Expression of Th-17 Related Cytokines (IL-17A & F) in Severe Asthma

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July 2010

A Thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

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Acknowledgments

I would like to thank my supervisor Dr. Qutayba Hamid for his continuous support, understanding and advice throughout my MSc program training. His wisdom and enthusiasm in research and medicine has influenced greatly my academic life.

My study was supported by the Fonds de la Recherche en Sante du Quebec through the national award of Dr. Hamid, and the Canadian Institute for Health research, I am grateful to these two organisations.

I also wish to thank my advisors Dr. John Richardson and Dr. Simon Rousseau for their guidance, and insightful comments. Special thanks to my program director Dr. Edith Zorychta for her endless support and motivation.

I would like to thank Dr. Ron Olivenstein & Cathy Fugere for their help with recruitment of the patients and collection of tissues and blood.

I am very appreciative to Patrice Vaillaicourt, Sawsan Al-Mot, Elsa Schotman, Fazila Chouiali, Severine Audusseau, Andrea Mogas, Alejandro Vazquez-Tello, and all my colleagues in the Meakins-Christie Laboratories for their assistance and support.

Finally, I am thankful to my parents Sohair Al-Ramli & Laila Al-Jajeh for their never ending support, patience and love.
Contribution of Co-Authors

The contribution of co-authors in the paper entitled “Th-17 associated cytokines (IL-17A and F) in severe asthma” published in the Journal of Allergy and Clinical Immunology which used data from this thesis are as follows:

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   - Manuscript writing and submission
2) Prefontaine D
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3) Chouiali F
   - Helped in supervising LCM experiments
4) Martin JG, Olivenstein R, and Lemière C
   - Responsible for recruiting patients and performing bronchoscopy
5) Hamid Q
   - Project supervisor
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
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<tr>
<td>AHR</td>
<td>Airway hyperresponsiveness</td>
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<td>ASMC</td>
<td>Airway smooth muscle cell</td>
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<tr>
<td>ATS</td>
<td>American Thoracic Society</td>
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<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
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<tr>
<td>CCR</td>
<td>Chemokine receptor</td>
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<tr>
<td>DAB</td>
<td>3,3’-Diaminobenzidine</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FEV₁</td>
<td>Forced Expiratory Volume in the first second</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<tr>
<td>Ig-</td>
<td>Immunoglobulin-</td>
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<tr>
<td>IL-</td>
<td>Interleukin-</td>
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<td>INF-γ</td>
<td>Interferon-gamma</td>
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<tr>
<td>MCP</td>
<td>Monocyte chemoattractant protein</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PMNL</td>
<td>Polymorphonuclear leukocytes</td>
</tr>
<tr>
<td>ROR-γt</td>
<td>Retinoic acid-related orphan nuclear hormone receptor-γt</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real-Time Polymerase Chain Reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
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<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
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<tr>
<td>Th-</td>
<td>T helper-</td>
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<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-alpha</td>
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Abstract

Inflammation and airway remodelling are hallmarks of severe asthma pathology. Although severe asthma represents 10% of the asthmatic population, this subset of patients has greater morbidity, mortality and a disproportionate need for health care services. Severe asthma appears to have a different inflammatory pattern compared to mild and moderate asthma. There is an increase in neutrophil infiltration, upregulation of IL-8 and disregulation of Th-2 cytokines. IL-17A and F are two proinflammatory and profibrotic cytokines produced primarily by Th-17 cells. These cytokines can act on a broad range of cell types to induce cytokines, chemokines and metalloproteinases as well as have important roles in neutrophil activity. We hypothesize that IL-17A and F are expressed in asthmatic airways and are increased in severe disease. The objective of our study is to investigate the expression of IL-17A and F in biopsies of severe asthmatics, and compare them with moderate and mild asthmatics as well as control subjects and relate this expression to that of IL-8 and neutrophilia. The expression of IL-17A and F mRNA and protein was evaluated in bronchial biopsies using immunocytochemistry and real-time PCR. The expression of both IL-17A and F were shown to be increased in severe asthmatic airways compared to mild asthmatics and control subjects. IL-17A was mostly expressed in mononuclear cells present in the subepithelial tissue within clusters of inflammatory cells, whereas IL-17F was expressed not only in inflammatory cells but also in epithelial cells. This observation was confirmed using laser capture microdissection. Our results are consistent with the possible role of IL-17 family in steroid hyperresponsive disorders, including rheumatoid arthritis, psoriasis and refractory asthma. We suggest that both IL-17A and F contribute to severe asthma inflammatory and
airway remodelling pathology. Our findings further the knowledge on severe asthma disease and open new options to further investigate the role of IL-17 as a target for asthma therapy.
Abrégé

Le processus inflammatoire et le remodelage des voies respiratoires sont typiquement liés à la pathologie de l’asthme réfractaire. Bien que l’asthme réfractaire n’affecte que 10% des asthmatiques, ce sous-groupe de patients est caractérisé par une plus grande morbidité, mortalité ainsi qu’un besoin disproportionné des services des soins de santé. Il semble que l’asthme réfractaire utilise un mécanisme d’inflammation distinct de celui associé à l’asthme léger et modéré. Il y a une augmentation de l’infiltration des neutrophiles, une régulation positive de l’IL-8 ainsi qu’une dérégulation des cytokines Th-2. Les cytokines IL-17A et F sont pro-inflammatoires, pro-fibrotiques et sont principalement produites par les cellules Th-17. Ces cytokines peuvent agir sur plusieurs types de cellules afin d’induire la production de cytokines, chémokines et métalloprotéinases. De plus, ces deux cytokines jouent un rôle prédominant dans l’activité des neutrophiles. Nous faisons l’hypothèse que l’IL-17A et F sont exprimées dans les voies respiratoires des asthmatiques et qu’elles sont augmentées dans l’asthme réfractaire. Cette étude a pour but d’investiguer l’expression de l’IL-17A et F sur biopsies provenant d’asthmatiques réfractaires et d’en faire la comparaison à celles provenant d’asthmatiques légers, modérés ainsi que de sujets sains et de relier cette expression à celle de l’IL-8 et de la neutrophilie. L’expression des ARNm et des protéines de l’IL-17A et F a été évaluée sur biopsies par immunocytochimie et ‘real-time PCR’. Il a été démontré que l’expression de l’IL-17A et F est augmentée dans les voies aériennes des asthmatiques réfractaires en comparaison aux asthmatiques légers et sujets sains. L’IL-17A est principalement exprimée par les cellules mononucléaires des tissus des régions sous-épithéliales parmi les grappes de cellules inflammatoires. L’IL-17F n’est pas
uniquement exprimée par les cellules inflammatoires mais également par les cellules épithéliales. Cette observation a été confirmée par microdissection au laser. Nos résultats sont en accord avec le rôle potentiel de la famille IL-17 dans les désordres associés à l’hyperréactivité aux stéroïdes incluant: l’arthrite rhumatoïde, le psoriasis et l’asthme réfractaire. Nous suggérons que l’IL-17-A et F contribuent chacun à la pathologie inflammatoire de l’asthme réfractaire ainsi qu’au remodelage des voies respiratoires. Nos résultats contribuent à approfondir notre compréhension de l’asthme réfractaire et sont susceptibles d’ouvrir la voie à de nouvelles investigations de l’IL-17 qui pourrait jouer un rôle dans le traitement de l’asthme.
Chapter 1: Introduction

Asthma: General Overview

Asthma is a chronic respiratory disease that affects both male and female individuals of different racial backgrounds and age groups. The incidence of the disease varies in different countries which might be due to social, genetic and/or environmental risk factors (Gold 2005). Asthma presents a significant burden on the health care system and cost as well as on the patient’s lifestyle. The Global Initiative for Asthma (GINA) estimates that approximately 300 million people worldwide currently suffer from asthma (Global Strategy for Asthma Management and Prevention 2009).

