10.1242/dev.129122 (2016).

79 Hoffmann, F. *et al.* Origin, Localization, and Immunoregulatory Properties of Pulmonary Phagocytes in Allergic Asthma. *Frontiers in immunology* **7**, 107, doi:10.3389/fimmu.2016.00107 (2016).
80 Fricker, M. & Gibson, P. G. Macrophage dysfunction in the pathogenesis and treatment of asthma. *The European respiratory journal* **50**, doi:10.1183/13993003.00196-2017 (2017).
81 Melgert, B. N. *et al.* More alternative activation of macrophages in lungs of asthmatic patients. *The Journal of allergy and clinical immunology* **127**, 831-833, doi:10.1016/j.jaci.2010.10.045 (2011).
82 Girodet, P. O. *et al.* Alternative Macrophage Activation Is Increased in Asthma. *American journal of respiratory cell and molecular biology* **55**, 467-475, doi:10.1165/rcmb.2015-0295OC (2016).
83 van Helden, M. J. & Lambrecht, B. N. Dendritic cells in asthma. *Current opinion in immunology* **25**, 745-754 (2013).
84 Gaurav, R. & Agrawal, D. K. Clinical view on the importance of dendritic cells in asthma. *Expert review of clinical immunology* **9**, 899-919, doi:10.1586/1744666x.2013.837260 (2013).
85 Batista, F. D. & Harwood, N. E. The who, how and where of antigen presentation to B cells. *Nature reviews. Immunology* **9**, 15-27, doi:10.1038/nri2454 (2009).
86 Valitutti, S., Coombs, D. & Dupre, L. The space and time frames of T cell activation at the immunological synapse. *FEBS letters* **584**, 4851-4857, doi:10.1016/j.febslet.2010.10.010 (2010).
87 Benvenuti, F. The Dendritic Cell Synapse: A Life Dedicated to T Cell Activation. *Frontiers in immunology* **7**, 70, doi:10.3389/fimmu.2016.00070 (2016).
88 Verboogen, D. R. *et al.* The dendritic cell side of the immunological synapse. *Biomolecular concepts* **7**, 17-28, doi:10.1515/bmc-2015-0028 (2016).
89 Monticelli, L. A. *et al.* Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nature immunology* **12**, 1045-1054, doi:10.1031/ni.2131 (2011).
90 Zook, E. C. & Kee, B. L. Development of innate lymphoid cells. *Nature immunology* **17**, 775-782, doi:10.1038/ni.3481 (2016).
91 Yu, S., Kim, H. Y., Chang, Y. J., DeKruyff, R. H. & Umetsu, D. T. Innate lymphoid cells and asthma. *The Journal of allergy and clinical immunology* **133**, 943-950; quiz 951, doi:10.1016/j.jaci.2014.02.015 (2014).
92 Ramos-Barbon, D., Presley, J. F., Hamid, Q. A., Fixman, E. D. & Martin, J. G. Antigen-specific CD4+ T cells drive airway smooth muscle remodeling in experimental asthma. *The Journal of clinical investigation* **115**, 1580-1589, doi:10.1172/jci19711 (2005).
93 Almeida, L., Lochner, M., Berod, L. & Sparwasser, T. Metabolic pathways in T cell activation and lineage differentiation. *Seminars in immunology* **28**, 514-524, doi:10.1016/j.smim.2016.10.009 (2016).
94 Zuniga-Pflucker, J. C. T-cell development made simple. *Nature reviews. Immunology* **4**, 67-72, doi:10.1038/nri1257 (2004).
95 DuPage, M. & Bluestone, J. A. Harnessing the plasticity of CD4(+) T cells to treat immune-mediated disease. *Nature reviews. Immunology* **16**, 149-163, doi:10.1038/nri.2015.18 (2016).
96 Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. & Coffman, R. L. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *Journal of immunology (Baltimore, Md. : 1950)* **136**, 2348-2357 (1986).
97 Choi, J., Enis, D. R., Koh, K. P., Shiao, S. L. & Pober, J. S. T lymphocyte-endothelial cell interactions. *Annual review of immunology* **22**, 683-709, doi:10.1146/annurev.immunol.22.012703.104639 (2004).
98 Dopfer, E. P., Minguet, S. & Schamel, W. W. A new vampire saga: the molecular mechanism of T cell trogocytosis. *Immunity* **35**, 151-153, doi:10.1016/j.immuni.2011.08.004 (2011).

99 Dustin, M. L. Signaling at neuro/immune synapses. *The Journal of clinical investigation* **122**, 1149-1155, doi:10.1172/jci58705 (2012).
100 Rainy, N. *et al.* H-Ras transfers from B to T cells via tunneling nanotubes. *Cell death & disease* **4**, e726, doi:10.1038/cddis.2013.245 (2013).
101 Al Heialy, S. *et al.* Nanotubes connect CD4+ T cells to airway smooth muscle cells: novel mechanism of T cell survival. *Journal of immunology (Baltimore, Md. : 1950)* **194**, 5626-5634, doi:10.4049/jimmunol.1401718 (2015).
102 Zaiss, D. M. *et al.* Amphiregulin, a TH2 cytokine enhancing resistance to nematodes. *Science (New York, N.Y.)* **314**, 1746, doi:10.1126/science.1133715 (2006).
103 Turner, M. D., Nedjai, B., Hurst, T. & Pennington, D. J. Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease. *Biochimica et biophysica acta* **1843**, 2563-2582, doi:10.1016/j.bbamcr.2014.05.014 (2014).
104 Abounit, S. & Zurzolo, C. Wiring through tunneling nanotubes--from electrical signals to organelle transfer. *Journal of cell science* **125**, 1089-1098, doi:10.1242/jcs.083279 (2012).
105 Kimura, S., Hase, K. & Ohno, H. The molecular basis of induction and formation of tunneling nanotubes. *Cell and tissue research* **352**, 67-76, doi:10.1007/s00441-012-1518-1 (2013).
106 Rustom, A., Saffrich, R., Markovic, I., Walther, P. & Gerdes, H. H. Nanotubular highways for intercellular organelle transport. *Science (New York, N.Y.)* **303**, 1007-1010, doi:10.1126/science.1093133 (2004).
107 Ohno, H., Hase, K. & Kimura, S. M-Sec: Emerging secrets of tunneling nanotube formation. *Communicative & integrative biology* **3**, 231-233 (2010).
108 Lokar, M., Iglic, A. & Veranic, P. Protruding membrane nanotubes: attachment of tubular protrusions to adjacent cells by several anchoring junctions. *Protoplasma* **246**, 81-87, doi:10.1007/s00709-010-0143-7 (2010).
109 Hase, K. *et al.* M-Sec promotes membrane nanotube formation by interacting with Ral and the exocyst complex. *Nature cell biology* **11**, 1427-1432, doi:10.1038/ncb1990 (2009).
