The Role of Immunoglobulin E in Airway Remodeling in Asthma

Omer Qibi

Department of Medicine
Division of Experimental Medicine, Meakins-Christie Laboratories
McGill University, Montreal
January 31, 2017

©Omer Qibi, 2017
Acknowledgments

I would like to thank all the people who have helped me to complete my research project. They are my supervisor Dr. Qutayba Hamid, co-supervisor Dr. Bruce Mazer, academic advisor Dr. Elizabeth Fixman and all the committee members whose help, support and guidance were essential for me to overcome the difficulties I have faced during my study. I would also like to thank Dr. Carolyn Baglole for her help and generous support; her comments and advices were pivotal to advance my project to the next step and to help me overcome very challenging situations. I thank all members of Dr. Hamid’s laboratory (i.e. Severine Audusseau, Alice Panariti, Andrea Mogas, Kevin Dessalle, Narayanan Venkatesan, Fazmina Zamzamir and Alexandre Haddad), who were very encouraging and always ready to help. Ms. Severine Audusseau gave me a tremendous amount of help and her contribution to my learning and supervision inside the lab was exceptional. Furthermore, I would like to thank Drs. David Eidelman and Mara Ludwig for their valuable opinions and constructive comments. Last but not least, I want to thank my family who is oversea for their support and motivation.
# TABLE OF CONTENTS

LIST OF ABBREVIATIONS ........................................................................................................... 4

ABSTRACT ........................................................................................................................................... 5

CHAPTER 1: INTRODUCTION .............................................................................................................. 8
  1.1 Asthma ............................................................................................................................................. 8
  1.1.1 Prevalence of asthma ................................................................................................................... 8
  1.1.2 Definition and disease severity of asthma .................................................................................... 8
  1.1.3 Cellular players in asthma .......................................................................................................... 10
  1.1.4 Airway remodeling in asthma ..................................................................................................... 10
  1.2 IgE Immunoglobulin, structure and production ............................................................................ 12
  1.3 IgE Receptors .................................................................................................................................... 13
  1.3.1 IgE Receptors ................................................................................................................................ 13
  1.3.2 FcεRI upregulation by IgE ............................................................................................................ 13
  1.3.3 Crosslinking and signaling of FcεRI ............................................................................................. 14
  1.3.4 Expression and function of FcεRI in other cell types .................................................................. 15
  1.3.5 The low affinity receptor for IgE (FceRII) ................................................................................... 16
  1.4 Anti-IgE therapy (omalizumab) ..................................................................................................... 17

CHAPTER 2: HYPOTHESIS AND AIMS ............................................................................................... 18

CHAPTER 3: EXPERIMENTAL PROCEDURES ..................................................................................... 19
  3.1 Cell culture and Treatment ............................................................................................................ 19
  3.2 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) Test ............................. 20
  3.3 Immunohistochemistry (IHC) ....................................................................................................... 20
  3.4 Flow Cytometry ............................................................................................................................... 21
  3.5 Real-Time PCR (qPCR) .................................................................................................................. 22
  3.6 Enzyme-Linked Immunosorbent Assay (ELISA) .......................................................................... 24
  3.7 Statistics ......................................................................................................................................... 24

CHAPTER 4: CONTRIBUTION OF CO-AUTHORS ................................................................................. 25

CHAPTER 5: RESULTS .......................................................................................................................... 26
  4.1 Sample characteristics .................................................................................................................... 26
  4.2 FcεRI is not expressed by human bronchial epithelial cells ............................................................ 26
  4.3 Treatment with IgE did not upregulate FcεRI in human bronchial epithelial cells of asthmatic subjects or U937 cells ................................................................................................................. 27
  4.4 Crosslinking FcεRI receptor did not increase the release of TGF-β1 or TSLP from human bronchial epithelial cells of asthmatic subjects ............................................................................................................. 28

CHAPTER 6: DISCUSSION ..................................................................................................................... 29
  5.1 Absence of FcεRI expression in bronchial epithelial cells in asthma ............................................ 29
  5.2 IgE did not upregulate FcεRI expression in human bronchial epithelial cells of asthmatic subjects 30
  5.3 Implications of an absence of FcεRI expression in asthma ............................................................ 31
  5.4 Future directions ............................................................................................................................. 32

TABLES AND FIGURES ....................................................................................................................... 33

CHAPTER 7: REFERENCES .................................................................................................................... 45
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgE</td>
<td>Immunoglobulin E</td>
</tr>
<tr>
<td>FcεRI</td>
<td>Fc epsilon RI</td>
</tr>
<tr>
<td>FcεRII</td>
<td>Fc epsilon RII</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced vital capacity</td>
</tr>
<tr>
<td>FEV1</td>
<td>Forced expiratory volume in the first second</td>
</tr>
<tr>
<td>ASMC</td>
<td>airway smooth muscle cells</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>NAEPP</td>
<td>National Asthma Education and Prevention Program</td>
</tr>
<tr>
<td>PEFR</td>
<td>peak expiratory flow rate</td>
</tr>
<tr>
<td>FEV₁</td>
<td>forced expiratory volume in one second</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>ITAM</td>
<td>immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>SYK</td>
<td>spleen tyrosine kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinases</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>extracellular signal-regulated kinases 1/2</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>BFA</td>
<td>brefeldin A</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>siRNA</td>
<td>short interfering ribonucleic acid</td>
</tr>
<tr>
<td>TBEC</td>
<td>tracheal bronchial epithelial cells</td>
</tr>
<tr>
<td>HLP</td>
<td>histidine leucine proline</td>
</tr>
<tr>
<td>TSLP</td>
<td>thymic stromal lymphopoietin</td>
</tr>
<tr>
<td>HDM</td>
<td>house dust mite</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>FVC</td>
<td>forced vital capacity</td>
</tr>
<tr>
<td>BEGM</td>
<td>bronchial epithelial cell growth medium</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>BALF</td>
<td>bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>RBM</td>
<td>reticular basement membrane</td>
</tr>
<tr>
<td>IIAM</td>
<td>International Institute for the Advancement of Medicine</td>
</tr>
<tr>
<td>GINA</td>
<td>Global Initiative for Asthma</td>
</tr>
<tr>
<td>ATS</td>
<td>American Thoracic Society</td>
</tr>
<tr>
<td>ICS</td>
<td>inhaled corticosteroids</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>CL</td>
<td>constant domain</td>
</tr>
<tr>
<td>VL</td>
<td>variable region of the light chain</td>
</tr>
<tr>
<td>VH</td>
<td>variable region of the heavy chain</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
</tr>
</tbody>
</table>
ABSTRACT

Immunoglobulin E (IgE) plays an important role in the pathogenesis of asthma, a chronic disease of the airways characterized often by inflammation and remodeling. In a large proportion of asthmatic patients, anti-IgE therapy is effective in controlling their symptoms. The exact target sites of anti-IgE are not well characterized; however, it has been proposed that the therapeutic effects of anti-IgE come from its intervention of the inflammation and airway remodeling process. IgE acts on two types of receptors: a high affinity receptor and a low affinity receptor. The high affinity receptor or Fc epsilon RI (FcεRI), has been reported to express on inflammatory cells such as mast cells, basophils and eosinophils. An allergen, upon re-exposure, cross-links the membrane bound IgE-receptor complex and leads to the degranulation and release of mediators including histamine and prostaglandins, which in turn drive airway inflammation in asthma. In addition to inflammatory cells, FcεRI has also been reported to express on airway structural cells such as airway smooth muscle cells, where it enhances the production of extracellular matrix (ECM) proteins, proliferation of airway smooth muscle cells (ASMC) and release of cytokines. Airway epithelial cells are involved in the remodeling process by producing remodeling associated cytokines such as transforming growth factor–beta (TGF)-β and thymic stromal lymphopoietin (TSLP). Although FcεRI has also been reported to express on airway epithelial cells, the findings are elusive. To gain an insight on how anti-IgE therapy improves asthma symptoms, in this study we aimed to validate the expression of FcεRI on airway epithelial cells and demonstrate its role in airway remodeling. We measured the expression of FcεRI (1) in situ in bronchial biopsy tissues of asthmatic and control subjects using immunohistochemistry and (2) in vitro in primary human bronchial epithelial cells obtained from asthmatic subjects, at baseline and after treatment with human IgE, using qPCR and flow cytometry. FcεRI expression in situ was detected only in a very small number of cells
in the epithelium of bronchial biopsies of asthmatic and control subjects. *In vitro* measurement revealed no expression of the receptor both at baseline and after treatment with IgE. To convincingly conclude the absence of FcεRI in bronchial epithelial cells, we incubated cells with crosslinking antibody and examined the downstream effects of IgE (i.e. the release of TSLP and TGF-β cytokines) using ELISA. No significant difference in TSLP and TGF-β protein releases was detected between stimulated and unstimulated cells. Hence, our data conclusively indicated that bronchial epithelial cells do not express functional high affinity receptor for IgE. Anti-IgE is therefore likely to exert its therapeutic effects via other structural cell types.