Asthma is described as a heterogeneous disease with variable clinical outcomes implicating many cells and cellular elements in its pathogenesis. Asthma can be classified according to the aetiology (extrinsic, intrinsic, aspirin sensitive and occupational asthma) or according to the severity of the disease. Different types of asthma display different cellular patterns, nevertheless inflammation remains the hallmark of the disease.

The American Thoracic Society (ATS) has placed a series of guidelines and criteria to better classify asthmatic groups. The guidelines and criteria for mild, moderate and severe asthmatics are based on symptoms such as episodic breathlessness, wheezing, cough and chest tightness; lung function measurements and the level of disease control (ATS 1986; ATS 2000).

Genetic factors were extensively investigated and many genetic association studies linking several genes to asthma and its severity were published in the last 20 years. Although asthma is described as a complex disease that does not follow simple Mendelian inheritance features, over 100 genes have been identified and linked to asthma
(Ober 2006). Genes such as that for high affinity immunoglobulin (Ig)- E receptor, IL-4, IL-4 receptor (IL-4R)-α, transforming growth factor (TGF)-β1, and ADAM-33 are a few of many genes that were associated to asthma phenotype and severity (Ober 2006; Wenzel 2007). The functional understanding of gene-environment interactions is important in asthma development and remains to be investigated (Mutius 2008).

Environmental factors such as exposure to aeroallergens (Arbes 2007), pollution (Brauer 2007), and tobacco smoke (Bergeron 2007) are important risk factors associated with asthma pathology. Environmental factors underlying asthma exacerbations include allergen exposure in sensitized individuals, viral infections, exercise, and irritants, among others. Allergen exposures such as that to aeroallergens (e.g. animal dander and house dust mites) are important in asthma development and clinical manifestations (Celedon 2007). The allergen exposure in general triggers the generation of antigen-specific IgE antibody, which in atopic individuals leads to persistent airway inflammation (Platts-Mills 2008). Respiratory tract infections such as one induced by rhinovirus are also thought to be among the important factors in the development of asthma (Kusel 2007). Viral respiratory tract infections were reported to be involved in loss of lung function, exacerbation and response to treatment (Hogg 2001; Jackson 2008). Exercise is another important exacerbating factor. Intense physical activity induces strong bronchospasms and results in wheezing, coughing, and shortness of breath (Weiler 2007). The pathophysiology of exercise induced asthma may be associated to the thermodynamic changes in the body that respond to the active heat gain and water loss as well as the increased production of inflammatory mediators (Hallstrand 2005).

Advances in our understanding of asthma pathology are attributed partially to the use of bronchoscopy and the use of molecular pathology to study samples obtained by
bronchoscopy such as biopsies and bronchoalveolar lavage (BAL) cells. Moreover, sputum induction developed in the last ten years has also contributed to our improved understanding of asthma pathology and allowed for phenotyping asthmatic subjects and monitoring response to treatment (Rasmussen 2002; Hargreave 2009).

For the past fifteen years, much of the research on asthma has been focused on the mild and moderate groups. Tissues obtained from mild and moderate asthmatics are associated with T cell activation, eosinophil accumulation, Th-2 type cytokine production and airway remodelling (Bradley 1991; Robinson 1992; Bentley 1993; Ying 1995). The present medications have been successful in controlling symptoms of these two subgroups, and the mortality and morbidity have decreased significantly over the years (Pearce 2007).

1.2 Severe Asthma

1.2.1 Epidemiology and Burden of Disease

Currently, there are approximately 10% of the asthmatic population who have the severe phenotype. The symptoms of this subgroup are uncontrolled despite using various optimal therapies including high doses of inhaled and/or systemic corticosteroids (Proceedings of the ATS Workshop on Refractory Asthma 2000). Although severe asthma represents only a small portion of the asthmatic population, this subset of patients presents with significant morbidity, mortality and utilize a large part of health care resource and cost (Wenzel 2007).

The direct and indirect economic cost of asthma is related to the severity of disease and is estimated to exceed those of tuberculosis and HIV/AIDS combined (Serra-Batllés 1998; Braman 2006). It was reported that severe asthmatic patients representing
the minority of the asthmatic population had a greater total asthma expenditure compared
to mild and moderate asthma (Beasley 2002). This is not surprising since the indirect
economic costs associated with loss of school or work days, lost productivity and
premature retirement are higher than the direct medical cost, as a consequence of
morbidity associated with the severity of the disease (Serra-Batlles 1998).

1.2.2. Physiology and Clinical Manifestations

The classical way to assess asthma physiology is by means of pulmonary function
tests measuring lung functions (Spirometry), changes in airway reactivity (PC$_{20}$) and
changes in elastic recoil properties of the lung as well as collapsibility of the small
airways (Wenzel 2003 Dec). Spirometry is today’s most commonly utilized respiratory
instrument that allows measurements of air volume and air flow. Most important
parameters measured by the device include vital capacity (VC), forced vital capacity
(FVC), and forced expiratory volume in 1 second (FEV$_1$) among others. Spirometry
results can be generally reported as a raw data (litres, litres per second), a ratio of two
parameters and/or as a percentage of predicted value considering patient’s age, sex,
height, weight and race (Miller 2005). Another important test is the PC$_{20}$ (provocative
concentrations causing a 20% fall in FEV$_1$) test, which permits the assessment of
bronchial responsiveness to varying concentrations of bronchoconstricting agents
(Cockcroft 1977).

Change in airflow limitation is an established physiologic component of severe
asthma. Severe asthma disease is believed to develop as a result of dynamic increase in
airflow restriction that may be initially present at onset of disease or one that may develop
over a period of time, in which it becomes irreversible (Wenzel 2003 Dec). In general,
normal healthy subjects have \% FEV$_1$ (ratio of FEV1/FVC) in the range of 80-98\%, whereas that of asthmatic patients is lower than 80\% and decreases with respect to disease severity (Rasmussen 2002). Accordingly, the decrease in value is often the result of reduced FEV$_1$, a reflection of increased airway resistance to expiratory flow.

Change in airway reactivity is the other physiologic component in asthma severity. Severe asthmatics display an increased bronchial hyperresponsiveness in response to metacholine or histamine challenge observed by PC$_{20}$ results. Airway reactivity relates to airflow instability and describes an important feature of severe asthma symptomatology (Weiss 2000). Airway structural changes (see section 1.2.4 airway remodelling) also contribute to disease severity and lead to irreversible airway obstruction (Tillie-Leblond 2008).

Physiological changes in elastic recoil properties of the lung and collapsibility of the small airways have been recognized as important factors in asthma severity. The elastic recoil properties of the lung are described to be abnormal in asthmatics and compliance was observed to increase with asthma worsening (Gelb 2000). It has been suggested that asthmatic airways may be more collapsible than in normal healthy individuals as observed by the reduced FVC/VC ratio in severe asthmatics (Wenzel 2003 Dec). It was reported that severe asthmatics had greater earlier closure of small airways compared to milder asthmatics (in’t Veen 2000).

There are several clinical manifestations associated with severe asthma. These include the standard asthma symptoms and ones occurring periodically with more severe episodes, as well as disruption in the standard of living (such as performing normal lifestyle activities, including gardening, walking up the stairs, and sleeping). Impaired
lung function and the use of several types of medications are also important clinical signs associated with this disease. That being said, there is now good evidence that these clinical manifestations can be controlled with appropriate treatments (Reddel 1999).

1.2.3. Pathology: Inflammation

Severe asthma is a complex heterogenic disorder with variable clinical and pathological features. Several factors including poor compliance to medications, lack of recognition of associated conditions, and biological resistance to treatment were proposed to explain the difficulty in management for this subtype of the disease (Barnes 1998). Extensive research has been done in the last few years to identify the inflammatory profile (Wenzel 1997) and the extent of structural changes (Benayoun 2003) in an effort to better understand and define severe asthma.

The pattern of airway inflammation typically identified in mild and moderate asthmatics is of T helper (Th)-2 type immune response that results in the recruitment of eosinophils into airways, which usually respond to corticosteroid treatments (Naseer 1997). Nevertheless, some asthmatic patients continue to be resistant or hyporesponsive to steroids (Leung 1995; Hauk 2002; Li 2004; Wang 2010).