110 Wang, Y., Cui, J., Sun, X. & Zhang, Y. Tunneling-nanotube development in astrocytes depends on p53 activation. *Cell death and differentiation* **18**, 732-742, doi:10.1038/cdd.2010.147 (2011).
111 Luchetti, F. *et al.* Fas signalling promotes intercellular communication in T cells. *PloS one* **7**, e35766, doi:10.1371/journal.pone.0035766 (2012).
112 Kadiu, I. & Gendelman, H. E. Human immunodeficiency virus type 1 endocytic trafficking through macrophage bridging conduits facilitates spread of infection. *Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology* **6**, 658-675, doi:10.1007/s11481-011-9298-z (2011).
113 Kadiu, I. & Gendelman, H. E. Macrophage bridging conduit trafficking of HIV-1 through the endoplasmic reticulum and Golgi network. *Journal of proteome research* **10**, 3225-3238, doi:10.1021/pr200262q (2011).
114 Gousset, K. *et al.* Prions hijack tunnelling nanotubes for intercellular spread. *Nature cell biology* **11**, 328-336, doi:10.1038/ncb1841 (2009).
115 He, K. *et al.* Long-distance intercellular connectivity between cardiomyocytes and cardiofibroblasts mediated by membrane nanotubes. *Cardiovascular research* **92**, 39-47, doi:10.1093/cvr/cvr189 (2011).
116 Wang, X., Veruki, M. L., Bukoreshtliev, N. V., Hartveit, E. & Gerdes, H. H. Animal cells connected by nanotubes can be electrically coupled through interposed gap-junction channels. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 17194-17199, doi:10.1073/pnas.1006785107 (2010).
117 Arkwright, P. D. *et al.* Fas stimulation of T lymphocytes promotes rapid intercellular exchange of death signals via membrane nanotubes. *Cell research* **20**, 72-88, doi:10.1038/cr.2009.112 (2010).

118 Chauveau, A., Aucher, A., Eissmann, P., Vivier, E. & Davis, D. M. Membrane nanotubes facilitate long-distance interactions between natural killer cells and target cells. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 5545-5550, doi:10.1073/pnas.0910074107 (2010).
119 Haimovich, G. *et al.* Intercellular mRNA trafficking via membrane nanotube-like extensions in mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America* **114**, E9873-e9882, doi:10.1073/pnas.1706365114 (2017).
120 Climent, M. *et al.* TGFbeta Triggers miR-143/145 Transfer From Smooth Muscle Cells to Endothelial Cells, Thereby Modulating Vessel Stabilization. *Circulation research* **116**, 1753-1764, doi:10.1161/circresaha.116.305178 (2015).
121 Hsiung, F., Ramirez-Weber, F. A., Iwaki, D. D. & Kornberg, T. B. Dependence of Drosophila wing imaginal disc cytonemes on Decapentaplegic. *Nature* **437**, 560-563, doi:10.1038/nature03951 (2005).
122 Quinn, T. A. *et al.* Electrotonic coupling of excitable and nonexcitable cells in the heart revealed by optogenetics. *Proceedings of the National Academy of Sciences of the United States of America* **113**, 14852-14857, doi:10.1073/pnas.1611184114 (2016).
123 Chinnery, H. R., Pearlman, E. & McMenamin, P. G. Cutting edge: Membrane nanotubes in vivo: a feature of MHC class II+ cells in the mouse cornea. *Journal of immunology (Baltimore, Md. : 1950)* **180**, 5779-5783 (2008).
124 Lou, E. *et al.* Imaging Tunneling Membrane Tubes Elucidates Cell Communication in Tumors. *Trends in cancer* **3**, 678-685, doi:10.1016/j.trecan.2017.08.001 (2017).
125 Tardivel, M. *et al.* Tunneling nanotube (TNT)-mediated neuron-to neuron transfer of pathological Tau protein assemblies. *Acta neuropathologica communications* **4**, 117, doi:10.1186/s40478-016-0386-4 (2016).
126 Vidulescu, C., Clejan, S. & O'Connor K, C. Vesicle traffic through intercellular bridges in DU 145 human prostate cancer cells. *Journal of cellular and molecular medicine* **8**, 388-396 (2004).
127 Li, X. *et al.* Mitochondrial transfer of induced pluripotent stem cell-derived mesenchymal stem cells to airway epithelial cells attenuates cigarette smoke-induced damage. *American journal of respiratory cell and molecular biology* **51**, 455-465, doi:10.1165/rcmb.2013-0529OC (2014).
128 Yang, H. *et al.* Biochip-based study of unidirectional mitochondrial transfer from stem cells to myocytes via tunneling nanotubes. *Biofabrication* **8**, 015012, doi:10.1088/1758-5090/8/1/015012 (2016).
129 Hanna, S. J. *et al.* The Role of Rho-GTPases and actin polymerization during Macrophage Tunneling Nanotube Biogenesis. *Scientific reports* **7**, 8547, doi:10.1038/s41598-017-08950-7 (2017).
130 Zaccard, C. R. *et al.* CD40L induces functional tunneling nanotube networks exclusively in dendritic cells programmed by mediators of type 1 immunity. *Journal of immunology (Baltimore, Md. : 1950)* **194**, 1047-1056, doi:10.4049/jimmunol.1401832 (2015).
131 Sowinski, S. *et al.* Membrane nanotubes physically connect T cells over long distances presenting a novel route for HIV-1 transmission. *Nature cell biology* **10**, 211-219, doi:10.1038/ncb1682 (2008).
132 Ramos-Barbon, D. *et al.* T Cells localize with proliferating smooth muscle alpha-actin+ cell compartments in asthma. *American journal of respiratory and critical care medicine* **182**, 317-324, doi:10.1164/rccm.200905-0745OC (2010).
133 Wilson, C. B., Rowell, E. & Sekimata, M. Epigenetic control of T-helper-cell differentiation. *Nature reviews. Immunology* **9**, 91-105, doi:10.1038/nri2487 (2009).
134 Murphy, K. M. & Stockinger, B. Effector T cell plasticity: flexibility in the face of changing circumstances. *Nature immunology* **11**, 674-680, doi:10.1038/ni.1899 (2010).

135 Robinson, D. S. The role of the T cell in asthma. *The Journal of allergy and clinical immunology* **126**, 1081-1091; quiz 1092-1083, doi:10.1016/j.jaci.2010.06.025 (2010).
136 Paul, W. E. & Zhu, J. How are T(H)2-type immune responses initiated and amplified? *Nature reviews. Immunology* **10**, 225-235, doi:10.1038/nri2735 (2010).
137 Fahy, J. V. Type 2 inflammation in asthma--present in most, absent in many. *Nature reviews. Immunology* **15**, 57-65, doi:10.1038/nri3786 (2015).