**RÉSUMÉ**

L'immunoglobuline E (IgE) joue un rôle important dans la pathogenèse de l'asthme, une maladie chronique des voies respiratoires souvent caractérisée par l'inflammation et le remodelage. Pour un grand nombre de patients asthmatiques, la thérapie anti-IgE est efficace pour contrôler leurs symptômes. Les sites cibles de l’anti-IgE ne sont pas bien caractérisés; Cependant, il a été proposé que les effets thérapeutiques de l’anti-IgE viennent de son intervention dans processus de inflammation et remodelage des voies aériennes. L'IgE agit sur deux types de récepteurs: un récepteur à haute affinité et un récepteur à faible affinité. Il a été publié que le récepteur à haute affinité ou Fc epsilon RI (FcεRI) est exprimé sur des cellules inflammatoires telles que des mastocytes, des basophiles et des éosinophiles. Durant une ré-exposition, un allergène se lie de manière spécifique au récepteur IgE qui est lié à la membrane, et mène à la dégranulation et à la libération de médiateurs, comme l'histamine et les prostaglandines, qui entraînent une inflammation des voies respiratoires dans l'asthme. En plus des cellules inflammatoires, il a également été publié que le FcεRI est exprimé sur les cellules structurales des voies aériennes telles que les cellules des muscles lisses des voies aériennes, où il augmente la production de
protéines de matrice extracellulaire (ECM), la prolifération des cellules musculaires lisses des voies aériennes (ASMC) et la libération de cytokines. Les cellules épithéliales des voies aériennes sont impliquées dans le processus de remodelage en produisant des cytokines associées au remodelage telles que le facteur de croissance transformant-bêta (TGF) -β et la lymphopoïétine stromale thymique (TSLP). Bien que l’expression du FcεRI sur les cellules épithéliales des voies aériennes ait également été publiée, les résultats ne sont pas clairs. Dans cette étude, nous avons cherché à valider l'expression de FcεRI sur les cellules épithéliales des voies respiratoires et à démontrer son rôle dans le remodelage des voies aériennes. Nous avons mesuré l'expression de FcεRI (1) in situ dans les tissus de biopsie bronchique de sujets asthmatiques et sujets contrôles en utilisant l'immunohistochimie et (2) in vitro dans des cellules épithéliales bronchiques humaines primaires obtenues à partir de sujets asthmatiques, sans traitement et après traitement avec de l’IgE humaine, en utilisant la qPCR et la cytométrie en flux. L'expression de FcεRI in situ n'a été détectée que dans un très petit nombre de cellules dans l'épithélium de biopsies bronchiques d'asthmatiques et de sujets contrôles. Les résultats in vitro n'ont révélé aucune expression du récepteur sans et après traitement avec IgE. Pour conclure de façon convaincante l'absence de FcεRI dans les cellules épithéliales bronchiques, nous avons incubé des cellules avec un anticorps qui lui est spécifique et examiné les effets en aval de l'IgE (c'est-à-dire la libération de cytokines TSLP et TGF-β) par ELISA. Aucune différence significative dans les libérations des protéines TSLP et TGF-β n'a été détectée entre les cellules stimulées et non stimulées. Par conséquent, nos données indiquent de façon concluante que les cellules épithéliales bronchiques n'expriment pas de récepteur fonctionnel à haute affinité pour les IgE. L'anti-IgE est donc susceptible d'exercer ses effets thérapeutiques via d'autres cellulaires structurels.
CHAPTER 1: INTRODUCTION

1.1.1 Prevalence of asthma

Asthma is a chronic disease of the airways often characterized by inflammation, structural changes and heightened sensitivity to bronchoconstrictor stimuli. The burden of this disease is increasing dramatically in many populations (1). The prevalence of asthma in children and young adults has the sharpest increase in countries such as the United Kingdom, Australia, Canada and other westernized countries (2). In Canada, a report published in 2010 calculated that the prevalence of asthma, in all ages, has increased from 8.5% to 13.3% between 1996 and 2005, a relative increase of 55.1% (p < 0.001) (3).

1.1.2 Definition and disease severity of asthma

According to the Global Initiative for Asthma (GINA) 2015 guidelines, asthma is a heterogeneous respiratory disease defined by two main criteria: the first is a history of variable respiratory complaints such as chest tightness, shortness of breath, cough and wheeze that differ in severity and vary from time to time. The second is a variable expiratory airflow limitation (4). For the more severe cases, The American Thoracic Society (ATS) 2013 defined severe asthma as an “asthma which requires treatment with high dose inhaled corticosteroids (ICS) plus a second controller (and/or systemic CS) to prevent it from becoming “uncontrolled” or which remains “uncontrolled” despite this therapy.” (5).

The National Asthma Education and Prevention Program (NAEPP) classified asthma according to severity (6). It is based on assessing clinical symptoms and lung function of an asthma patient before the onset of therapy to determine the severity of his disease. This system
involves three elements: day symptoms, night symptoms and lung function (Table 1) and each element has four grades of severity. Asthma severity in a patient is determined according to the element with greatest severity. For example, if a patient has a continuous daytime symptoms, he will be categorized as having severe persistent asthma, even if his night symptoms is less than two per week and his lung function is normal (6).

**Table 1.** NAEPP classification of asthma severity

<table>
<thead>
<tr>
<th>Symptoms Severity</th>
<th>Days with Symptoms</th>
<th>Nights with Symptoms</th>
<th>PEFR FEV1</th>
<th>PEF Variability</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Step 4</strong> Severe Persistent</td>
<td>Continual</td>
<td>Frequent</td>
<td>≤ 60 %</td>
<td>&gt; 30 %</td>
</tr>
<tr>
<td><strong>Step 3</strong> Moderate Persistent</td>
<td>Daily</td>
<td>&gt; 1 / week</td>
<td>&gt; 60% - &lt; 80 %</td>
<td>&lt; 30 %</td>
</tr>
<tr>
<td><strong>Step 2</strong> Mild Persistent</td>
<td>&gt;2/ week, but &lt; 1 / day</td>
<td>&gt; 2 / month</td>
<td>≥ 80 %</td>
<td>20-30 %</td>
</tr>
<tr>
<td><strong>Step 1</strong> Mild Intermittent</td>
<td>≤ 2 / week</td>
<td>≤ 2 / month</td>
<td>≥ 80 %</td>
<td>&lt; 20 %</td>
</tr>
</tbody>
</table>

1.1.3 Cellular players in asthma

Asthma is a complex trait and at the cellular level it is characterized by the interactions among airway structural cells such as epithelial cells and infiltrating inflammatory cells (e.g. eosinophils, mast cells, basophils, antigen presenting cells (APC) and type 2 helper (Th2) cells). Indeed, the number of eosinophils in the lung is associated with disease severity (7), and has been used to assign clinical phenotypes and dictate guiding therapy. Increases in neutrophils have also been found in patients with severe, persistent asthma.

Bronchial epithelial cells play a role in innate immunity. In asthma the airway epithelium is a source of cytokines and mediators production which together regulate the airway immune response (8). Th2 cells and the cytokines they produced (e.g. interleukin (IL)-4, IL-5 and IL-13) play a significant role in the pathogenesis of asthma (9-11). Th17 cells are important in the development of neutrophilic steroid-resistant asthma (12). Eosinophils, basophils and mast cells are key effector cells present in the inflammatory process of asthma. Eosinophils are major producers of TGF-β which could play an important role in airway remodeling (13). Degranulation of mast cells and basophils in response to FcεRI stimulations lead to the release of mediators that induce bronchoconstriction and mucus production.