Severe poorly controlled asthmatic patients have recently been the focus of study, and comparison with mild and moderate asthmatics has revealed important differences. A recent report showed that severe asthmatics had more airway inflammation as observed by increased recruitment of eosinophils and neutrophils in sputum compared to moderate asthmatics (Lemiere 2006). Similarly, it was shown that BAL fluid and endobronchial as well as transbronchial biopsy samples from severe asthmatics have increased neutrophils compared to other asthmatics (Wenzel 1997 and 1999). Moreover, important mediators of
eosinophilia and neutrophilia were shown to be present in airway wall biopsy of asthmatics. Specifically, interleukin (IL)-8 was highly expressed in both epithelial and subepithelial surfaces of bronchial biopsy from severe asthmatics (Shannon 2008). IL-8 is a known potent chemoattractant for neutrophils (Pease 2002), and its increased expression in airways of severe asthmatics perhaps reflects the excess neutrophils observed in the sputum. The effects of IL-8 are not restricted to neutrophil recruitment. IL-8 has been reported to cause airway smooth muscle cell (ASMC) contraction and migration that have important implication in airway remodelling (Govindaraju 2006). Corticosteroid resistance or hyporesponsiveness observed in severe asthmatics are associated with increased expression of both IL-8 and neutrophils (Chakir 2002; Fukakusa 2005). Another important difference was documented between severe and moderate asthmatics, in that a dysregulation in Th-1/Th-2 cytokine expression existed. More specifically, it was shown that in severe asthma IL-4, a Th-2 cytokine, was downregulated compared to moderate asthma, whereas INF-γ, a cytokine that belongs to Th-1 cells, was upregulated (Shannon 2008). Figure 1 summarizes Shannon et al. results.
1.2.4. Pathology: Airway Remodelling

Airway remodelling refers to the long-term pathological changes in the airway epithelium and subepithelium in response to ongoing chronic inflammation that is associated with asthma severity (Chetta 1997). Structural changes such as epithelial alteration (Naylor 1962; Laitinen 1985), thickening of the lamina reticularis (Roche 1989), subepithelial fibrosis (Elias 1999), goblet cell hyperplasia and hypertrophy (Aikawa 1992; Carroll 1993), increased smooth muscle mass (Carroll 1993), among other
21

features are found in both the large and small airways of asthmatics of different severity. Figure 2 summarizes airway remodelling features. These features ultimately cause increase in bronchial wall thickness that affects the severity of physiological and clinical parameters of asthmatic patients (Bento 1998). Airway remodelling has been suggested to explain the associated decrease in lung function, increased airway hyperresponsiveness (AHR), and greater use of medications by severe asthma patients (Lazaar 2003; Chen 2009, Kościuch 2009, Margulis 2009, Tang 2009).

Figure 2: Diagram illustrating features of airway remodelling in asthma.
Airway remodelling features include: 1) epithelial alteration, 2) goblet and mucous gland hyperplasia, 3) subepithelial fibrosis, 4) increased smooth muscle mass, 5) angiogenesis, 6) loss of cartilage integrity and 7) inflammation.
Documented features of remodelling include epithelial cell detachment, loss of ciliated cells, goblet cell hyperplasia, and increase in receptor expression and release of several mediators. Airway epithelium functionally provides a physical protective barrier against inhaled particles such as allergens (Hackett 2008). The degree of epithelial damage has been described to correlate with AHR and asthma severity (Jeffery 1989). Nevertheless, epithelial desquamation observed in bronchial biopsies is a topic of controversy and of debate, as some researchers suggest that the shedding to be merely an artifact of physical tissue sampling (Wiggs 1997; Ordonez 2000). Next, goblet cells secrete mucin glycoproteins that have important role in host defence. Nevertheless, pathological increase in goblet cell numbers that is a characteristic feature observed in asthmatic airway epithelium (Ordonez 2001) can lead to excess mucin release. Consequently, this excess mucin can form mucus plugs in the small and medium airways that contribute to airflow limitation, air trapping and airway obstruction in severe asthma (Rogers 2007; Neveu 2009). The exact mechanism of how goblet cell abnormally multiplies in asthma is unclear, however it is suggested that activation of mucin genes may lead to the conversion of nongranulated secretory cells to goblet cells (Alimann 2000) and/or through up-regulation of a calcium-activated chloride channel (HCLCA1) that regulates the expression of soluble gel-forming mucins, such as MUC5A/C (Toda 2002). Finally, epithelial cells express various types of receptors and cytokines that are associated with inflammation and remodelling which is increased in severe asthma phenotype (Joubert 2008; Shannon 2008; Li 2010).
Subepithelial fibrosis is an important feature of airway remodelling that occurs as a result of increased deposition of extracellular matrix (ECM) proteins (such as collagens I, III and V; fibronectin and tenascin) by fibroblasts and other cells in the lamina reticularis layer located below the basement membrane (Roche 1989; Wilson 1997; Huang 1999). This deposition in turn results in increased thickening of the lamina reticularis that is associated with airway hyperresponsiveness and asthma severity (Elias 1999; Little 2002). Respectively, severe asthmatics were described to have increased collagen deposition and higher extent of subepithelial fibrosis compared to less severe asthmatics and control subjects (Chakir 2003; Pepe 2005). It has been shown in asthmatics that an imbalance between proteases and antiproteases responsible for ECM protein degradation and protection, respectively contributes to fibrosis (Vignola 1998 Dec; Akers 2000; Margulis 2009 Feb). Cellular sources for these proteases and antiproteases include macrophages and neutrophils among other type of cells (Bergeron 2009), which are present in severe asthma at significant levels. Equally important are the matrix metalloproteinases (MMPs) family, which play important roles in airway remodelling and asthma inflammation. MMP-9 is the most important MMP in asthma and is implicated in collagen degradation (Vignola 1998 Dec; Suzuki 2001), angiogenesis (Johnson 2003 Feb), smooth muscle hyperplasia (Johnson 2003 Jan) as well as airway obstruction and airway inflammation (Wenzel 2003 June). MMP-9 has been described to be significantly elevated in asthmatic patients compared with control subjects (Vignola 1998 Dec) and its increased level is associated with a decrease in FEV₁ as well as the severity of asthma (Wenzel 2003 June). Excessive formation of fibrous tissue is detrimental to lung function and thus to respiration. Nevertheless, remodelling of the airway wall may also be beneficial in some cases. It has been proposed by independent
research groups that airway wall thickening and ECM protein deposition in the subepithelium surface protects against bronchoconstriction (Palmans 2000; Milanese 2001). The deposition of proteins result in airway wall stiffening that can inhibit narrowing of airways and smooth muscle shortening (Pepe 2005). This concept is supported by animal model experiments demonstrating that airway reactivity may amplify as a result of airway inflammation, but may attenuate with airway deposition of fibronectin and collagen (Palmans 2000).

Changes in ASMCs constitute an important component of asthma pathophysiology and of the remodelling process. ASMCs have many properties including secretory, proliferative (hypertrophy and hyperplasia) and migratory that can modulate airway tone (Carroll 1993; Ebina 1993; Cohen 1997; Johnson 2004; Joubert 2005 Sept). Increase in airway smooth muscle mass as a result of hypertrophy and hyperplasia contribute to airway hyperresponsiveness and asthma severity (Lambert 1993; Benayoun 2003). Smooth muscle remodelling causes the mechanical narrowing of the lumen as a consequence of greater space occupancy by ASMCs within the airway wall (James 1989). It is recognized that ASMCs contribute to asthma inflammation and remodelling activities through the release of proinflammatory mediators, deposition of ECM proteins and their migration within the subepithelial surface (Johnson 2001; Panettieri 2002). Recently, migration of ASMCs has been described as a feature of airway remodelling that could contribute to airflow obstruction and to asthma severity (Joubert 2005 Aug; Takeda 2009). Morphometric studies demonstrated that severe asthmatics have both increased ASM mass and a significantly closer smooth muscle bundle to the epithelium compared to moderate asthmatics (Pepe 2005).
1.3 Eosinophils and Neutrophils in Asthma

Eosinophils and neutrophils form an important part of the host defence and provide protection against invading pathogens. Nevertheless, at high number and over a long period of time these granulocytes lead to inflammation, tissue damage and clinical disease. Eosinophils and neutrophils are described as proinflammatory cells found in high numbers in allergic airway disease including asthma, particularly in severe disease. Both granulocytes have been studied in bronchial mucosa, BAL and sputum of asthmatics and their numbers were described to increase in relation to the severity of asthma (Jatakanon 1999; Louis 2000; Shannon 2008).