138 Bosnjak, B., Stelzmueller, B., Erb, K. J. & Epstein, M. M. Treatment of allergic asthma: modulation of Th2 cells and their responses. *Respiratory research* **12**, 114, doi:10.1186/1465-9921-12-114 (2011).
139 Finotto, S. *et al.* Development of spontaneous airway changes consistent with human asthma in mice lacking T-bet. *Science (New York, N.Y.)* **295**, 336-338, doi:10.1126/science.1065544 (2002).
140 Nakamura, Y. *et al.* Gene expression of the GATA-3 transcription factor is increased in atopic asthma. *The Journal of allergy and clinical immunology* **103**, 215-222 (1999).
141 Stockinger, B. & Omenetti, S. The dichotomous nature of T helper 17 cells. *Nature reviews. Immunology* **17**, 535-544, doi:10.1038/nri.2017.50 (2017).
142 Wang, Y. H. & Wills-Karp, M. The potential role of interleukin-17 in severe asthma. *Current allergy and asthma reports* **11**, 388-394, doi:10.1007/s11882-011-0210-y (2011).
143 Wang, Q., Li, H., Yao, Y., Xia, D. & Zhou, J. The overexpression of heparin-binding epidermal growth factor is responsible for Th17-induced airway remodeling in an experimental asthma model. *Journal of immunology (Baltimore, Md. : 1950)* **185**, 834-841, doi:10.4049/jimmunol.0901490 (2010).
144 Martin-Orozco, E., Norte-Munoz, M. & Martinez-Garcia, J. Regulatory T Cells in Allergy and Asthma. *Frontiers in pediatrics* **5**, 117, doi:10.3389/fped.2017.00117 (2017).
145 Michalek, R. D. *et al.* Cutting edge: distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory CD4+ T cell subsets. *Journal of immunology (Baltimore, Md. : 1950)* **186**, 3299-3303, doi:10.4049/jimmunol.1003613 (2011).
146 Hartl, D. *et al.* Quantitative and functional impairment of pulmonary CD4+CD25hi regulatory T cells in pediatric asthma. *The Journal of allergy and clinical immunology* **119**, 1258-1266, doi:10.1016/j.jaci.2007.02.023 (2007).
147 Koch, S., Sopel, N. & Finotto, S. Th9 and other IL-9-producing cells in allergic asthma. *Seminars in immunopathology* **39**, 55-68, doi:10.1007/s00281-016-0601-1 (2017).
148 Eyerich, S. *et al.* Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. *The Journal of clinical investigation* **119**, 3573-3585, doi:10.1172/jci40202 (2009).
149 Betts, R. J. & Kemeny, D. M. CD8+ T cells in asthma: friend or foe? *Pharmacology & therapeutics* **121**, 123-131, doi:10.1016/j.pharmthera.2008.09.001 (2009).
150 van Rensen, E. L. *et al.* Bronchial CD8 cell infiltrate and lung function decline in asthma. *American journal of respiratory and critical care medicine* **172**, 837-841, doi:10.1164/rccm.200504-619OC (2005).
151 Stock, P. *et al.* CD8(+) T cells regulate immune responses in a murine model of allergen-induced sensitization and airway inflammation. *European journal of immunology* **34**, 1817-1827, doi:10.1002/eji.200324623 (2004).
152 Wells, J. W., Cowled, C. J., Giorgini, A., Kemeny, D. M. & Noble, A. Regulation of allergic airway inflammation by class I-restricted allergen presentation and CD8 T-cell infiltration. *The Journal of allergy and clinical immunology* **119**, 226-234, doi:10.1016/j.jaci.2006.09.004 (2007).
153 Wu, Y. L. *et al.* gammadelta T cells and their potential for immunotherapy. *International journal of biological sciences* **10**, 119-135, doi:10.7150/ijbs.7823 (2014).

154 Ramstead, A. G. & Jutila, M. A. Complex role of gammadelta T-cell-derived cytokines and growth factors in cancer. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* **32**, 563-569, doi:10.1089/jir.2012.0073 (2012).
155 Zheng, R. & Yang, Q. The role of the gamma delta T cell in allergic diseases. *Journal of immunology research* **2014**, 963484, doi:10.1155/2014/963484 (2014).
156 McMenamin, C., Pimm, C., McKersey, M. & Holt, P. G. Regulation of IgE responses to inhaled antigen in mice by antigen-specific gamma delta T cells. *Science (New York, N.Y.)* **265**, 1869-1871 (1994).
157 Isogai, S. *et al.* The effects of CD8+gammadelta T cells on late allergic airway responses and airway inflammation in rats. *The Journal of allergy and clinical immunology* **112**, 547-555 (2003).
158 Cooper, M. D. The early history of B cells. *Nature reviews. Immunology* **15**, 191-197, doi:10.1038/nri3801 (2015).
159 Nemazee, D. Mechanisms of central tolerance for B cells. *Nature reviews. Immunology* **17**, 281-294, doi:10.1038/nri.2017.19 (2017).
160 Pieper, K., Grimbacher, B. & Eibel, H. B-cell biology and development. *The Journal of allergy and clinical immunology* **131**, 959-971, doi:10.1016/j.jaci.2013.01.046 (2013).
161 Cornaby, C. *et al.* B cell epitope spreading: mechanisms and contribution to autoimmune diseases. *Immunology letters* **163**, 56-68, doi:10.1016/j.imlet.2014.11.001 (2015).
162 Hoffman, W., Lakkis, F. G. & Chalasani, G. B Cells, Antibodies, and More. *Clinical journal of the American Society of Nephrology : CJASN* **11**, 137-154, doi:10.2215/cjn.09430915 (2016).
163 Mitzner, W. Airway smooth muscle: the appendix of the lung. *American journal of respiratory and critical care medicine* **169**, 787-790, doi:10.1164/rccm.200312-1636PP (2004).
164 Gunst, S. J. & Tang, D. D. The contractile apparatus and mechanical properties of airway smooth muscle. *The European respiratory journal* **15**, 600-616 (2000).
165 Canning, B. J., Woo, A. & Mazzone, S. B. Neuronal modulation of airway and vascular tone and their influence on nonspecific airways responsiveness in asthma. *Journal of allergy* **2012**, 108149, doi:10.1155/2012/108149 (2012).
166 Amrani, Y. & Panettieri, R. A. Airway smooth muscle: contraction and beyond. *The international journal of biochemistry & cell biology* **35**, 272-276 (2003).
167 Hautmann, M. B., Madsen, C. S. & Owens, G. K. A transforming growth factor beta (TGFbeta) control element drives TGFbeta-induced stimulation of smooth muscle alpha-actin gene expression in concert with two CArG elements. *The Journal of biological chemistry* **272**, 10948-10956 (1997).
168 Berair, R., Saunders, R. & Brightling, C. E. Origins of increased airway smooth muscle mass in asthma. *BMC medicine* **11**, 145, doi:10.1186/1741-7015-11-145 (2013).