1.1.4 Airway remodeling in asthma

Airway remodeling refers to the structural changes seen in asthmatic airways (Figure 1) and is associated with asthma severity and disease outcomes. These changes include epithelial destruction, goblet cell hyperplasia, increased mucus gland size, increased mucus production, increased thickness of reticular basement membrane, subepithelial fibrosis, increased smooth muscle cell mass, airway wall edema and vascular changes (14). Both the large and small
airways are affected in the remodeling process and the large airways are significantly and predominantly affected in the severe cases of asthma (14). In a study that compared various parameters of airway remodeling (e.g. total wall area, smooth muscle cell area, mucus gland area and cartilage area) in the small and large airways of fatal cases of asthma, nonfatal cases and control subjects, it was found that the large airways (more than 4 mm in perimeter) had significantly greater total wall area and smooth muscle area in fatal cases of asthma as compared to nonfatal and control subjects, while smaller airways (less than 4 mm) had significantly greater total wall area and smooth muscle area in both fatal and nonfatal cases as compared to control subjects (14). Mucus glands and cartilage areas in the large airways were significantly greater in fatal cases as compared to control subjects (14).

IgE, in addition to its inflammatory role in the pathogenesis of asthma, has recently been implicated to affect airway remodeling. Incubation of ASMC with human IgE resulted in an increase in cell proliferation and deposition of total ECM protein by ASMC (15). Bronchial epithelial cells are major effector cells in the remodeling process where they secrete profibrotic cytokines (e.g. TGF-β) and induce fibrotic proteins production by fibroblasts (16,17). In asthma, baseline TGF-β1 level in BAL fluid is significantly higher in clinically stable patients with atopic asthma as compared to control subjects following endobronchial allergen exposure, and TGF-β1 is significantly higher in allergen challenged as compared to saline challenged locations (18). Furthermore, in a murine model of ovalbumin induced airway remodeling, treatment of mice with anti-TGF-β suppresses ovalbumin induced subepithelial collagen deposition (19). TSLP is a cytokine discovered in the supernatants of thymic stromal cell line that resembles IL-7. It is produced by immune and structural cells. In vitro Production of TSLP by human airway epithelial cells at the baseline level has been tested in asthmatic and control cells. Asthmatic
airway epithelial cells produce higher amounts of TSLP compared to control cells (41). In a murine model of house dust mite (HDM) induced airway remodeling, pretreatment of the mice with intranasal anti-TSLP antibodies one hour before HDM has been shown to reduce the depth of HDM induced peri-bronchial collagen deposition and goblet cell coverage (42).

![Figure 1](image_url)

Figure courtesy of GJ Berry, Stanford University California and taken from Gally SJ et al. Nature 454: 445(20

Figure 1. H&E staining of cross sections of an airway of a non-asthmatic subject (panel a) and a patient with severe asthma (panel b). M= mucus, SM= bronchial smooth muscles, black arrows in (a and b) refer to goblet cells, asterisks in (a and b) refer to lamina reticularis, green arrows in (b) refer to eosinophils in the submucosa. Specimens were obtained from lung resections and sections were obtained from the same area of the tissue.

1.2 Structure and production of IgE

IgE is composed of two identical heavy epsilon chains and two identical light chains. Each light chain has one constant domain (CL) and one variable region (VL) at the N terminus; each heavy chain has four constant domains (Cε1- Cε4) and one variable region (VH) at the N terminus. These domains are linked by disulfide bonds (20). While the variable regions bind to the epitopes of an antigen, the constant domains of the heavy chains bind to the IgE receptors. IgE exists as either membrane bound or secretory. The former is expressed on B cells after class
switching to IgE and secretory IgE is produced by IgE plasma cell either in the lymph nodes or locally in the mucosa (21).

1.3 IgE receptors

IgE binds to two types of receptors, the high affinity receptor (FcεRI) and the low affinity receptor (FcεRII). FcεRI exists as either a tetrameric form composed of an alpha, beta and two gamma chains or a trimeric form composed of one alpha and two gamma chains (22). The tetrameric receptor is expressed by mast cells, basophils, eosinophils and ASMC, and the trimeric receptor is expressed by antigen presenting cells including monocytes, macrophages and dendritic cells (22). Each subunit of FcεRI has its own function: the alpha subunit is an immunoglobulin like chain and contains two domains that bind to the Fc portion of an IgE molecule; the beta subunit amplifies the signal initiated at the gamma subunit; and the gamma subunit is responsible for signal transduction (22). Furthermore, the alpha and gamma subunits have immunoreceptor tyrosine-based activation motifs (ITAM) for signal transduction (22).

1.3.2 FcεRI upregulation by IgE

Binding of FcεRI to the Fc portion of IgE is an early step in receptor activation. The crystal structure of IgE-Fc demonstrated that the alpha subunit binds the Fc portion at two asymmetrical binding sites and that FcεRI binds to IgE in a 1:1 ratio (23). IgE has been shown to upregulate the expression of FcεRI (24) and that it can occur without changing the baseline transcriptional level of the receptor (24); however, it needs an ongoing synthesis of the receptor proteins at a baseline level (24). Furthermore, IgE upregulates the trimeric FcεRI as efficiently
as the tetrameric FcεRI, suggesting that the presence of the beta subunit is not necessary for the IgE mediated upregulation of the receptor (24).

1.3.3 Cross-linking and signaling of FcεRI

Upon re-exposure to an allergen, IgE expressed on the surface of mast cells and basophils would recognize the allergen and binds to it. An allergen binds to more than one membrane bound IgE on the same cell and triggers a series of signaling cascade starting with tyrosine phosphorylation of the gamma subunit of FcεRI by tyrosine-protein kinase LYN (25). Phosphorylation of the gamma chain enhances the phosphorylation of tyrosine-protein kinase SYK and subsequent downstream events lead to calcium flux into the cytosol. It has been shown that the tetrameric form of FcεRI exhibited a greater signaling capability as compared to the trimeric form, and such difference was attributed to the presence of the beta subunit. Subsequently, initial calcium flux is greater in the tetrameric model compared to the trimeric model (25). Given that the two forms of FcεRI are expressed by different cells; tetrameric FcεRI is expressed by mast cells and basophils, and trimeric FcεRI is expressed by antigen presenting cells, the different signaling capabilities may be required by their functions in the body. Tetrameric receptor mediates degranulation of mast cells and basophils while trimeric receptor mediates internalization of allergens into APCs.
1.3.4 Expression and function of FceRI in other cell types

In addition to mast cells and basophils, FceRI also expresses in other cell types including eosinophils, monocytes and ASMC. In eosinophils, crosslinking the receptor using anti-FceRIα leads to the release of eosinophil peroxidase, suggesting a role of receptor activation in defense against certain parasitic infections (26). Its role in defense is further supported by the finding that incubation of eosinophils with Schistosoma mansoni in the presence of antigen specific IgE induced cell lysis (26).

In monocytes, FcεRI is likely to play a role in mounting an inflammatory response since crosslinking anti-IgE has been shown to induce the release of tumor necrosis factor- alpha (TNF-α), IL-6 and IL-10 from monocytes, and decrease phagocytic function and ability to clear apoptotic debris; however, bacterial killing capacity has not been changed (27).

With regards to structural cells, ASMC have been shown to express FcεRI. Incubation of ASMC with IgE led to a dose dependent increase in the release of the cytokines IL-4, IL-5, IL-13 and eotaxin (28). Furthermore, treatment with IgE on ASMC has been shown to induce cell proliferation and total ECM proteins deposition (15). Pretreatment with omalizumab, a monoclonal antibody against IgE, significantly inhibited IgE mediated ASMC proliferation and deposition of ECM proteins, suggesting a probable mechanism underlying the therapeutic effect of omalizumab (15). The role of FcεRI in ASMC in asthma is further demonstrated when human asthmatic and non-asthmatic ASMC were incubated with atopic serum and proliferation of ASMC was observed (29). The induced proliferation was significantly higher in asthmatic than non-asthmatic cells and pre-incubation with omalizumab reduced the effect of atopic serum on
asthmatic type I collagen deposition by ASMC. Molecularly, ERK1/2 MAPK plays a crucial role in IgE induced ASMC proliferation and ECM proteins deposition (29, 15).