The accumulation of proinflammatory cells and more importantly their release of pre-stored proteins might lead to exacerbations. Eosinophils exert their biological effects through the synthesis and release of different types of mediators. On activation, eosinophils can release a wide variety of compounds including cytotoxic proteins, oxygen free radicals, lipid mediators, as well as a range of cytokines and chemokines that mediate asthma development and worsening (Saeed 2002). Cytotoxic proteins released by eosinophils include eosinophil cationic protein, eosinophil peroxidase, and major basic protein, all of which have proinflammatory and tissue destructive functions (van Dalen 2001; Trautmann 2002; Swaminathan 2005). Similarly, oxygen free radicals have damaging cellular and structural effects mediated by their inflammatory capacities (Pawliczak 2003). Lipid mediators such as leukotrienes (LTB₄, and LTC₄) expressed and released by activated eosinophils are important contributors in asthma pathology. Specifically, leukotrienes can potently induce SMC contraction, increase microvascular permeability, and mucous hypersecretion in human airways (Csoma 2002), among other
effects. Eosinophils also express an array of cytokines including IL-1 to -6, IL-10 to -13, TGF-β, tumor necrosis factor (TNF)-α and granulocyte-macrophage colony-stimulating factor (GM-CSF); as well as chemokines such as, IL-8, RANTES and MIP-1α (Rothenberg 2006). Airway inflammation and remodelling described in asthmatics can be explained to some degree by these synthesized and released compounds from eosinophils, which have themselves cytotoxic, proinflammatory and profibrotic properties.

Neutrophils could also play important roles in allergic processes in general, and in asthma in particular and their role in inflammation includes phagocytosis, and the release of several arrays of mediators that contribute to asthma pathology (Gasperini 1999; Scapini 2001; Yoshimura 2007). Phagocytosis is an essential mechanism in the innate immune system to remove pathogens and cell debris, which in turn assists in the reduction of the inflammatory process. Neutrophils can express elastase, a strong secretagogue molecule that has been found to actively promote hypersecretion of mucous in asthma (Vignola 1998 Feb; Nadel 1999). This molecule can also contribute to bronchial hyperresponsiveness and to tissue remodelling (Sampson 2000; Zhu 2000). In addition, neutrophil elastase was reported to be found beneath the bronchial mucosa in severe asthma (Wenzel 1999). Neutrophils can release tumour necrosis factor (TNF)-α, a strong proinflammatory cytokine that contributes to airway hyperreactivity and to the chemoattraction of neutrophils via IL-8 (Xiu 1995; Keatings 1996). Neutrophils themselves have the ability to release IL-8 in response to inflammatory stimuli present in asthmatic airways including eosinophil granule major basic protein (Page 1999) that leads to more of its recruitment and perpetuation of inflammation.
Thus, both eosinophils and neutrophils can be associated with airway gland hypersecretion, bronchial hyperreactivity and airway remodelling. Discovery of new cells and mediators as well as investigation of their properties will certainly redefine our knowledge about the pathogenesis of asthma.

1.4 Cytokines in Asthma

Morphological and functional abnormalities associated with airways and lungs of asthmatics can be related to the expression and release of a family of small glycosylated proteins, referred to as cytokines. Within this family of cytokines, another group of smaller size proteins exist and are referred to as chemokines. Together, this family of proteins carry out important roles in cell development (growth, differentiation, and proliferation), signalling, chemotaxis, as well as Ig-isotype switching and apoptosis (Lukacs 1999; Kim 2009; Komai 2009); all of which regulates different inflammatory and remodelling processes that take place in asthma pathology.

Cytokines can mediate their effects in an autocrine, paracrine and/or an endocrine fashion by binding to their corresponding cell surface receptors expressed on target cells (Onishi 1998; Cuneo 2009). Inflammatory cells such as T lymphocytes, eosinophils and neutrophils among other inflammatory cells, as well as structural cells including epithelial, endothelial and ASM cells are able to express and release various cytokines. There is also a redundancy in cytokine expression among different cells. So far, over 30 different cytokines have been discovered and implicated in asthma pathology, all of which participate in mediating allergic, cytotoxic, cell-mediated, and/or humoral immunity (Commins 2010). Many cytokines display pleiotropic and overlapping functions thereby capable of building up an intense pathological response (Onishi 1998).
Cytokines could be classified based on their functional activities and are subdivided into eosinophil-associated cytokines, IgE-mediated cytokines, remodelling-associated cytokines and immunomodulatory cytokines.

1.4.1 Eosinophil-Associated Cytokines

By far the most important cytokine for eosinophil development and function is IL-5 (Weltman 2000). In addition to being expressed by various types of cells including, T helper cells, cytotoxic T lymphocytes, and mast cells, IL-5 is also significantly produced and released by eosinophils (Borish 2003; Larche 2003). Indeed, airway challenge experiments show that eosinophils are able to express significantly high levels of IL-5 mRNA and protein in lung fluid (Ohnishi 1993). IL-5 is described to stimulate the production and release of eosinophils from the bone marrow and participates in their chemoattraction. Moreover, IL-5 activates mature eosinophils and stimulates their secretory and cytotoxic capacity. Survival of these cells is also provided by IL-5 by inhibiting apoptosis (Weltman 2000). The administration of IL-5 was shown to accumulate and activate eosinophils within the lung, ultimately leading to AHR (Eum 1995; Mould 2000; Tanaka 2004). Recently, two independent studies showed that patients with refractory eosinophilic asthma benefited from anti-IL-5 treatments, as it lowers eosinophil numbers in blood and sputum and improves asthma control (Leckie 2000; Haldar 2009; Nair 2009).

In addition to IL-5, IL-3 (Rothenberg 1988) and GM-CSF (Owen 1987) play important roles in eosinophil activity and development. IL-3 is described as a pluripotent growth factor that stimulates stem cells to differentiate and proliferate into several cells including eosinophils, neutrophils, and monocytes among many other cells. IL-3 is
expressed primarily by T cells, but it can also be produced by eosinophils and mast cells during pathological conditions (Wallaert 1995, Hawwari 2002). It was demonstrated by Rothernberg et al. that coculture of eosinophils with IL-3 leads to prolonged eosinophil survival, enhanced eosinophil cytotoxicity as well as improved functional properties. These changes in eosinophil features are pathologically significant as they may perpetuate the inflammatory process in asthma and cause extensive damage to airway structural cells. Similarly, GM-CSF is a pluripotent growth factor involved in the stimulation and development of granulocytes and monocytes. GM-CSF is involved in the activation, accumulation and survival of eosinophils and has been associated with the development of airway remodelling of asthma (Saha 2009). Eosinophils have been also described to increase their degranulation, cytotoxicity and response to chemoattraction as a result of GM-CSF stimulation (Commins 2010). Animal studies have shown that chronic eosinophilia in the lung as a result of incorporation of the GM-CSF gene leads to irreversible fibrosis (Xing 1996; Adach 2002). The expression of GM-CSF has been also reported to be increased in both bronchial biopsies and airway fluids of asthmatics (Woolley 1994; Dente 2006; Erin 2008).