169 Pascoe, C. D., Wang, L., Syyong, H. T. & Pare, P. D. A Brief History of Airway Smooth Muscle's Role in Airway Hyperresponsiveness. *Journal of allergy* **2012**, 768982, doi:10.1155/2012/768982 (2012).
170 Wang, S. W. *et al.* Amphiregulin expression in human mast cells and its effect on the primary human lung fibroblasts. *The Journal of allergy and clinical immunology* **115**, 287-294, doi:10.1016/j.jaci.2004.11.037 (2005).
171 Blotnick, S., Peoples, G. E., Freeman, M. R., Eberlein, T. J. & Klagsbrun, M. T lymphocytes synthesize and export heparin-binding epidermal growth factor-like growth factor and basic fibroblast growth factor, mitogens for vascular cells and fibroblasts: differential production and release by CD4+ and CD8+ T cells. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 2890-2894 (1994).
172 Tliba, O. & Panettieri, R. A., Jr. Noncontractile functions of airway smooth muscle cells in asthma. *Annual review of physiology* **71**, 509-535, doi:10.1146/annurev.physiol.010908.163227 (2009).

173 Zuyderduyn, S., Sukkar, M. B., Fust, A., Dhaliwal, S. & Burgess, J. K. Treating asthma means treating airway smooth muscle cells. *The European respiratory journal* **32**, 265-274, doi:10.1183/09031936.00051407 (2008).
174 Amishima, M. *et al.* Expression of epidermal growth factor and epidermal growth factor receptor immunoreactivity in the asthmatic human airway. *American journal of respiratory and critical care medicine* **157**, 1907-1912, doi:10.1164/ajrccm.157.6.9609040 (1998).
175 Ganesan, S. & Sajjan, U. S. Repair and Remodeling of airway epithelium after injury in Chronic Obstructive Pulmonary Disease. *Current respiratory care reports* **2**, doi:10.1007/s13665-013-0052-2 (2013).
176 Takeyama, K. *et al.* Epidermal growth factor system regulates mucin production in airways. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 3081-3086 (1999).
177 Takeyama, K. *et al.* Activation of epidermal growth factor receptors is responsible for mucin synthesis induced by cigarette smoke. *American journal of physiology. Lung cellular and molecular physiology* **280**, L165-172 (2001).
178 Takeyama, K. *et al.* Oxidative stress causes mucin synthesis via transactivation of epidermal growth factor receptor: role of neutrophils. *Journal of immunology (Baltimore, Md. : 1950)* **164**, 1546-1552 (2000).
179 Kohri, K., Ueki, I. F. & Nadel, J. A. Neutrophil elastase induces mucin production by ligand-dependent epidermal growth factor receptor activation. *American journal of physiology. Lung cellular and molecular physiology* **283**, L531-540, doi:10.1152/ajplung.00455.2001 (2002).
180 Burgel, P. R. *et al.* Human eosinophils induce mucin production in airway epithelial cells via epidermal growth factor receptor activation. *Journal of immunology (Baltimore, Md. : 1950)* **167**, 5948-5954 (2001).
181 Vargaftig, B. B. & Singer, M. Leukotrienes mediate part of Ova-induced lung effects in mice via EGFR. *American journal of physiology. Lung cellular and molecular physiology* **285**, L808-818, doi:10.1152/ajplung.00377.2002 (2003).
182 Wong, D. T. *et al.* Human eosinophils express transforming growth factor alpha. *The Journal of experimental medicine* **172**, 673-681 (1990).
183 Zaiss, D. M. *et al.* Amphiregulin enhances regulatory T cell-suppressive function via the epidermal growth factor receptor. *Immunity* **38**, 275-284, doi:10.1016/j.immuni.2012.09.023 (2013).
184 Shao, M. X., Ueki, I. F. & Nadel, J. A. Tumor necrosis factor alpha-converting enzyme mediates MUC5AC mucin expression in cultured human airway epithelial cells. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 11618-11623, doi:10.1073/pnas.1534804100 (2003).
185 Greenlee, K. J., Werb, Z. & Kheradmand, F. Matrix metalloproteinases in lung: multiple, multifarious, and multifaceted. *Physiological reviews* **87**, 69-98, doi:10.1152/physrev.00022.2006 (2007).
186 Hamilton, L. M. *et al.* The role of the epidermal growth factor receptor in sustaining neutrophil inflammation in severe asthma. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* **33**, 233-240 (2003).
187 Schneider, M. R. & Wolf, E. The epidermal growth factor receptor ligands at a glance. *Journal of cellular physiology* **218**, 460-466, doi:10.1002/jcp.21635 (2009).
188 Tengroth, L. *et al.* Functional effects of Toll-like receptor (TLR)3, 7, 9, RIG-I and MDA-5 stimulation in nasal epithelial cells. *PloS one* **9**, e98239, doi:10.1371/journal.pone.0098239 (2014).
189 Wieduwilt, M. J. & Moasser, M. M. The epidermal growth factor receptor family: biology driving targeted therapeutics. *Cellular and molecular life sciences : CMLS* **65**, 1566-1584, doi:10.1007/s00018-008-7440-8 (2008).

190 Garrett, T. P. *et al.* The crystal structure of a truncated ErbB2 ectodomain reveals an active conformation, poised to interact with other ErbB receptors. *Molecular cell* **11**, 495-505 (2003).
191 Higashiyama, S., Abraham, J. A., Miller, J., Fiddes, J. C. & Klagsbrun, M. A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. *Science (New York, N.Y.)* **251**, 936-939 (1991).
192 Prince, R. N. *et al.* The heparin-binding domain of HB-EGF mediates localization to sites of cell-cell contact and prevents HB-EGF proteolytic release. *Journal of cell science* **123**, 2308-2318, doi:10.1242/jcs.058321 (2010).
193 Fen, Z. *et al.* Structural organization and chromosomal assignment of the gene encoding the human heparin-binding epidermal growth factor-like growth factor/diphtheria toxin receptor. *Biochemistry* **32**, 7932-7938 (1993).
194 Pathak, B. G. *et al.* Mouse chromosomal location of three EGF receptor ligands: amphiregulin (Areg), betacellulin (Btc), and heparin-binding EGF (Hegfl). *Genomics* **28**, 116-118, doi:10.1006/geno.1995.1116 (1995).
195 Naglich, J. G., Metherall, J. E., Russell, D. W. & Eidels, L. Expression cloning of a diphtheria toxin receptor: identity with a heparin-binding EGF-like growth factor precursor. *Cell* **69**, 1051-1061 (1992).
196 Singh, B., Carpenter, G. & Coffey, R. J. EGF receptor ligands: recent advances. *F1000Research* **5**, doi:10.12688/f1000research.9025.1 (2016).