1.3.5 The low affinity receptor for IgE (FceRII)

The low affinity receptor for IgE (FceRII) is composed of three C-lectin heads. One C-lectin head binds to one heavy chain at the interface between Ce3 and Ce4 domains, which lies at the opposite site of FceRI binding site. Binding of FceRII to IgE-Fc induces a conformational change in the Fc portion that prevents binding of FceRI to its binding sites. In addition, two heads of FceRII bind to one IgE-Fc in a ratio of 2:1, while one FceRI binds to one IgE-Fc in a ratio of 1:1 (30). FceRII is expressed by immune cells such as B cells and by structural cells such as ASMC and airway epithelial cells (28). On B cells, FceRII plays a role in regulating IgE production (31).

Expression of FceRII by airway epithelial cells has been demonstrated on primary tracheal bronchial epithelial cells and epithelial cell lines (calu-3) at both the mRNA and protein levels (32). The receptors are expressed on the brush border of the apical membrane of calu-3 cells and co-localized with IgE. Polarized calu-3 cells on a transwell insert are capable of transporting IgE or IgE-antigen complex through them. Incubation of human IgE with soluble FceRII inhibited the transport of IgE across calu-3 cells significantly (32).
1.4 Anti-IgE therapy (omalizumab)

Anti-IgE therapy (omalizumab) is a humanized antibody that is used in the treatment of asthma. Total IgE levels in mucosal homogenates are higher in atopic and non-atopic asthmatic individuals as compared to healthy subjects (33) and omalizumab has been shown to reduce the free serum IgE level and expression of FcεRI by basophils (34). More recently, the effect of omalizumab on FcεRI expression by dendritic cells has been shown. Omalizumab treated and placebo control groups were monitored for their free serum IgE levels and FcεRI expression on dendritic cells (DC) surface. In the omalizumab treatment group there was a 95% decrease in the mean free IgE level on day 3 and a decrease in FcεRI expression in two dendritic cells subsets (pDC1 and pDC2) on day 7 (35).

Omalizumab binds to IgE at the Fc portion of the receptor hence preventing IgE from binding to its receptors. The epitope of omalizumab on IgE-Fc is composed of the three amino acids (i.e. histidine, leucine and proline) located within the Cε3 domain and it overlaps with the two FcεRI binding sites (36).
CHAPTER 2: HYPOTHESIS AND AIMS

HYPOTHESIS

IgE plays a role in airway remodeling in asthma through stimulation of the high affinity receptor for IgE on bronchial epithelial cells

OBJECTIVES:

1- To determine if FcεRI is expressed on human bronchial epithelial cells
2- To examine the effect on FcεRI expression after IgE treatment in bronchial epithelial cells
3- To examine the downstream effects of FcεRI stimulation in asthmatic human bronchial epithelial cells

AIMS:

1- To measure in situ FcεRI expression on human bronchial epithelial cells
2- To measure in vitro FcεRI expression on asthmatic bronchial epithelial cells after IgE treatment
3- To measure the release of TSLP and TGF-β1 from asthmatic human bronchial epithelial cells after FcεRI activation
CHAPTER 3: EXPERIMENTAL PROCEDURES

3.1 Cell culture and treatment schemes

Asthmatic human airway epithelial cells were grown in bronchial epithelial cell medium (BEGM) (Lonza, Walkersville Inc.) in T75 flasks until cells were ≥ 60 % confluent, then seeded either in T25 flasks or in 6 well plates for flow cytometry, or in 12 well plates for ELISA and real time PCR. For flow cytometry, cells (passage 2-4) were treated with azide-free human IgE (DIA HE1-1, BioPorto) at 1 and 5μg/ml, mouse IgG (Sigma, Saint-Louis, MO) at 1 and 5μg/ml, vehicle (PBS) at 5μl/ml in BEGM, or BEGM alone for 24 hours, before being treated by accutase enzyme (catalogue # CR005, Millipore, Temecula, CA 92590) at 0.1ml/cm² for cell detachment. To serve as a positive control for the upregulation of FcεRI expression by IgE, U937 cells were placed in two T25 flasks containing growth medium at a density of 250,000 cells/5ml medium and treated with IgE at 1 or 5μg/ml for 24 hours. To assess whether accutase treatment affects FcεRI expression on cells, THP-1 cells were washed with PBS, incubated with 2.5ml accutase or 2.5ml growth medium/ 500,000 cells for 10 minutes in the incubator at 37°C.

For ELISA, cells in 12 well plates were treated for 24 hours with IgE at 5μg/ml or left untreated. IgE treated and untreated cells were treated with anti-FcεRIα (BioLegend, San Diego, CA) at 0.5μg/ml, IgG isotype control (BioLegend, San Diego, CA) at the same concentration, vehicle (PBS) at 0.5μl/ml, or BEGM alone for 24 hours.
3.2 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) Test

Vehicle (0.01 M phosphate buffer + 0.09% sodium azide + 0.15 M NaCl) was filtered with a 0.2µm syringe filter. MTT solution (Sigma, Saint-Louis, MO) was diluted to 5mg/ml in PBS and filtered using a 0.2µm syringe filter. Commercially purchased asthmatic human airway epithelial cells (catalog # 00194911, Lonza, Walkersville Inc.) were seeded in 96 well plates, using a repeater at 16000 cells in 200µl BEGM/well and cultured for two days. Seeded cells were treated with either azide containing IgE (BioPorto) or vehicle at various concentrations (0.005, 0.05, 0.5 and 5µg/ml). Cells cultured in BEGM alone were used as the controls. Cells were incubated for 48 or 72 hours. Four hours prior to the end of the incubation period, MTT solution was added to each well at 1:10 dilution (24µl/well). After incubation, medium was removed and 100µl of dimethyl sulfoxide (Sigma, USA) was added to each well. Cells were homogenized for 5 minutes and the optical density of each well was measured using a microplate reader set to 550nm. The optical density readout was subtracted from that of the treatment condition and normalized to the cells cultured in BEGM alone, which was considered as 100% viable.

3.3 Immunohistochemistry (IHC)

Bronchial biopsy sections were stained using the Ventana Discovery autostainer (Protocol 1, attached). Briefly, the process began with a deparaffinization step using a Triton-based buffer, followed by an antigen retrieval step using an ethylenediaminetetraacetic acid (or commonly known as EDTA) like buffer, then a blocking step using a serum-free blocking buffer (Dako). Blocking of endogenous peroxidase activity was done using an inhibitor. Mouse anti-
FcεRIα antibody at a dilution of 1:200 (clone 9E1, Abcam, Toronto, ON) was manually added and the Ventana autostainer diluted the applied antibody 5 times to a final dilution of 1:1000. Further staining procedure included application of Hapten (HQ) conjugated antibody, application of horseradish peroxidase conjugated anti-HQ antibody and color development using 3,3’-Diaminobenzidine substrate. Counterstaining was done with hematoxylin and a bluing reagent and slides were washed in water with drops of dish washing detergent and mounted. A biopsy tissue of the inferior turbinate of an allergic rhinitis subject was used as a positive control. Negative controls were prepared by using isotype matched mouse IgG (Cedarlane, Burlington, ON) to the primary antibody or by the omission of the primary antibody from the staining process.

3.4 Flow Cytometry (FACS)

Cells were washed with phosphate buffered saline (PBS) and detached from the flask using accutase enzyme at 0.1ml/cm² concentration for 10 minutes at 37 °C in an incubator. Following detachment, cells were transferred to 15ml tubes for centrifugation and supernatants were removed. Cells were then transferred into FACS tubes, washed with PBS and stained with viability dye (efluor660) (eBioSciences, Affymetrix, San Diego, CA 92121, USA) at 0.1µl/100 µl of PBS. Cells were incubated in the dark for 15 minutes at room temperature. Cells were washed with PBS-bovine serum albumin (BSA) (0.2%) and incubated in the dark with rabbit anti-FcεRIα antibody (catalog # 06-725, EMD Millipore, Etobicoke, ON) diluted in PBS-BSA (0.2%) (0.25µl in 100µl PBS-BSA) for 30 minutes at 4°C. Cells were washed again with PBS-BSA (0.2%) and incubated in the dark with a goat anti-rabbit Alexa Fluor® 488 conjugated
antibody (Molecular Probes, Life Technology) (0.33µl in 100µl PBS-BSA (0.2%)) at 4°C for 30 minutes. Cells were washed again with PBS-BSA (0.2%) and passed through a filter for disaggregation. Stained cells were read using the flow cytometer Accuri C6 machine and results were analyzed using the flowJo software. Human monocyte cell lines (U937 and THP-1) were used as positive controls. Cells stained without the primary antibody was used as the negative control. Viability dye compensation control was produced by killing 250,000 monocytes by incubating them in a dry bath at 65°C for 15 minutes. Killed cells were mixed with the same number of live cells. Cells were washed with PBS and stained with viability dye only. No primary or secondary antibodies were added to the viability dye compensation control. Alexafluor488 compensation control was prepared using positive and negative beads as the following: one drop of positive beads (coated with anti-mouse Ig) was added to one drop of negative beads in a FACS tube then incubated with Alexa Fluor® 488 conjugated mouse IgG (0.33µl in 100µl PBS-BSA (0.2%)) antibody in the dark for 30 minutes at 4°C. Cells were then washed with PBS-BSA (0.2%).