1.4.2 IgE-Mediated Cytokines

Allergen exposure triggers hypersensitivity reactions in atopic individuals characterized by increase IgE antibody production by B cells, a common feature observed in allergic induced asthma (Oettgen 1999). In response to antigen presentation, B cells generally activate specific Th2 cells to produce a number of cytokines important for B cell development, activation, and function. Specifically, IgE-associated cytokines such as IL-4, IL-9 and IL-13 are of important interest (Hamid 2009). The binding of IgE
antibodies to the high affinity IgE receptor FcεRI expressed on mast cells, eosinophils and basophils leads to their sensitization to antigen exposure (Oettgen 1999; Girodet 2005). Subsequently, these cells are prompted to form and release proinflammatory and cytotoxic compounds (e.g. eicosanoids, histamine and reactive oxygen species) with the aim to eradicate the pathogen. Nevertheless, the release of these compounds also leads to airway inflammation, smooth muscle constriction, mucous hypersecretion and vasodilatation, all of which contribute to asthma pathology (Hamid 2009).

IL-4 is an important Th2 cytokine that is essential for the regulation of B cell development and activity (Tangye 2002) among other functions. IL-4 signals through its specific cell-surface receptor composed of the IL-4Rα chain and the γ common chain. It has been shown that IL-4 potentiates IgE production by B cells and preferentially stimulates isotype switching to IgE (Lebman 1988). Moreover, IL-4 is able to increase IgE receptor expression on inflammatory cells (Vercelli 1988), thus possibly intensifying IgE-mediated response in the airways. In contrast, Th1-cytokine IFN-γ can downregulate the IL-4 production of IgE by B cells through suppression of isotype switch recombination (Xu 1994); as well as inhibit IgE receptor expression induced by IL-4 (Denoroy 1990). IL-4 can promote the recruitment of various inflammatory cells from the blood into the sites of inflammation through upregulation of VCAM-1 on endothelial cells (Schleimer 1992; Chung 1999). Murine model experiments demonstrate that blockade of IL-4R significantly reduces antigen-induced AHR and decreases goblet cells and BAL eosinophils, all of which are hallmarks of asthma (Gavett 1997). Nevertheless, blocking IL-4 does not result in similar attenuation of asthma features, suggesting that other cytokines may signal through the same receptor.
IL-13 is a Th2 cytokine that shares sequence homology and receptor binding with IL-4. More specifically, IL-4 and IL-13 share approximately 30% of their amino acid sequences, and bind a heterodimer receptor composed of IL-4Rα chain and IL-13Rα (Zurawski 1993; Zurawski 1994). Thus, some degree of functional overlap may exist between these cytokines. Indeed, IL-13 is described to have similar activities to IL-4 such as stimulation of IgE production, inflammation, mucus hypersecretion, and eosinophilia (Zhu 1999). The quality that distinguishes IL-13 from IL-4 is observed after antigen challenge when blocking individual cytokines and observing the consequential diminishing of AHR that only occurs for IL-13 (Wills-Karp 1998). Hence, IL-13 is proposed to be essential in the development and stimulation of allergen-induced AHR. Nevertheless, IL-13 has been suggested to have another effect on allergic response in addition to that traditionally associated with its eosinophils and IgE mediated effects in that it induces effects on epithelial and SMCs (Wills-Karp 2003). In accordance, a recent study describes IL-13 and IL-4 important modulatory roles on human airway smooth muscle cells (Moynihan 2008) that may be important for airway remodelling in asthma.

IL-9 is a Th2-type cytokine that is also involved in the pathogenesis of asthma. IL-9 can be produced by several inflammatory cells including mast cells, eosinophils, and neutrophils, however the major sources of this cytokine are T helper cells (Hauber 2003). Several mediators are involved in the regulation of IL-9 production, in particular, Th1 cytokine IL-2. IL-9 synthesis was shown to be induced and dependent on IL-2 (Kajiyama 2007). IL-9 induces IgE production by B cells (Petit-Frère 1993), and stimulates mucus secretion by bronchial epithelial cells (Louahed 2000). Moreover, IL-9 was also shown to potentiate the IL-4 induced IgE production by B lymphocytes (Dugas 1993). Studies on transgenic mice demonstrate an IL-9 role in the increased expression of eotaxin and
monocyte chemoattractant protein (MCP) in airway epithelial cells (Dong 1999), which is important for asthma pathology. Furthermore, murine model experiments show that IL-9 overexpression leads to several features of asthma, including eosinophilic airway inflammation, mucous overproduction and AHR (McLane 1998; Temann 1998; Vink 1999). The expression of IL-9 in airways of asthmatics was also shown to inversely correlate with the value of FEV₁ and the degree of AHR (Shimbara 2000). Other activities of IL-9 include stimulation of protease and FcεRI expression by mast cells (Louahed 1995) thereby priming and enhancing these cells to respond to allergen challenge.

1.4.3 Remodelling-Associated Cytokines

Structural changes in the airways and lungs of asthmatics (as described in section 1.2.4 Pathology: Airway remodelling) are a significant cause of asthma worsening and management difficulties. The pathological alterations are associated with functional consequences including AHR, chronic reduction in airway diameter and steroid resistance. The mechanisms involved in airway remodelling are not entirely elucidated, but associated expression of particular cytokines including TGF-β, platelet-derived growth factor (PDGF), and IL-11 are well documented in this process.

TGF-β is described as a remodelling-associated cytokine having powerful proinflammatory and profibrotic properties. TGF-β is primarily expressed by eosinophils, but other cells including lymphocytes, fibroblasts, epithelial cells, and mast cells also express TGF-β (Makinde 2007). Levels of TGF-β were shown to be increased in airway fluids (Redington 1997) and tissues of asthmatics and were related to the severity of asthma (Minshall 1997). TGF-β plays important roles in the regulation of differentiation,
survival and proliferation of various cells. It was shown to stimulate the synthesis of ECM proteins and proliferation of ASMCs (Black 1996; Coutts 2001; Chen 2006), thereby contributing to the SMC hypertrophy and hyperplasia in airway remodelling. Moreover, TGF-β can induce the proliferation and survival of fibroblasts (Khalil 2005) as well as their differentiation to myofibroblasts (Qing 2000; Hashimoto 2001) that essentially contributes to subepithelial fibrosis observed in asthmatics. TGF-β can also stimulate and inhibit the production and release of many proinflammatory and profibrotic cytokines from different cells as well as act as a chemoattactant for a range of cells (Halwani 2010). It was also shown that it has angiogenic capacity (Hyman 2002; Wang 2004), which also contributes to airway remodelling. Thus, TGF-β displays a pleiotropic nature and has a multifunctional role in the development of airway remodelling.

PDGF is a well known mitogen and an important remodelling-associated cytokine. It can be produced by a wide range of cells, including structural cells such as epithelial cells and endothelial cells as well as by several inflammatory cells including macrophages and eosinophils (Floege 1993). PDGF is thought to work with other remodelling cytokines, in specific TGF-β, to cause structural changes in airways of asthmatics. It was described to have the ability to promote the development of tissue-structural cells including fibroblasts, epithelial cells, and vascular smooth muscle cells (Ito 2009; Kouzaki 2009), which is of major importance in airway remodelling. PDGF has been shown to facilitate the migration of ASMCs through the regulation of MMP and tissue inhibitors of metalloproteinase (TIMP) (Ito 2009). Moreover, it was shown that PDGF can stimulate fibroblasts to proliferate, express collagen as well as contract collagen matrix (Clark 1989). Interestingly, in severe asthmatics the airway fibroblasts
exhibited a more synthetic phenotype compared to fibroblasts from milder asthma patients and healthy subjects (Lewis 2005).