197 Vinante, F. *et al.* Granulocyte-macrophage colony-stimulating factor induces expression of heparin-binding epidermal growth factor-like growth factor/diphtheria toxin receptor and sensitivity to diphtheria toxin in human neutrophils. *Blood* **94**, 3169-3177 (1999).
198 Powell, P. P., Klagsbrun, M., Abraham, J. A. & Jones, R. C. Eosinophils expressing heparin-binding EGF-like growth factor mRNA localize around lung microvessels in pulmonary hypertension. *The American journal of pathology* **143**, 784-793 (1993).
199 De Vos, J. *et al.* Identifying intercellular signaling genes expressed in malignant plasma cells by using complementary DNA arrays. *Blood* **98**, 771-780 (2001).
200 Dluz, S. M., Higashiyama, S., Damm, D., Abraham, J. A. & Klagsbrun, M. Heparin-binding epidermal growth factor-like growth factor expression in cultured fetal human vascular smooth muscle cells. Induction of mRNA levels and secretion of active mitogen. *The Journal of biological chemistry* **268**, 18330-18334 (1993).
201 Yoshizumi, M. *et al.* Tumor necrosis factor increases transcription of the heparin-binding epidermal growth factor-like growth factor gene in vascular endothelial cells. *The Journal of biological chemistry* **267**, 9467-9469 (1992).
202 Hashimoto, K. *et al.* Heparin-binding epidermal growth factor-like growth factor is an autocrine growth factor for human keratinocytes. *The Journal of biological chemistry* **269**, 20060-20066 (1994).
203 Kinugasa, Y., Hieda, M., Hori, M. & Higashiyama, S. The carboxyl-terminal fragment of pro-HB-EGF reverses Bcl6-mediated gene repression. *The Journal of biological chemistry* **282**, 14797-14806, doi:10.1074/jbc.M611036200 (2007).
204 Dong, J. *et al.* The membrane-anchoring domain of epidermal growth factor receptor ligands dictates their ability to operate in juxtacrine mode. *Molecular biology of the cell* **16**, 2984-2998, doi:10.1091/mbc.E04-11-0994 (2005).
205 Wang, X. *et al.* Cytoplasmic domain phosphorylation of heparin-binding EGF-like growth factor. *Cell structure and function* **31**, 15-27 (2006).
206 Agache, I. & Akdis, C. A. Endotypes of allergic diseases and asthma: An important step in building blocks for the future of precision medicine. *Allergology international : official journal of the Japanese Society of Allergology* **65**, 243-252, doi:10.1016/j.alit.2016.04.011 (2016).

207 Yarden, Y. The EGFR family and its ligands in human cancer. signalling mechanisms and therapeutic opportunities. *European journal of cancer (Oxford, England : 1990)* **37 Suppl 4**, S3-8 (2001).
208 Wang, Z. Transactivation of Epidermal Growth Factor Receptor by G Protein-Coupled Receptors: Recent Progress, Challenges and Future Research. *International journal of molecular sciences* **17**, doi:10.3390/ijms17010095 (2016).
209 Giri, D. K. *et al.* Endosomal transport of ErbB-2: mechanism for nuclear entry of the cell surface receptor. *Molecular and cellular biology* **25**, 11005-11018, doi:10.1128/mcb.25.24.11005-11018.2005 (2005).
210 Li, L. Y. *et al.* Nuclear ErbB2 enhances translation and cell growth by activating transcription of ribosomal RNA genes. *Cancer research* **71**, 4269-4279, doi:10.1158/0008-5472.can-10-3504 (2011).
211 Sithanandam, G. & Anderson, L. M. The ERBB3 receptor in cancer and cancer gene therapy. *Cancer gene therapy* **15**, 413-448, doi:10.1038/cgt.2008.15 (2008).
212 Sundvall, M. *et al.* Role of ErbB4 in breast cancer. *Journal of mammary gland biology and neoplasia* **13**, 259-268, doi:10.1007/s10911-008-9079-3 (2008).
213 Minutti, C. M. *et al.* Epidermal Growth Factor Receptor Expression Licenses Type-2 Helper T Cells to Function in a T Cell Receptor-Independent Fashion. *Immunity* **47**, 710-722.e716, doi:10.1016/j.immuni.2017.09.013 (2017).
214 Liu, H. *et al.* MicroRNA-338-3p regulates osteogenic differentiation of mouse bone marrow stromal stem cells by targeting Runx2 and Fgfr2. *Journal of cellular physiology* **229**, 1494-1502, doi:10.1002/jcp.24591 (2014).
215 Carraro, G. *et al.* miR-17 family of microRNAs controls FGF10-mediated embryonic lung epithelial branching morphogenesis through MAPK14 and STAT3 regulation of E-Cadherin distribution. *Developmental biology* **333**, 238-250, doi:10.1016/j.ydbio.2009.06.020 (2009).
216 Uchiyama, K. *et al.* Carbon monoxide enhance colonic epithelial restitution via FGF15 derived from colonic myofibroblasts. *Biochemical and biophysical research communications* **391**, 1122-1126, doi:10.1016/j.bbrc.2009.12.035 (2010).
217 Goldfarb, M. *et al.* Fibroblast growth factor homologous factors control neuronal excitability through modulation of voltage-gated sodium channels. *Neuron* **55**, 449-463, doi:10.1016/j.neuron.2007.07.006 (2007).
218 Schoorlemmer, J. & Goldfarb, M. Fibroblast growth factor homologous factors are intracellular signaling proteins. *Current biology : CB* **11**, 793-797 (2001).
219 Wu, Q. F. *et al.* Fibroblast growth factor 13 is a microtubule-stabilizing protein regulating neuronal polarization and migration. *Cell* **149**, 1549-1564, doi:10.1016/j.cell.2012.04.046 (2012).
220 Ornitz, D. M. & Itoh, N. The Fibroblast Growth Factor signaling pathway. *Wiley interdisciplinary reviews. Developmental biology* **4**, 215-266, doi:10.1002/wdev.176 (2015).
221 Turner, N. & Grose, R. Fibroblast growth factor signalling: from development to cancer. *Nature reviews. Cancer* **10**, 116-129, doi:10.1038/nrc2780 (2010).
222 Bissonnette, E. Y. *et al.* Fibroblast growth factor-2 is a sputum remodeling biomarker of severe asthma. *The Journal of asthma : official journal of the Association for the Care of Asthma* **51**, 119-126, doi:10.3109/02770903.2013.860164 (2014).
223 Vock, C., Hauber, H. P. & Wegmann, M. The other T helper cells in asthma pathogenesis. *Journal of allergy* **2010**, 519298, doi:10.1155/2010/519298 (2010).
224 Taylor, S. R., Markesbery, M. G. & Harding, P. A. Heparin-binding epidermal growth factor-like growth factor (HB-EGF) and proteolytic processing by a disintegrin and metalloproteinases (ADAM): a regulator of several pathways. *Seminars in cell & developmental biology* **28**, 22-30, doi:10.1016/j.semcdb.2014.03.004 (2014).