3.5 Real time PCR

Primers specific to the alpha subunit of FcεRI (FcεRIα) were purchased from Life Technology and human GAPDH primers were kindly donated by Dr. Baglole. Primers sequences are listed in table 2. RNA extraction was done using NucleoSpin® RNA extraction kit (Clontech, Takara Bio Group, Germany) according to the manufacturer. Immediately after extraction, RNA purity and concentration were determined by spectrophotometry using the Epoch Spectrophotometer System. Aliquots were prepared to measure RNA purity, quality and
concentration using Experion™ RNA StdSens kit (Bio-Rad, Mississauga, ON) according to the supplier’s manual. 400 ng of RNA from each sample were then reverse transcribed to cDNA using iscript reverse transcription kit (Bio-Rad, Mississauga, ON) and nexus machine according to the supplier’s manual. cDNA was stored at -20°C until the day of the experiment.

Thermal gradient test was done to determine the optimal annealing temperature of the primers. Human peripheral blood mononuclear cells (PBMC) were used as the positive control; reaction mix without cDNA was used as the negative control; and human GAPDH primers were used as the house keeping gene. cDNA samples of all donors were diluted (1:2) in polymerase chain reaction (PCR) grade H₂O and 10µl reaction mix was used in the real-time PCR experiment (3.5µl H₂O, 0.25µl forward primer, 0.25µl reverse primer, 5µl power SybrGreen and 1 µl cDNA). The annealing step of the PCR was set at 6 temperatures (52, 54, 56, 58, 60 and 62°C) for each primer pair (FcεRIα and hGAPDH) and the optimal annealing temperature was determined to be 60°C for both genes. The experiment was done using the StepOnePlus Real-Time PCR System. Standard curve was generated to determine primers efficiency with 8 points at 1:2 dilution series starting from no dilution in triplicates. Experimental procedure and controls were done as described above. The primers were tested on the positive control only since no expression of FcεRIα was detected on epithelial cells as determined by the thermal gradient. Mean quantitation cycle (Cq) value of each point was plotted against the log10 of its dilution. Slopes of the plots were used to determine reaction efficiency of the primers using QPCR Standard Curve Slope to Efficiency Calculator. Reaction efficiency was 90.66% for the FcεRIα primers and 96.14% for the hGAPDH primers. The samples were run on the real time PCR machine using the same experimental procedure and controls as described above in the thermal gradient experiment.
**Table 2.** Primer sequences used in real time PCR

<table>
<thead>
<tr>
<th></th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>FceRIαF</em></td>
<td>5’-CTCCATTACAAATGCCACAGTTG-3’</td>
</tr>
<tr>
<td><em>FceRIαR</em></td>
<td>CACGCGGAGCTTTTATTACAGTA</td>
</tr>
<tr>
<td><em>hGAPDH F</em></td>
<td>GTCTCCTCTGACTTCAACAGC</td>
</tr>
<tr>
<td><em>hGAPDH R</em></td>
<td>ACCACCCTGGTGGCTGTAGCCA</td>
</tr>
</tbody>
</table>

3.6 **Enzyme-linked immunosorbent assay (ELISA)**

Cell-conditioned media were collected, centrifuged at 2000rpm for 10 minutes at 4°C and cell-free supernatants were stored in aliquots at -80°C. TGF-β1 and TSLP ELISA kits were purchased from R & D systems (Minneapolis, MN 55413, USA). ELISA was done according to the manufacturer’s protocol.

3.7 **Statistics**

One way ANOVA and t-tests were used to assess the statistical significance among experimental groups using the software Graphpad Prism 5.
CHAPTER 4: CONTRIBUTION OF COAUTHORS

**Omer Qibi**: M.Sc student, McGill University. Conducted the original work of this project. Designed, performed and carried out analysis of experiments, and wrote this thesis.

**Severine Audessue**: Research assistant in Dr. Hamid’s lab, McGill University. Helped in immunohistochemical stains for detection of FceRI in bronchial biopsy sections and conducting ELISA experiments used to detect TSLP in cell supernatants.

**Dr. Abdelilah Soussi Gounni**: Professor, Department of Immunology, College of Medicine, University of Manitoba. Gave advice regarding the treatment of cells with IgE and the choice of control.

**Alice Panariti**: Postdoctoral fellow, Dr. Hamid Lab, McGill University. Helped in designing the MTT test and data analysis.

**Dr. Carolyn Baglole**: Associate professor, Department of Medicine, McGill University. Helped in supervising this project and designing experiments to show expression of FceRI *in vivo*.

**Dr. David Eidelman**: Dean, Faculty of Medicine, McGill University. Provided guidance and feedback.

**Dr. Bruce Mazer**: Professor, Department of Pediatrics, McGill University. Cosupervised this project with Dr. Hamid.

**Dr. Qutayba Hamid**: Professor in the Department of Medicine and director of Meakins-Christie Laboratories, McGill University, thesis supervisor.
CHAPTER 4: RESULTS

4.1 Samples characteristics

Endobronchial biopsy sections from 16 subjects (4 non-asthmatic, 4 mild asthmatic, 4 moderate asthmatic and 4 severe asthmatic) were obtained from the Tissue Bank of the Respiratory Health Network of the FRQ-S, demographic data are shown in table 3. For the in vitro experiments, human bronchial epithelial cells from two asthmatic donors were used, demographic data in table 4. Cells of the first donor were purchased commercially (00194911, Lonza, Walkersville Inc.). Cells of the second donor were isolated in our laboratory. Consent for research was confirmed by the International Institute for the Advancement of Medicine (IIAM) coordinator.

4.2 FcεRI is not expressed by human bronchial epithelial cells

At baseline, in situ expression of FcεRI by immunohistochemistry was detected in a small number of cells in the epithelium of the biopsy tissues (Figure 2A). When quantified, there was no significant difference among the groups, however; the number of FcεRI+ cells in the epithelium per unit length of the basement membrane was higher in mild asthmatic subjects as compared to other groups, but did not reach statistical significance (Figure 2B). Staining of two serial sections of a bronchial biopsy from an asthmatic subject, one for FcεRIα and the other for common marker for leukocytes, CD45, detected the presence of inflammatory cells in the epithelium which are likely the cells expressing FcεRIα (Figure 2C).

At baseline, cell viability and expression of FcεRI in vitro measured by flow cytometry was tested in U937 cells and primary human bronchial epithelial cells from two donors. Cell viability was 99.2% in U937 cells and 97% in epithelial cells (Figure 3 A and B). In the
positive control cell type, U937 cells expressed FcεRI (Figure 3 C and E), while in epithelial cells no significant difference was found in the mean fluorescence intensity of stained cells compared to unstained cells (negative control) (Figure 3 D and F (p=0.2)). An effect of accutase on FcεRI expression was demonstrated by a 11% reduction of the percentage of U937 cells expressing FcεRI in cells treated with accutase as compared to the non-treated as measured by flow cytometry (Figure 4A) and a greater reduction was observed when measured using mean fluorescence intensity (MFI) (Figure 4 B and C). Furthermore, no mRNA expression of FcεRIα was detected in cells from the same donors (primary human bronchial epithelial cells of asthmatic subjects) (Figure 5).