IL-11 is described as a pleiotropic cytokine with key functions in airway and lung pathology. It is generally found in low levels in normal lung tissue and a variety of primary lung cells, however during pathological conditions it was reported to be increased in fibroblasts, epithelial cells, ASMCs, and eosinophils (Elias 1994 March; Elias 1994 Sept; Elias 1997; Minshall 2000). IL-11 is associated with airway remodelling and is increased in patients with asthma, particularly in those with severe disease (Chakir 2003). Its remodelling effects include fibrosis and collagen deposition (Molet 2003) and it can induce airway obstruction and AHR (Kuhn 2000). It was reported that IL-11 expression in the airways of severe asthma patients was increased compared to mild asthma patients and control subjects. More specifically, IL-11 mRNA and protein expression was localized in the epithelium and subepithelium compartments of the airway walls as well as the infiltrating eosinophils. Moreover, IL-11 expression was reported to inversely correlate with FEV1 of severe asthmatics (Minshall 2000). These results are in parallel with IL-11 transgenic mice experiments that demonstrate thickening of airway wall, enlarged alveoli, and fibrosis of subepithelial and adventitial tissue as well as increased collagen deposition and proinflammatory cells (Wang 2000; Chen 2001; Lee 2001; Zhu 2001).

1.4.4 Immunomodulatory Cytokines

The imbalance between Th1 and Th2 cytokines is suggested to be central in the development of asthma (Barnes 2001). As was described, the majority of pathological consequences resulting in the progression and exacerbation of asthma are a result of the
Th2-type inflammatory response. Shifting the immunological response from a Th2 to a Th1 type reaction by immunomodulatory cytokines such as IL-10, IL-12 and IFN-γ may improve the symptoms of asthma and prevent the initial onset of the disease.

IL-10 is described as an immunoregulatory cytokine having both immunosuppressive and anti-inflammatory effects important to the control of asthma symptoms (O'Garra 2008). IL-10 is produced by lymphocytic cells as well as by activated monocytes, mast cells and macrophages (Borish 2003). In general, it is found in normal lungs, whereas its expression is reported to be significantly reduced in asthmatic patients (Borish 1996). IL-10 limits the effects and production of proinflammatory mediators, as well as T cell activation and proliferation (John 1998; Pietrzak 2009). Its inhibitory action is described to be both direct and indirect. IL-10 inhibits the development and interferes with the functions of monocytes and macrophages (Pietrzak 2009) as well as inhibits IgE synthesis through downregulation of IL-4 induced isotype switching of activated B cells (Chung 1999). In addition to modulating antigen presentation and dampening Th2 response, IL-10 can also block reactive oxygen species release from inflammatory cells (Cunha 1992; O'Garra 2008). In view of these immunosuppressive capacities by IL-10, it is under investigation for use as treatment against inflammatory diseases, including asthma (Zhou 2010).

IFN-γ is the hallmark cytokine of Th1 cells with a significant role in cell-function regulations and cell-mediated immunity. It has an essential function in the balance between Th1/Th2 cells; in that it promotes Th1 cell development while strongly inhibits that of Th2 cells (de Pater-Huijsen 2002; Romagnani 2006). IFN-γ is produced by cytotoxic T cells and natural killer (NK) cells, but primary sources of this cytokine are Th cells (Boehm 1997). IFN-γ is involved in the reduction of allergic reactions through
inhibition of IgE production and class switching by B cells (Romagnani 1989). Other activities of IFN-γ include the stimulation of growth, maturation and differentiation of several types of cells as well as enhancement of their productivity and activity (Perussia 1983; Young 1995; Boehm 1997). Modulation of cell function by IFN-γ includes an increase in cytokine production, stimulation of phagocytic and adherence capacity, as well as enhancement of respiratory burst and nitric oxide release. All these heightened biological activities can be translated into either improved immune responses or strong damaging abilities by those cells. Nevertheless, evidence suggests a therapeutic effect for IFN-γ. In support of this is the animal study by Lack et al. demonstrating that subsequent to IFN-γ delivery there is a reduction of allergen-specific IgE, Th2 cytokine expression, AHR and lung eosinophilia (Lack 1996).

IL-12 is described as a multifunctional cytokine with properties bridging innate and adaptive immunity. Similar to IFN-γ, IL-12 acts as a key regulator of cell-mediated immune responses via stimulation of Th1 cells. IL-12 is produced by a vast range of cells including B cells, monocytes, macrophages and dendritic cells, among other cells (Del Vecchio 2007). IL-12 is similar to IFN-γ as it can also inhibit T cell differentiation toward a Th2-mediated response (Manetti 1993) and prevent the synthesis of IgE (Kiniwa 1992). In sensitized and allergen-challenged mice, IL-12 shows similar effects as IFN-γ in reducing asthma features (Kips 1996; Lee 1999), however IL-12 effects were time dependent on its administration (Hofstra 1998). In asthmatics, IL-12 administration was found to decrease peripheral blood and sputum level of eosinophils and to some degree AHR, however it had no significant effect on the late-phase asthmatic responses (Bryan 2000).
1.4.5 Chemokines

Chemokines are a subfamily of cytokines that are 8 to 10 kDa in size. They have a broad range of biological activities, but primarily function in the process of chemotaxis, by which they attract and regulate cell trafficking into tissues (Rollins 1997). Over 40 chemokines have been identified, and all bind and signal through seven transmembrane spanning G-protein coupled receptors (Neote 1993; Gerard 1994; Murphy 1994). Chemokines have been identified and categorized into four subgroups according to their structure, i.e. CXC, CC, C and Cx3C. The two main groups are CXC also referred to as α chemokines (IL-8 and IP-10) and CC also referred to as β chemokines (Eotaxin, RANTES, MCP-1–MCP-4, MIP-1α, and MIP-1β). The α-chemokines primarily target neutrophils, whereas the β-chemokines target monocytes, T cells, and eosinophils (Ward 1998). Chemokine receptors (CCR) can be bound by several types of chemokines; however some chemokines can only bind one specific receptor. Moreover, chemokines and CCRs are expressed on a broad range of cells including inflammatory and structural cells (Ward 1998). Similar to cytokines, chemokines were also reported to be in higher levels in both airway fluids and tissues of asthmatic patients compared to control subjects (Borish 2003). Given the fact that both cytokines and chemokines perform integral roles in coordinating, maintaining, and intensifying many pathological conditions, their specific targeting may be of strategy in the treatment of asthma and other diseases.

1.5 T Cells & Cytokines

Subsequent to antigen exposure, the adaptive immune response primes naive CD4+ T cells to develop into specialized effector subsets such as Th-1, Th-2 or Th-17 cells. Each T helper cell type is distinguished by its specific mode of regulation and its
unique production of a set of cytokines. The selection of a T helper cell type and the
constitutive production of their corresponding cytokines depends on environmental and/or
genetic factors (Abbas 1996; Sawyer 2004; Bessoles 2008; Hung 2010).

1.5.1 Th1 and Related Cytokines

Th1 cells function in mediating phagocyte-dependent inflammatory immune
responses against intracellular bacteria and some viruses. The protection against disease-
causing agents by Th1 cells is facilitated by the production of interferon (INF)-γ, IL-2 and
TNF-α that influence the assembly of antibodies by B cells, macrophage’s activity, cell
cytotoxicity and stimulation of cell mediated immunity (Romagnani 2000). Nonetheless,
persistent Th-1 responses may lead to different inflammatory and autoimmune diseases
such as contact dermatitis, acute allograft rejection, and rheumatoid arthritis to name a
few (Kapsenberg 1991; Simon 1994; Steele 1994).

Differentiation of naïve T cells to Th-1 cells is initially cued by pathogens
stimulating dendritic cells to release IL-12. The subsequent maturation stage of these cells
depends on signals provided by INF-γ (Romagnani 2000; Hildenbrand 2008; Rathinam
2008). The initial binding of IL-12 to its receptor expressed on naïve T cells leads to
selective tyrosine phosphorylation of the signal transducer and activator of transcription
(STAT)-4, which interestingly also regulates INF-γ gene (Kaplan 1996). Moreover, other
interactions such as that from IL-18 and its downstream signalling pathway are also
important in the activation of the IFN-γ gene (Romagnani 2000).