225 Hirata, Y. *et al.* BCL6 degradation caused by the interaction with the C-terminus of pro-HB-EGF induces cyclin D2 expression in gastric cancers. *British journal of cancer* **100**, 1320-1329, doi:10.1038/sj.bjc.6605010 (2009).
226 Nanba, D., Mammoto, A., Hashimoto, K. & Higashiyama, S. Proteolytic release of the carboxy-terminal fragment of proHB-EGF causes nuclear export of PLZF. *The Journal of cell biology* **163**, 489-502, doi:10.1083/jcb.200303017 (2003).
227 Fooksman, D. R. *et al.* Functional anatomy of T cell activation and synapse formation. *Annual review of immunology* **28**, 79-105, doi:10.1146/annurev-immunol-030409-101308 (2010).
228 Benson, R. A. *et al.* Antigen presentation kinetics control T cell/dendritic cell interactions and follicular helper T cell generation in vivo. *eLife* **4**, doi:10.7554/eLife.06994 (2015).
229 Fazilleau, N., McHeyzer-Williams, L. J., Rosen, H. & McHeyzer-Williams, M. G. The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding. *Nature immunology* **10**, 375-384, doi:10.1038/ni.1704 (2009).
230 Tubo, N. J. *et al.* Single naive CD4+ T cells from a diverse repertoire produce different effector cell types during infection. *Cell* **153**, 785-796, doi:10.1016/j.cell.2013.04.007 (2013).
231 Crotty, S., Johnston, R. J. & Schoenberger, S. P. Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation. *Nature immunology* **11**, 114-120, doi:10.1038/ni.1837 (2010).
232 Arima, M. *et al.* A putative silencer element in the IL-5 gene recognized by Bcl6. *Journal of immunology (Baltimore, Md. : 1950)* **169**, 829-836 (2002).
233 Ogasawara, T. *et al.* Development of chronic allergic responses by dampening Bcl6-mediated suppressor activity in memory T helper 2 cells. *Proceedings of the National Academy of Sciences of the United States of America* **114**, E741-e750, doi:10.1073/pnas.1613528114 (2017).
234 Dent, A. L., Shaffer, A. L., Yu, X., Allman, D. & Staudt, L. M. Control of inflammation, cytokine expression, and germinal center formation by BCL-6. *Science (New York, N.Y.)* **276**, 589-592 (1997).
235 Arima, M., Fukuda, T. & Tokuhisa, T. Role of the Transcriptional Repressor BCL6 in Allergic Response and Inflammation. *The World Allergy Organization journal* **1**, 115-122, doi:10.1097/WOX.0b013e31817dc522 (2008).
236 Doran, E. *et al.* Interleukin-13 in Asthma and Other Eosinophilic Disorders. *Frontiers in medicine* **4**, 139, doi:10.3389/fmed.2017.00139 (2017).
237 Takatsu, K. & Nakajima, H. IL-5 and eosinophilia. *Current opinion in immunology* **20**, 288-294, doi:10.1016/j.coi.2008.04.001 (2008).
238 Robinson, D. S. *et al.* Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *The New England journal of medicine* **326**, 298-304, doi:10.1056/nejm199201303260504 (1992).
239 Greenfeder, S., Umland, S. P., Cuss, F. M., Chapman, R. W. & Egan, R. W. Th2 cytokines and asthma. The role of interleukin-5 in allergic eosinophilic disease. *Respiratory research* **2**, 71-79 (2001).
240 Badr, G. *et al.* Type I interferon (IFN-alpha/beta) rescues B-lymphocytes from apoptosis via PI3Kdelta/Akt, Rho-A, NFkappaB and Bcl-2/Bcl(XL). *Cellular immunology* **263**, 31-40, doi:10.1016/j.cellimm.2010.02.012 (2010).
241 Koshikawa, N. *et al.* Proteolytic activation of heparin-binding EGF-like growth factor by membrane-type matrix metalloproteinase-1 in ovarian carcinoma cells. *Cancer science* **102**, 111-116, doi:10.1111/j.1349-7006.2010.01748.x (2011).
242 Suzuki, M., Raab, G., Moses, M. A., Fernandez, C. A. & Klagsbrun, M. Matrix metalloproteinase-3 releases active heparin-binding EGF-like growth factor by cleavage at a specific juxtamembrane site. *The Journal of biological chemistry* **272**, 31730-31737 (1997).

243 Kivisaari, A. K. *et al.* Matrix metalloproteinase-7 activates heparin-binding epidermal growth factor-like growth factor in cutaneous squamous cell carcinoma. *The British journal of dermatology* **163**, 726-735, doi:10.1111/j.1365-2133.2010.09924.x (2010).
244 Jain, C. V. *et al.* Trophoblast survival signaling during human placentation requires HSP70 activation of MMP2-mediated HBEGF shedding. *Cell death and differentiation* **24**, 1772-1783, doi:10.1038/cdd.2017.104 (2017).
245 Goishi, K. *et al.* Phorbol ester induces the rapid processing of cell surface heparin-binding EGF-like growth factor: conversion from juxtacrine to paracrine growth factor activity. *Molecular biology of the cell* **6**, 967-980 (1995).
246 Raab, G. *et al.* Biosynthesis and processing by phorbol ester of the cells surface-associated precursor form of heparin-binding EGF-like growth factor. *Biochemical and biophysical research communications* **204**, 592-597 (1994).
247 Dethlefsen, S. M. *et al.* Extracellular calcium influx stimulates metalloproteinase cleavage and secretion of heparin-binding EGF-like growth factor independently of protein kinase C. *Journal of cellular biochemistry* **69**, 143-153 (1998).
248 Raab, G. *et al.* Mouse preimplantation blastocysts adhere to cells expressing the transmembrane form of heparin-binding EGF-like growth factor. *Development (Cambridge, England)* **122**, 637-645 (1996).
249 Barnes, P. J. Effect of corticosteroids on airway hyperresponsiveness. *The American review of respiratory disease* **141**, S70-76 (1990).
250 van Rensen, E. L. *et al.* Effect of inhaled steroids on airway hyperresponsiveness, sputum eosinophils, and exhaled nitric oxide levels in patients with asthma. *Thorax* **54**, 403-408 (1999).
251 Morimoto, Y. *et al.* Amphiregulin-Producing Pathogenic Memory T Helper 2 Cells Instruct Eosinophils to Secrete Osteopontin and Facilitate Airway Fibrosis. *Immunity* **49**, 134-150.e136, doi:10.1016/j.immuni.2018.04.023 (2018).
252 Wegrowski, Y. *et al.* Cell surface proteoglycan expression during maturation of human monocytes-derived dendritic cells and macrophages. *Clinical and experimental immunology* **144**, 485-493, doi:10.1111/j.1365-2249.2006.03059.x (2006).