4.3 IgE did not upregulate FcεRI expression in human bronchial epithelial cells of asthmatic subjects or U937 cells

Epithelial cells were treated with IgE at 1 and 5µg/ml for 24 hours based on a published report that a concentration between 1 and 5 (1.5µg/ml) was used successfully to upregulate FcεRI in rat basophilic leukemia cells and the highest amount of the receptor was found at a time point between 24 and 28 hours (37). Mouse IgG was used as a negative control for IgE treatment. Epithelial cells treated with azide containing IgE had greatly reduced viability and MTT assays demonstrated that cell viability was decreased after treatment with either azide containing IgE or azide – containing vehicle in a time and dose dependent manner, p< 0.05 (Figure 6); hence, subsequent experiments were done using azide-free human IgE. U937 cells were used as positive control for FcεRIα expression with 99% viability (Figure 7A) and expressed FcεRIα (Figure 7C and E). Epithelial cells treated with azide-free human IgE, mouse IgG or vehicle remained viable (Figure 7B) did not express significantly higher FcεRIα as
compared to the control cells (p=0.2) (Figure 7D, F). At baseline, U937 cells expressed FcεRIα as shown above; however, treatment with or without azide free IgE at 1 and 5µg/ml for 24 hours did not change the expression level of FcεRIα (Figure 8).

4.4 Crosslinking FcεRI did not increase the release of TGF-β1 or TSLP from human bronchial epithelial cells of asthmatic subjects

No significant differences in the protein levels of TGF-β1 or TSLP under various stimulation schemes as compared to the unstimulated cells (Figure 9 A and B).
CHAPTER 5: DISCUSSION

5.1 Absence of FcεRI expression in bronchial epithelial cells in asthma

Asthma pathogenesis is often characterized by airway inflammation and remodeling. IgE plays a role in the pathogenesis of allergic diseases such as asthma, yet its role in airway remodeling is not very clear. FcεRI is expressed by immune cells such as mast cells, basophils and monocytes of atopic subjects; however, its expression in ASMC, a structural cell type, has recently been reported (28). In this study we investigated whether bronchial epithelial cells also express FcεRI and our data indicated that they do not. Our findings are in contrast to a previously published report demonstrating that human bronchial epithelial cells expressed FcεRI using RT-PCR and immunohistochemistry (38). Several factors may explain the inconsistent conclusions. First, cell viability. In Campbell AM et al., the epithelial cells were obtained from bronchial brushing with less than 50% viability for both the allergic asthmatic and control cells (16% and 43%, respectively) (38). In contrast, the mean cell viability in this study was closed to 100% for various experiments (Figure 7B). Second, cell types heterogeneity. Number of inflammatory cells such as eosinophils, mast cells and macrophages is increased in the bronchial epithelium of asthmatic compared to control subjects (43). FcεRI expresses on eosinophils, mast cells and some antigen presenting cells such as monocytes and dendritic cells (26, 27, 35). In Campbell AM et al., the epithelial cells contained other cell types (mainly macrophages and a small amount of metachromatic cells) which expressed FcεRI and may have produced false positive results. Third, validations. Campbell AM et al., used immunohistochemistry to detect FcεRI expression in epithelial cells and they are known to have non-specific staining which may have produced false positive results. Furthermore, the PCR data did not correlate strongly with the immunohistochemistry data. In Campbell AM et al., where the band of the alpha subunit of
FcεRI in the PCR data was much brighter than that of the housekeeping gene (GAPDH) in most of the control subjects, yet the IHC data showed FcεRI expression in only half of the asthmatic subjects. In addition, there was no clear counter staining shown in the tissue sections. In our study, we investigated the expression of FcεRI on bronchial epithelial cells from both the control and asthmatic subjects and validated the negative results using various techniques such as immunohistochemistry, flow cytometry and PCR. In conclusion, we do not have evidence that human bronchial epithelial cells express FcεRI. Our data suggest that anti-IgE therapy likely has no effect on bronchial epithelial cells mediated through FcεRI.

5.2 IgE did not upregulate FcεRI expression in human bronchial epithelial cells of asthmatic subjects.

Treatment with IgE is expected to upregulate the level of FcεRI on cell surface provided that there is ongoing synthesis of the receptor subunits inside the cell at a baseline level (24). Absence of FcεRI on bronchial epithelial cells even after treatment with IgE further confirmed the absence of FcεRI expression at a baseline level. Initially cells were treated with azide containing IgE which resulted in a great reduction of cell viability. MTT test revealed that azide was killing the airway epithelial cells in the first set of experiments; therefore, azide free IgE was used in the next set of experiments to stimulate epithelial cells. Accutase was used to detach adherent cells while preserving the integrity of membrane structures; yet when we incubated monocytes with accutase before staining them for flow cytometry, accutase reduced the number of cells expressing the receptor by about 11% and reduced the amount of the receptor on the cells by greater percentage. However, there was still a reasonable amount of the receptor expressed
on the cell surface. IgE was used to upregulate FcεRI; to confirm that IgE was not interfering with staining by blocking the binding of the primary antibody with FcεRI, monocytes were incubated with IgE then stained. FcεRI was still expressed even after treatment with IgE; however, unexpectedly IgE did not upregulate FcεRI expression on monocytes. This could simply be due to the low number of experiments, one experiment, n=1.

5.3 Implications of an absence of FcεRI expression in asthma

Anti-IgE such as omalizumab, an anti-IgE recombinant humanized monoclonal antibody, works to control asthma symptoms by targeting IgE which in turn reduces IgE driven inflammation. The epitope of omalizumab lies in the Fc portion of human IgE and overlaps with the FcεRI binding site, thus preventing IgE from binding to FcεRI (36). In addition to immune cells, FcεRI has been reported to express on ASMC and that FcεRI stimulation by IgE mediates the remodeling process such as cell proliferation and release of ECM proteins from ASMC (15). Omalizumab inhibits this effect of IgE significantly. Given that FcεRI has been reported to express in bronchial epithelial cells (38), it is plausible that IgE may mediate the remodeling process via epithelial cells. However, we could not confirm the expression of FcεRI in human bronchial epithelial cells, as previously reported in the literature. Our data suggest that anti-IgE therapy likely has no effect on bronchial epithelial cells mediated through FcεRI.

An absence of FcεRI on human airway epithelial cells, as our results suggest, does not imply that there is no interaction between IgE and airway epithelial cells. In fact, expression of the low affinity receptor of IgE (FcεRII) has been shown on airway epithelial cells in vitro using airway epithelial cell lines and primary cells and that it mediates transcytosis of IgE or immune complex (IgE plus antigen) (32). Hence, FcεRII could play a role in asthma by increasing the availability of allergens to the infiltrating immune cells in the submucosa. At present, whether
FcεRII in airway epithelial cells plays a role in airway remodeling is not known and this receptor has not been well investigated.

5.4 Future directions

Anti-IgE therapy has been shown to interfere with the airway remodeling process (39,40); however, its mechanisms have not been extensively investigated. As aforementioned, FcεRII is a plausible target to study. Downstream effects of FcεRII stimulation by IgE, such as cytokines release, rate of proliferation, apoptosis and ECM proteins deposition in bronchial epithelial cells can be investigated and the effects of omalizumab can also be investigated in these experiments.
TABLES AND FIGURES