1.5.2 Th2 and Related Cytokines

Th-2 cells mediate specific immune responses against extracellular bacteria,
parasites and toxins. The immune responses are mediated through expression of IL-4, IL-
5, IL-6, IL-9, IL-10, and IL-13 that activate and induce several components of the immune system. More specifically, IL-4 and IL-13 are involved in stimulating B cells to produce IgE antibody and epithelial cells, endothelial cells and fibroblasts to produce eotoxin (Doucet 1998); IL-4 and IL-10 stimulate mast cell development, IL-5 causes eosinophil accumulation (Fakhri 2002), and IL-9 and IL-13 leads to mucus hypersecretion and mucus cell hyperplasia (Longphre 1999; Zhu 1999), among many other actions that each cytokine encompasses. Th-2 cells and cytokines have a central role in allergic diseases including asthma. It was shown by cell transfer experiments that mice receiving Th2 cells and cytokines had exaggerated allergic asthmatic symptoms including accumulation of eosinophils in the airways, mucus hypersecretion, and airway hyperresponsivness (Romagani 2000).

Th2 cells differentiate and proliferate from naïve T cells in response to early IL-4 expression. Interestingly, naïve T cells produce IL-4 at low levels, and its concentration increases as a lymphocyte activates. In this manner, when IL-4 reaches the necessary threshold to overtake other cytokine concentrations it allows Th cells to differentiate into Th2 subset (Paul 1994). The engagement of IL-4 with its receptor leads to the selective tyrosine phosphorylation of STAT6 and activation of downstream signalling pathways needed for the development of Th2 cells (Shimoda 1996). GATA-3 is a transcription factor expressed by T cells that is involved in the general induction and maintenance of the Th-2 pattern of cytokine secretion (Zheng 1997) and one that down-regulates Th1 cytokines and regulator IFN-γ (Ferber 1999). Moreover, c-maf is another transcription factor important in Th2 development, but one that is specific for IL-4 in that it transactivates its promoter (Szabo 1997; Kim 1999).
1.5.3 TH17 and Related Cytokines

A recent discovery of a new class of CD4+ T cells that produce IL-17 has been identified and designated as Th-17 cells (Cua 2003). This unique IL-17 producing subset has been implicated in promoting tissue inflammation and autoimmunity (Langrish 2005). Th-17 cells are characterized by the production of IL-17A, IL-17F, IL-6 and TNF-α (Langrish 2005; Steinman 2007; Tesmer 2008).

The two main cytokines that characterize Th-17 cells, namely IL-17A and IL-17F have a high degree of sequence homology and share biologic properties. IL-17A and F are themselves associated with pro-inflammatory and pro-fibrotic properties, and possess the ability to stimulate and recruit a broad range of cells (e.g. neutrophils, eosinophils and structural cells) and mediators (IL-1, IL-6, TNF-α, CXCL8, G-CSF, and GM-CSF) (Kolls 2004; Liang 2006; Ouyang 2008). Moreover, both IL-17A and F belong to a family of cytokines that includes IL-17B to E (Aggarwal 2002; Ouyang 2008; Tesmer 2008). These two cytokines signal through a multimeric receptor complex composed of two IL-17RA subunits and one IL-17RC subunit. It was reported in humans that both IL-17A and F had similar affinities for IL-17RC, whereas IL-17A had a much higher affinity than IL-17F for IL-17RA (Kuestner 2007; Shen 2008). The next group of cytokines expressed by Th-17 cells consists of IL-6 and TNF-α. Both of these cytokines are involved in inflammation, airway remodelling and the induction of the acute-phase reaction among other roles. Furthermore, IL-6 and TNF-α are typically found at elevated levels in BAL fluid and biopsy specimens from both allergic and asthmatic individuals (Keatings 1996; Babu 2004; Becknel 2005; Hamid 2005).

The regulation of Th-17 cells is controlled by various mediators at different regulatory levels. It was shown in vitro that the differentiation of IL-17 producing cells
from naïve T cells required only the combination of both IL-6 and TGF-β (Bettelli 2006; Veldhoen 2006), two cytokines that are increased in severe asthma. At the next maturation stage, it was demonstrated that IL-23 is the essential mediator for Th-17 proliferation and survival (Aggarwal 2003; Bettelli 2006; Steinman 2007). Although the second stage of maturation required only IL-23, the expression of IL-23 receptors (IL-23R) on the Th-17 cells are upregulated by IL-6 or the combination of IL-6 and TGF-β (Bettelli 2007). Hence, an inflammatory environment similar to that of severe asthma could induce the differentiation of Th-17 cells from naïve T cells and cause an upregulation in IL-23R expression. In contrast, IL-4 disrupts the IL-23 mediated proliferation and maintenance of Th-17 cells, as evident by IL-4 neutralizing experiments (Pflanz 2002; Harrington 2005). It was reported that one of the marker for Th-17 cells, CCR-6 was important in the development of an animal model of allergic respiratory diseases (Laan 1999; Lukacs 2001; Hirota 2007). Transcription factors are the other essential regulatory component in Th-17 subset development. This is seen by the recruitment of STAT3 and to a lesser extent STAT1 during the binding of IL-6 to its receptor; while phosphorylation of SMAD molecules occurs in response to TGF-β receptor binding. Additionally, IL-23 recruits both STAT3 and STAT4 during its signalling for the expansion of Th-17 cells (Batten 2006). It was reported that the combination of IL-6 and TGF-β leads to the upregulation of retinoic acid-related orphan nuclear hormone receptor-γt (RORγt) transcription factor that is important in the development of Th-17 cells (Korn 2007). RORγt was described to be expressed in IL-17 producing T cells present in the intestinal lamina propria and transduction of naïve T cells with the transcription factor induced IL-17 production. Moreover, IL-17A and IL-17F genes were shown to be directly transactivated by RORγt (Ivanov 2006). Thus,
RORγt is an important component in the differentiation of Th-17 cells, and is suggested to be used as a marker for this T cell subset (Ivanov 2006).

1.6 Rationale

Given that severe asthma pathogenesis is not well understood and that much of the affected population remains unresponsive or hyporesponsive to steroid treatments, this study is important in unveiling possible new effector molecules in this disease that may be targeted for therapy. It has been shown that severe asthmatics have a dysregulation in the Th-1/Th-2 cytokine expression, and proinflammatory and profibrotic properties of IL-17A and F implicate them in this disease. These cytokines as mentioned previously have the ability to induce other cells to express cytokines, chemokines and matrix metalloproteinases that can ultimately lead to severe asthma pathology. Moreover, both IL-17A and F are involved in neutrophil activities thereby implicating them in the pathogenesis of severe asthma. Thus, investigating the potential role of IL-17A and F in severe asthma is of important interest and may be of therapeutic value.

1.7 Hypothesis

We hypothesize that both IL-17A and F are present and are highly expressed in airway tissues of severe asthmatics compared to mild and moderate asthmatics as well as control subjects. We also hypothesize that their expression correlates to that of IL-8 and neutrophils.

1.8 Specific Objectives

We had several objectives: 1) To evaluate the expression of IL-17A and F protein and mRNA in biopsies of severe asthmatics; 2) to compare IL-17A and F expression in
severe asthmatics to mild, moderate asthmatics and control subjects; and 3) to correlate the expression of both IL-17A and F with neutrophil infiltration and IL-8 expression in these patients.
Chapter 2: Materials & Methods

2.1 Subject recruitment

Patients with well-defined moderate and severe asthma were selected based on the American Thoracic Society (ATS) workshop criteria for Refractory Asthma (ATS 2000; ATS 1986) and were recruited from the Montreal Chest Institute and Sacré Coeur Hospital. Patients with mild asthma (ATS 1987) and control subjects were also selected based on specific criteria described below and were recruited from the Montreal Chest Institute. Patient demographics are shown in Table 1. The protocols for the study were reviewed and approved by the Ethics Committees of both hospitals, and informed consent was obtained from all participating subjects.