253 McBrien, C. N. & Menzies-Gow, A. The Biology of Eosinophils and Their Role in Asthma. *Frontiers in medicine* **4**, 93, doi:10.3389/fmed.2017.00093 (2017).
254 Foster, P. S., Hogan, S. P., Ramsay, A. J., Matthaei, K. I. & Young, I. G. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *The Journal of experimental medicine* **183**, 195-201 (1996).
255 Humbles, A. A. *et al.* A critical role for eosinophils in allergic airways remodeling. *Science (New York, N.Y.)* **305**, 1776-1779, doi:10.1126/science.1100283 (2004).
256 Lee, J. J. *et al.* Defining a link with asthma in mice congenitally deficient in eosinophils. *Science (New York, N.Y.)* **305**, 1773-1776, doi:10.1126/science.1099472 (2004).
257 Eum, S. Y., Maghni, K., Tolloczko, B., Eidelman, D. H. & Martin, J. G. IL-13 may mediate allergen-induced hyperresponsiveness independently of IL-5 or eotaxin by effects on airway smooth muscle. *American journal of physiology. Lung cellular and molecular physiology* **288**, L576-584, doi:10.1152/ajplung.00380.2003 (2005).
258 Leckie, M. J. *et al.* Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet (London, England)* **356**, 2144-2148 (2000).
259 Castro, M. *et al.* Reslizumab for poorly controlled, eosinophilic asthma: a randomized, placebo-controlled study. *American journal of respiratory and critical care medicine* **184**, 1125-1132, doi:10.1164/rccm.201103-0396OC (2011).

260 L. Trevor, J. *et al. Mepolizumab Improves Lung Function and Exacerbation Rates in Severe Eosinophilic Asthma: The Musca Study - a Phase IIIb Randomized, Placebo-Controlled Tria*. Vol. 139 (2017).
261 Commins, S. P., Borish, L. & Steinke, J. W. Immunologic messenger molecules: cytokines, interferons, and chemokines. *The Journal of allergy and clinical immunology* **125**, S53-72, doi:10.1016/j.jaci.2009.07.008 (2010).
262 Zhang, H. *et al.* CD4(+) T cell-released exosomes inhibit CD8(+) cytotoxic T-lymphocyte responses and antitumor immunity. *Cellular & molecular immunology* **8**, 23-30, doi:10.1038/cmi.2010.59 (2011).
263 Dustin, M. L. A dynamic view of the immunological synapse. *Seminars in immunology* **17**, 400-410, doi:10.1016/j.smim.2005.09.002 (2005).
264 Sowinski, S., Alakoskela, J. M., Jolly, C. & Davis, D. M. Optimized methods for imaging membrane nanotubes between T cells and trafficking of HIV-1. *Methods (San Diego, Calif.)* **53**, 27-33, doi:10.1016/j.ymeth.2010.04.002 (2011).
265 Osborne, D. G. & Wetzel, S. A. Trogocytosis results in sustained intracellular signaling in CD4(+) T cells. *Journal of immunology (Baltimore, Md. : 1950)* **189**, 4728-4739, doi:10.4049/jimmunol.1201507 (2012).
266 Begueret, H. *et al.* Inflammation of bronchial smooth muscle in allergic asthma. *Thorax* **62**, 8-15, doi:10.1136/thx.2006.062141 (2007).
267 Keglowich, L. F. & Borger, P. The Three A's in Asthma - Airway Smooth Muscle, Airway Remodeling & Angiogenesis. *The open respiratory medicine journal* **9**, 70-80, doi:10.2174/1874306401509010070 (2015).
268 Wang, X. & Gerdes, H. H. Transfer of mitochondria via tunneling nanotubes rescues apoptotic PC12 cells. *Cell death and differentiation* **22**, 1181-1191, doi:10.1038/cdd.2014.211 (2015).
269 Burtey, A. *et al.* Intercellular transfer of transferrin receptor by a contact-, Rab8-dependent mechanism involving tunneling nanotubes. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **29**, 4695-4712, doi:10.1096/fj.14-268615 (2015).
270 Mukerji, J., Olivieri, K. C., Misra, V., Agopian, K. A. & Gabuzda, D. Proteomic analysis of HIV-1 Nef cellular binding partners reveals a role for exocyst complex proteins in mediating enhancement of intercellular nanotube formation. *Retrovirology* **9**, 33, doi:10.1186/1742-4690-9-33 (2012).
271 Fifadara, N. H., Beer, F., Ono, S. & Ono, S. J. Interaction between activated chemokine receptor 1 and FcepsilonRI at membrane rafts promotes communication and F-actin-rich cytoneme extensions between mast cells. *International immunology* **22**, 113-128, doi:10.1093/intimm/dxp118 (2010).
272 Seyed-Razavi, Y., Hickey, M. J., Kuffova, L., McMenamin, P. G. & Chinnery, H. R. Membrane nanotubes in myeloid cells in the adult mouse cornea represent a novel mode of immune cell interaction. *Immunology and cell biology* **91**, 89-95, doi:10.1038/icb.2012.52 (2013).
273 Zaccard, C. R., Rinaldo, C. R. & Mailliard, R. B. Linked in: immunologic membrane nanotube networks. *Journal of leukocyte biology*, doi:10.1189/jlb.4VMR0915-395R (2016).
274 Schiller, C., Huber, J. E., Diakopoulos, K. N. & Weiss, E. H. Tunneling nanotubes enable intercellular transfer of MHC class I molecules. *Human immunology* **74**, 412-416, doi:10.1016/j.humimm.2012.11.026 (2013).
275 Wang, X., Bukoreshtliev, N. V. & Gerdes, H. H. Developing neurons form transient nanotubes facilitating electrical coupling and calcium signaling with distant astrocytes. *PloS one* **7**, e47429, doi:10.1371/journal.pone.0047429 (2012).
276 Zhu, S., Victoria, G. S., Marzo, L., Ghosh, R. & Zurzolo, C. Prion aggregates transfer through tunneling nanotubes in endocytic vesicles. *Prion* **9**, 125-135, doi:10.1080/19336896.2015.1025189 (2015).

277 Hashimoto, M. *et al.* Potential Role of the Formation of Tunneling Nanotubes in HIV-1 Spread in Macrophages. *Journal of immunology (Baltimore, Md. : 1950)* **196**, 1832-1841, doi:10.4049/jimmunol.1500845 (2016).
278 Hausott, B., Schlick, B., Vallant, N., Dorn, R. & Klimaschewski, L. Promotion of neurite outgrowth by fibroblast growth factor receptor 1 overexpression and lysosomal inhibition of receptor degradation in pheochromocytoma cells and adult sensory neurons. *Neuroscience* **153**, 461-473, doi:10.1016/j.neuroscience.2008.01.083 (2008).