Table 3A. Demographic data of study population- Endobronchial biopsies

<table>
<thead>
<tr>
<th>Subject ID</th>
<th>Asthma status (Severity)</th>
<th>Allergy status</th>
<th>age</th>
<th>Blood eosinophils $10^9$/Liter</th>
<th>FEV1/FVC</th>
<th>Medication</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABB00075</td>
<td>Non</td>
<td>Non</td>
<td>20</td>
<td>0.15</td>
<td>0.78</td>
<td>-</td>
</tr>
<tr>
<td>ABB01607</td>
<td>Non</td>
<td>Non</td>
<td>19</td>
<td>-</td>
<td>0.77</td>
<td>-</td>
</tr>
<tr>
<td>ABB01373</td>
<td>Non</td>
<td>Non</td>
<td>27</td>
<td>0.14</td>
<td>0.81</td>
<td>-</td>
</tr>
<tr>
<td>ABB01363</td>
<td>Non</td>
<td>Non</td>
<td>29</td>
<td>0.07</td>
<td>0.84</td>
<td>-</td>
</tr>
<tr>
<td>ABB00035</td>
<td>Mild</td>
<td>Allergic</td>
<td>22</td>
<td>0.51</td>
<td>0.71</td>
<td>Ventolin</td>
</tr>
<tr>
<td>ABB01621</td>
<td>Mild</td>
<td>Allergic</td>
<td>29</td>
<td>1.46</td>
<td>0.78</td>
<td>Ventolin</td>
</tr>
<tr>
<td>ABB01668</td>
<td>Mild</td>
<td>Allergic</td>
<td>30</td>
<td>0.07</td>
<td>0.92</td>
<td>Bicanyl, Depo-provera</td>
</tr>
<tr>
<td>ABB04235</td>
<td>Mild</td>
<td>Allergic</td>
<td>25</td>
<td>0.09</td>
<td>0.79</td>
<td>Ventolin</td>
</tr>
<tr>
<td>ABB01644</td>
<td>Moderate</td>
<td>Allergic</td>
<td>31</td>
<td>-</td>
<td>0.76</td>
<td>Pulmicort, Bicanyl</td>
</tr>
<tr>
<td>ABB03066</td>
<td>Moderate</td>
<td>Allergic</td>
<td>26</td>
<td>0.16</td>
<td>0.85</td>
<td>Ventolin, Flovent HFA</td>
</tr>
<tr>
<td>ABB02400</td>
<td>Moderate</td>
<td>Allergic</td>
<td>30</td>
<td>0.39</td>
<td>0.71</td>
<td>Ventolin, Symbicort</td>
</tr>
<tr>
<td>ABB00109</td>
<td>Moderate</td>
<td>Allergic</td>
<td>37</td>
<td>-</td>
<td>0.85</td>
<td>Ventolin, Advair HFA, Prevacid</td>
</tr>
<tr>
<td>ABB03477</td>
<td>Severe</td>
<td>Allergic</td>
<td>35</td>
<td>0.19</td>
<td>0.53</td>
<td>Ventolin, Alvesco, Advair Diskus 500</td>
</tr>
<tr>
<td>ABB01268</td>
<td>Severe</td>
<td>Allergic</td>
<td>35</td>
<td>0.2</td>
<td>0.86</td>
<td>Ventolin, Advair Diskus 250, Singulair, Methotrexate</td>
</tr>
<tr>
<td>ABB00148</td>
<td>Severe</td>
<td>Allergic</td>
<td>29</td>
<td>-</td>
<td>0.63</td>
<td>Ventolin, Advair Diskus 500</td>
</tr>
<tr>
<td>ABB03338</td>
<td>Severe</td>
<td>Allergic</td>
<td>39</td>
<td>-</td>
<td>0.66</td>
<td>Alvesco, Ventolin, Singulair, Prednisone, Advair Diskus 500</td>
</tr>
</tbody>
</table>

Note: All subjects in table 2 are non-smokers
Table 3B. Demographic data of study population- Endobronchial biopsies

<table>
<thead>
<tr>
<th>Subject ID</th>
<th>PREDICTED FVC</th>
<th>OPTIMAL FVC for last 2 years (L,%),</th>
<th>PREDICTED FEV1</th>
<th>OPTIMAL FEV1 for last 2 years (L,%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABB00075</td>
<td>3.91</td>
<td>3.78 97</td>
<td>2.93</td>
<td>2.93 100</td>
</tr>
<tr>
<td>ABB01607</td>
<td>3.95</td>
<td>3.64 92</td>
<td>3.49</td>
<td>2.82 81</td>
</tr>
<tr>
<td>ABB01373</td>
<td>4.07</td>
<td>4.39 108</td>
<td>3.48</td>
<td>3.56 102</td>
</tr>
<tr>
<td>ABB01363</td>
<td>5.72</td>
<td>6.39 112</td>
<td>4.70</td>
<td>5.39 115</td>
</tr>
<tr>
<td>ABB00035</td>
<td>6.00</td>
<td>5.22 93</td>
<td>4.64</td>
<td>3.73 80</td>
</tr>
<tr>
<td>ABB01621</td>
<td>5.56</td>
<td>5.40 97</td>
<td>4.59</td>
<td>4.34 95</td>
</tr>
<tr>
<td>ABB01668</td>
<td>3.30</td>
<td>3.67 96</td>
<td>3.30</td>
<td>3.24 98</td>
</tr>
<tr>
<td>ABB04235</td>
<td>6.00</td>
<td>5.08 88</td>
<td>4.73</td>
<td>4.03 84</td>
</tr>
<tr>
<td>ABB01644</td>
<td>3.60</td>
<td>3.49 97</td>
<td>3.11</td>
<td>2.73 87</td>
</tr>
<tr>
<td>ABB03066</td>
<td>3.73</td>
<td>4.13 111</td>
<td>3.26</td>
<td>3.72 114</td>
</tr>
<tr>
<td>ABB02400</td>
<td>5.22</td>
<td>4.48 86</td>
<td>4.20</td>
<td>3.18 74</td>
</tr>
<tr>
<td>ABB00109</td>
<td>5.24</td>
<td>5.09 97</td>
<td>4.28</td>
<td>4.31 101</td>
</tr>
<tr>
<td>ABB03477</td>
<td>4.36</td>
<td>3.44 79</td>
<td>3.69</td>
<td>1.85 50</td>
</tr>
<tr>
<td>ABB01268</td>
<td>5.47</td>
<td>4.44 81</td>
<td>4.46</td>
<td>3.69 83</td>
</tr>
<tr>
<td>ABB00148</td>
<td>5.35</td>
<td>4.59 90</td>
<td>4.43</td>
<td>2.84 66</td>
</tr>
<tr>
<td>ABB03338</td>
<td>4.71</td>
<td>4.51 96</td>
<td>3.90</td>
<td>3.23 83</td>
</tr>
</tbody>
</table>

Table 4. Demographic data of study samples - Primary human airway epithelial cells

<table>
<thead>
<tr>
<th>Subject ID</th>
<th>age</th>
<th>Respiratory disease</th>
<th>Smoking history</th>
<th>sex</th>
<th>Medication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purchased cells</td>
<td>45</td>
<td>asthma</td>
<td>smoker</td>
<td>female</td>
<td>Albuterol</td>
</tr>
<tr>
<td>Locally isolated cells</td>
<td>40</td>
<td>asthma</td>
<td>ex-smoker</td>
<td>male</td>
<td>Dulara, Vasopressin, Levophed, Probiotic, Solumedrol,T4, Insulin, Heparin, Zosyn, Lasix, Mannitol, Rocuronium, Albumin</td>
</tr>
</tbody>
</table>
A- IHC detection of FcεRIα

Turbinate of allergic rhinitis subject

 Negative control

 FcεRIα
B- FcεRIα+ cells in the epithelium- Quantification

![Graph showing FcεRIα+ cells in the epithelium](image)

n=4 per group, p=0.06

C- IHC detection of CD45+ cells

![Image showing IHC detection of CD45+ cells](image)

Figure 2. **FcεRI is not expressed by human bronchial epithelial cells.** A, IHC detection of FcεRIα: (a&b) expression of FcεRIα by turbinate of allergic rhinitis subject. No expression of FcεRIα was detected in the epithelium of bronchial biopsies of control, mild asthmatic, moderate asthmatic, and severe asthmatic subjects (d,f,h,j), except for very few cells. Staining for FcεRIα was absent in the negative control (c,e,g,i) in which tissues were incubated with isotype matched control antibody. B, Quantification of FcεRIα+ cells in the bronchial epithelium: There was no significant difference between studied groups (control, mild asthmatic, moderate asthmatic, and severe asthmatic subjects). Results represent mean ± SEM of 4 subjects per group (p=0.06). C, IHC detection of CD45+ cells in bronchial epithelium of one asthmatic subject. Few cells were positive for FcεRIα in the bronchial epithelium (panel a) and few CD45+ cells were present in the bronchial epithelium (panel b).
A. Viability of U937- Quantification