279 Grothe, C., Haastert, K. & Jungnickel, J. Physiological function and putative therapeutic impact of the FGF-2 system in peripheral nerve regeneration--lessons from in vivo studies in mice and rats. *Brain research reviews* **51**, 293-299, doi:10.1016/j.brainresrev.2005.12.001 (2006).
280 Bottcher, R. T. & Niehrs, C. Fibroblast growth factor signaling during early vertebrate development. *Endocrine reviews* **26**, 63-77, doi:10.1210/er.2003-0040 (2005).
281 Kubota, Y. & Ito, K. Chemotactic migration of mesencephalic neural crest cells in the mouse. *Developmental dynamics : an official publication of the American Association of Anatomists* **217**, 170-179, doi:10.1002/(sici)1097-0177(200002)217:2<170::aid-dvdy4>3.0.co;2-9 (2000).
282 Webb, S. E., Lee, K. K., Tang, M. K. & Ede, D. A. Fibroblast growth factors 2 and 4 stimulate migration of mouse embryonic limb myogenic cells. *Developmental dynamics : an official publication of the American Association of Anatomists* **209**, 206-216, doi:10.1002/(sici)1097-0177(199706)209:2<206::aid-aja6>3.0.co;2-m (1997).
283 McCoy-Simandle, K., Hanna, S. J. & Cox, D. Exosomes and nanotubes: Control of immune cell communication. *The international journal of biochemistry & cell biology* **71**, 44-54, doi:10.1016/j.biocel.2015.12.006 (2016).
284 Beasley, R., Semprini, A. & Mitchell, E. A. Risk factors for asthma: is prevention possible? *Lancet (London, England)* **386**, 1075-1085, doi:10.1016/s0140-6736(15)00156-7 (2015).
285 Figard, L. & Sokac, A. M. A membrane reservoir at the cell surface: unfolding the plasma membrane to fuel cell shape change. *Bioarchitecture* **4**, 39-46, doi:10.4161/bioa.29069 (2014).
286 Dewitt, S. & Hallett, M. Leukocyte membrane "expansion": a central mechanism for leukocyte extravasation. *Journal of leukocyte biology* **81**, 1160-1164, doi:10.1189/jlb.1106710 (2007).
287 Majstoravich, S. *et al.* Lymphocyte microvilli are dynamic, actin-dependent structures that do not require Wiskott-Aldrich syndrome protein (WASp) for their morphology. *Blood* **104**, 1396-1403, doi:10.1182/blood-2004-02-0437 (2004).
288 Drubin, D. G., Feinstein, S. C., Shooter, E. M. & Kirschner, M. W. Nerve growth factor-induced neurite outgrowth in PC12 cells involves the coordinate induction of microtubule assembly and assembly-promoting factors. *The Journal of cell biology* **101**, 1799-1807 (1985).
289 Kim, B., Leventhal, P. S., Saltiel, A. R. & Feldman, E. L. Insulin-like growth factor-I-mediated neurite outgrowth in vitro requires mitogen-activated protein kinase activation. *The Journal of biological chemistry* **272**, 21268-21273 (1997).
290 Rabacchi, S. A. *et al.* BDNF and NT4/5 promote survival and neurite outgrowth of pontocerebellar mossy fiber neurons. *Journal of neurobiology* **40**, 254-269 (1999).
291 Morfini, G., DiTella, M. C., Feiguin, F., Carri, N. & Caceres, A. Neurotrophin-3 enhances neurite outgrowth in cultured hippocampal pyramidal neurons. *Journal of neuroscience research* **39**, 219-232, doi:10.1002/jnr.490390212 (1994).
292 Trian, T. *et al.* Bronchial smooth muscle remodeling involves calcium-dependent enhanced mitochondrial biogenesis in asthma. *The Journal of experimental medicine* **204**, 3173-3181, doi:10.1084/jem.20070956 (2007).
293 Johnson, P. R. *et al.* Airway smooth muscle cell proliferation is increased in asthma. *American journal of respiratory and critical care medicine* **164**, 474-477, doi:10.1164/ajrccm.164.3.2010109 (2001).

294 Hakonarson, H. *et al.* T lymphocyte-mediated changes in airway smooth muscle responsiveness are attributed to induced autocrine release and actions of IL-5 and IL-1beta. *The Journal of allergy and clinical immunology* **110**, 624-633 (2002).
295 Matusovsky, O. S., Nakada, E. M., Kachmar, L., Fixman, E. D. & Lauzon, A. M. CD4+ T cells enhance the unloaded shortening velocity of airway smooth muscle by altering the contractile protein expression. *The Journal of physiology* **592**, 2999-3012, doi:10.1113/jphysiol.2014.270843 (2014).
296 Arpaia, N. *et al.* A Distinct Function of Regulatory T Cells in Tissue Protection. *Cell* **162**, 1078-1089, doi:10.1016/j.cell.2015.08.021 (2015).
297 Bandukwala, H. S. *et al.* Signaling through Fc gamma RIII is required for optimal T helper type (Th)2 responses and Th2-mediated airway inflammation. *The Journal of experimental medicine* **204**, 1875-1889, doi:10.1084/jem.20061134 (2007).
298 Podolin, P. L. *et al.* T cell depletion protects against alveolar destruction due to chronic cigarette smoke exposure in mice. *American journal of physiology. Lung cellular and molecular physiology* **304**, L312-323, doi:10.1152/ajplung.00152.2012 (2013).
299 Terzano, C. *et al.* The smooth muscle and airway hyperresponsiveness. *European review for medical and pharmacological sciences* **7**, 9-26 (2003).
300 Onfelt, B. *et al.* Structurally distinct membrane nanotubes between human macrophages support long-distance vesicular traffic or surfing of bacteria. *Journal of immunology (Baltimore, Md. : 1950)* **177**, 8476-8483 (2006).
301 Austefjord, M. W., Gerdes, H. H. & Wang, X. Tunneling nanotubes: Diversity in morphology and structure. *Communicative & integrative biology* **7**, e27934, doi:10.4161/cib.27934 (2014).
302 Ahmad, T. *et al.* Miro1 regulates intercellular mitochondrial transport & enhances mesenchymal stem cell rescue efficacy. *The EMBO journal* **33**, 994-1010, doi:10.1002/embj.201386030 (2014).
303 Tamaoka, M. *et al.* The epidermal growth factor receptor mediates allergic airway remodelling in the rat. *The European respiratory journal* **32**, 1213-1223, doi:10.1183/09031936.00166907 (2008).
304 Katajisto, P. *et al.* Stem cells. Asymmetric apportioning of aged mitochondria between daughter cells is required for stemness. *Science (New York, N.Y.)* **348**, 340-343, doi:10.1126/science.1260384 (2015).
305 Patsoukis, N. *et al.* The role of metabolic reprogramming in T cell fate and function. *Current trends in immunology* **17**, 1-12 (2016).