B. Viability of epithelial cells- Quantification

C. FcεRIα (MFI)- U937 cells- Histogram

D. FcεRIα (MFI)- epithelial cells- Histogram

E. FcεRIα (MFI)- U937 cells- Quantification

F. FcεRIα (MFI)- epithelial cells- Quantification

Figure 3. FcεRIα is not expressed by primary human airway epithelial cells of asthmatic subjects in vitro using flow cytometry. A and B, Quantification of cell viability: Mean viability was 99.2% for U937 cells and 97% for epithelial cells. C and D, Histograms showing MFI of FcεRIα of the unstained negative control (orange) and cells stained with anti-FcεRIα (blue) of both U937 cells (C) and bronchial epithelial cells (D). E and F, Quantitation of FcεRIα (MFI) of both U937 cells (E) and bronchial epithelial cells (F): No significant difference was found between unstained and stained bronchial epithelial cells (p=0.2). Results are expressed as the mean ± SEM of 4 independent experiments.
A. FACS detection of FcεRIα in U937 cells- Dot plot graphs

B. Histogram graph

C. FcεRIα (MFI)- Quantification

Figure 4. **Effect of incubation with accutase on expression of FcεRIα by U937 cells**: A, Dot plot graphs showing the percentage of cells expressing FcεRIα in U937 cells. No staining for FcεRIα was detected in the negative control (panel i), in which the primary antibody (anti-FcεRIα) was omitted from staining. 98.6% of cells were positive for FcεRIα in cells incubated in growth medium only (panel ii). 87.4% of cells were positive for FcεRIα in cells incubated with accutase for 10 minutes before staining for flow cytometry. B, Histogram showing the mean fluorescence intensity of FcεRIα for the negative control (blue curve), cells incubated in growth medium (orange curve) or cells incubated with accutase (green curve). C. Quantitation of FcεRIα (MFI) with or without incubation with accutase. Results are obtained from one experiment.
Figure 5. **FceRIα is not expressed by primary human airway epithelial cells of asthmatic subjects in vitro using real time PCR.** The figure represents a quantification of delta Cq (Δ Cq) values of FceRIα mRNA expression in the positive control (PBMC), the negative control (reaction mix without cDNA) and in bronchial epithelial cells. Results are expressed as the mean ± SEM of 3 independent experiments.
Figure 6. Human airway epithelial cells were killed by azide containing IgE. A and B, quantification of viability of epithelial cells after incubation with various doses of azide containing IgE (0.005-5µg/ml) for 48 hours (A) or azide containing vehicle at the same doses (0.005-5 µl/ml) for 48 hours (B). Results in A and B are expressed as the mean ± SD of 4 replicates from 1 experiment. C and D, quantification of viability of epithelial cells after incubation with various doses of azide containing IgE (0.005-5µg/ml) for 72 hours (C) or azide containing vehicle at the same doses (0.005-5 µl/ml) for 72 hours (D). Results in C and D are expressed as the mean ± SD of 4 replicates from 1 experiment. Viability of epithelial cells was decreased as the dose of IgE or the vehicle increased in A, B, C and D, p<0.05.
A. Viability of U937 - Quantification

B. Viability of epithelial cells - Quantification

C. FcεRIα (MFI) - U937 cells - Histogram

D. FcεRIα (MFI) - epithelial cells - Histogram

E. FcεRIα (MFI) - U937 cells - Quantification

F. FcεRIα (MFI) - epithelial cells - Quantification

n=2, p=0.015
Figure 7. **IgE did not upregulate FceRI in human bronchial epithelial cells of asthmatic subjects or U937 cells.** A and B, Quantification of cell viability: Mean viability was 99.2% for U937 cells and 96.5% for epithelial cells. C and D, Histograms showing MFI of FcεRIα of the unstained negative control (blue) and stained cells of U937 cells (C) and bronchial epithelial cells (D). E and F, Quantitation of FcεRIα (MFI) of U937 cells (E) and bronchial epithelial cells with or without treatment with IgE or mouse IgG (F): No significant difference was found between the negative control (unstained) and the stained untreated epithelial cells group (p = 0.2). No significant difference was found between the untreated group compared to IgE and mouse IgG treated epithelial cells groups (p ≥ 0.6 for all). Results are expressed as mean ± SEM of 4 independent experiments.
Figure 8. **IgE did not block FcεRIα signal on the surface of U937 cells.**  
A. Histogram showing expression of FcεRIα (MFI) with or without treatment of U937 cells with IgE at 1 and 5µg/ml, green and orange curves; respectively. Blue curve is the unstained negative control.  
B. Quantification of histogram data. The results were obtained from one experiment.
Figure 9. Crosslinking FcεRI did not increase the release of TGF-β1 or TSLP from human bronchial epithelial cells of asthmatic subjects. A, Quantification of TGF-β1 in culture supernatants of the following treatment conditions (left to right): cells incubated with medium only for 48 hours (medium renewed each 24 hours); cells treated with IgE for 24 hours at 5μg/ml then incubated in growth medium for 24 hours; cells incubated in growth medium for 24 hours then treated with anti-FcεRIα at 0.5μg/ml for 24 hours; cells treated with IgE for 24 hours at 5μg/ml then anti-FcεRIα at 0.5μg/ml for 24 hours; cells incubated in growth medium for 24 hours then treated with vehicle of anti-FcεRIα (PBS) for 24 hours; cells treated with IgE for 24 hours at 5μg/ml then treated with vehicle of anti-FcεRIα (PBS) for 24 hours; cells incubated in growth medium for 24 hours then treated with isotype control matched to anti-FcεRIα at 0.5μg/ml for 24 hours; and cells treated with IgE at 5μg/ml for 24 hours then treated with isotype control matched to anti-FcεRIα at 0.5μg/ml for 24 hours. No significant difference was found between stimulated and un-stimulated groups (p≥ 0.11 for all). Results are expressed as mean ± SEM of 3 independent experiments. B, Quantification of TSLP protein of the same treatment conditions, as in TGF-β1. No significant difference was found between stimulated and un-stimulated cells (p≥ 0.3 for all). Results are expressed as mean ± SEM of 3 independent experiments.
CHAPTER 6: REFERENCES


43. Laitinen LA, Laitinen A, Haahtela T. Airway mucosal inflammation even in patients with newly diagnosed asthma. American Review of Respiratory Disease. 1993 Mar 1;147:697-.
ATTACHMENT

Protocol 1. Immunohistochemistry

1. Deparaffinization [Selected] **Triton based buffer**
2. Warmup Slide to [65 Deg C], and Incubate for [8 Minutes] (Cycle 1)
3. Incubate for [8 Minutes] (Cycle 2)
4. Incubate for [8 Minutes] (Cycle 3)
5. Pretreatment [Selected]
6. Cell Conditioning [Selected]
7. CC1 Reservoir [Selected] **EDTA like buffer for antigen retrieval**
8. Warmup Slide to [95 Deg C], and Incubate for 4 Minutes (Cell Conditioner #1)
9. CC1 8 Min [Selected]
10. CC1 16 Min [Selected]
11. CC1 24 Min [Selected]
12. Option [Selected]
13. Warmup Slide to [37 Deg C], and Incubate for 4 Minutes (Option)
14. Apply One Drop of [OPTION 1] (Option), and Incubate for [0 Hr 12 Min] **Blocking buffer**
15. Inhibitor [Selected]
16. Inhibitor CM [Selected] **Endogenous peroxidase Inhibitor**
17. Apply One Drop of Inhibitor CM, and Incubate for [8 Minutes]
18. Antibody [Selected]
19. 1st Antibody Manual Application [Selected] anti-FcεRI α  1/200 dilution
20. Warmup Slide to [37 Deg C] from Very Low Temperatures (Primary Antibody)
21. Hand Apply (Primary Antibody), and Incubate for [60 Minutes]
22. Linking Antibody [Selected]
23. 2nd Antibody [Selected]
24. Warmup Slide to [37 Deg C] from Very Low Temperatures (2nd Antibody)
25. Apply One Drop of [Anti-Mouse HQ] (Detection #1), and Incubate for [0 Hr 12 Min]
26. Enzyme conjugate [Selected]
27. Apply One Drop of [Anti-HQ HRP] (Conjugate #1), and Incubate for [12 Minutes]
28. DAB [Selected]
29. Counterstain [Selected]
30. Use RB for Counterstain [Selected]
31. Apply One Drop of [HEMATOXYLIN] (Counterstain), and Incubate for [8 Minutes]
32. Post Counterstain [Selected]
33. Use RB for Post Counterstain [Selected]
34. Apply One Drop of [BLUING REAGENT] (Post Counterstain), and Incubate for [4 Minutes]