Table 1: Subject demographics

<table>
<thead>
<tr>
<th>Groups</th>
<th>Severe (n = 15)</th>
<th>Moderate (n = 10)</th>
<th>Mild (n = 15)</th>
<th>Controls (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M:F)</td>
<td>8:7</td>
<td>4:6</td>
<td>7:8</td>
<td>6:9</td>
</tr>
<tr>
<td>Age (y)</td>
<td>44 (28-68)</td>
<td>40.5 (23-61)</td>
<td>24 (21-46)</td>
<td>28 (19-63)</td>
</tr>
<tr>
<td>Disease duration (y)</td>
<td>30 (20-60)</td>
<td>23 (5-40)</td>
<td>21 (6-40)</td>
<td>N/A</td>
</tr>
<tr>
<td>FEV₁ (L)</td>
<td>2.3 (1.01)</td>
<td>3.2 (0.7)</td>
<td>3.5 (0.6)</td>
<td>3.6 (1.1)</td>
</tr>
<tr>
<td>FEV₁% predicted</td>
<td>64.1 (21.8)</td>
<td>98.6 (13.8)</td>
<td>92.9 (14.1)</td>
<td>100.8 (12.8)</td>
</tr>
<tr>
<td>FVC (L)</td>
<td>3.4 (1.3)</td>
<td>4.2 (0.75)</td>
<td>4.4 (0.8)</td>
<td>4.6 (1.54)</td>
</tr>
<tr>
<td>FVC% predicted</td>
<td>75.9 (21.7)</td>
<td>104.7 (8.8)</td>
<td>98.6 (11.1)</td>
<td>104.5 (19.3)</td>
</tr>
<tr>
<td>FEV₁/FVC (%)</td>
<td>68.3 (7.6)</td>
<td>77.6 (10.9)</td>
<td>79.4 (14.1)</td>
<td>80.7 (7.3)</td>
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<tr>
<td>Groups*</td>
<td>Severe (n = 15)</td>
<td>Moderate (n = 10)</td>
<td>Mild (n = 15)</td>
<td>Controls (n = 15)</td>
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<td>---------</td>
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</tr>
<tr>
<td>Atopy (M:F)</td>
<td>8:7</td>
<td>2:4</td>
<td>7:8</td>
<td>1:3</td>
</tr>
</tbody>
</table>

M, Male; F, female. * Age and disease duration are shown as median (range). All other data are expressed as means ± SEM.

In order to be suitable for enrolment, patients with severe asthma had to fulfill at least 1 of 2 major criteria: (1) treatment with daily oral steroids for more than 50% of the previous 12 months; or (2) treatment with high-dose inhaled steroid (≥1000 μg fluticasone or equivalent per day) and at least 1 other add-on therapy (long-acting β2-agonist, leukotriene receptor antagonist, theophylline) continuously over the previous 12 months. In addition, 2 or more minor criteria were required: (1) daily use of short-acting β-agonist; (2) persistent airflow obstruction as documented by prebronchodilator FEV1 less than 70% and FEV1/FVC ratio less than 80% predicted; (3) at least 1 urgent care visit in the previous 12 months; (4) ≥3 steroid bursts in the previous 12 months; (5) prompt deterioration with less than 25% dose reduction of steroids; or (6) a near-fatal asthma event in the last 3 years.

Patients with moderate asthma were required to fulfill all of the following criteria: (1) well-controlled asthma on at least 200 μg/day of fluticasone or equivalent, but not exceeding 1000 μg/day, with or without the use of a long-acting β-agonist; (2) 2 or less steroid bursts in the previous 12 months and none in the past 3 months and the total number of days on oral steroids could not exceed 30 days in the previous 12 months; (3) FEV1 greater than 70% predicted and greater than 90% of personal best from the previous
2 years; and (4) no more than 1 nonscheduled urgent care or office visit in the previous 12 months.

Compliance with medication was assessed using the MDILog (Medtrac Technologies; Lakewood, Colo), an electronic device that was attached to each inhaler thereby permitting accurate monitoring of inhaler use. We included in the moderate and severe groups only those subjects who took at least the prescribed 4 inhalations of fluticasone and salmeterol (Advair 250/25 μg) per day on at least 70% of days in a month run-in period and were thus sufficiently compliant to be considered as having refractory asthma despite adequate treatment.

Patients with mild asthma had FEV\(_1\) readings greater than or equal to 70% postbronchodilator and required infrequent use of inhaled short-acting β\(_2\)-agonist medication alone. It was necessary for each patient with asthma to display either a 12% or greater improvement in FEV\(_1\), with an absolute increase of at least 200 mL from baseline, after administration of a β-adrenergic agonist or a PC\(_{20}\) of less than or equal to 8 mg/mL of methacholine.

Control subjects were healthy nonsmokers without history of respiratory disease or symptoms suggestive of asthma. They had normal spirometry and were well at time of recruitment.

Exclusion criteria included chronic obstructive pulmonary disease, the presence of any other known pulmonary disease, HIV, metastatic cancer, congestive heart failure, or any major comorbid disease that might affect asthma disease activity. It was necessary for all patients with asthma to be free from respiratory tract infection for at least 1 month before enrolment in the study. All subjects had pulmonary function tests and skin prick allergen tests performed before bronchoscopy.
2.2 Immunocytochemistry

To detect cytoplasmic protein expression of IL-17A and F in bronchial biopsies, bronchoscopy was performed under light sedation. Six endoscopic bronchial biopsies were obtained from the right lung at various segmental and subsegmental carinae using a biopsy forceps with a fenestrated cup (Olympus biopsy forceps 35C; Olympus Medical Systems Corp). Tissues were fixed in formalin and embedded in paraffin before 5-µm-thick sections were cut on a microtome. Following resection of the biopsies and preparation of tissue slides, immunocytochemistry technique was performed. Briefly, sections were deparaffinized in xylene, rehydrated through a graded alcohol series, and then washed in PBS. Antigen retrieval was performed for slides to be stained with IL-17F antibody (Ab) by boiling the sections in a 0.01 M EDTA buffer (pH 8.0) for 3 min. Sections were cooled in tap water then permeabilized with Triton (0.2% in PBS) and hydrogen peroxide (5% in PBS). The sections were then washed three times for 5 min in PBS followed by incubation with a universal blocking solution (DakoCytomation) for 20 min. Tissue sections were immunostained at 4°C overnight with polyclonal goat antihuman IL-17A or IL-17F Abs (R&D Systems, Minneapolis, MN) at a concentration of 0.83 or 0.33 µg/mL respectively. To confirm specificity of the IL-17 Abs, tissue sections were also stained with corresponding goat IgG isotype control Ab. Next day, the secondary Ab raised in rabbit (DakoCytomation) was applied at concentrations of 1:100 for 45 minutes at room temperature. The immunostains were developed using horseradish peroxidase (HRP) and diaminobenzidine (DAB) substrate as per the manufacturer’s instructions (DakoCytomation). Sections were counterstained with hematoxylin and mounted. Slides were examined and images were acquired by a BX51 Olympus.
microscope attached to a CoolSNAP-Pro color digital camera (Carsen Group) using the Image Pro-plus 4.0 system (Media Cybernetics).

To detect cytoplasmic protein expression of IL-17A and F in neutrophils, cells were first isolated and purified from venous blood then cytospun on slides (see section 2.5 Blood & Neutrophil isolation). Briefly, slides were washed twice in PBS and incubated with a universal blocking solution (DakoCytomation) for 15 min. Cytospun samples were immunostained at 4°C overnight with polyclonal goat antihuman IL-17A or IL-17F Abs (R&D Systems, Minneapolis, MN) at a concentration of 1:200 and 1:250 respectively. To confirm specificity of the IL-17 Abs, corresponding goat IgG isotype controls were also performed. Next day, secondary Ab raised in rabbit (DakoCytomation) was applied at concentrations of 1:100 for 45 minutes at room temperature. The immunostains were developed using HRP and DAB substrate as per the manufacturer’s instructions (DakoCytomation). Sections were counterstained with hematoxylin and mounted. Slides were examined and images were acquired as described above.

2.3 RNA extraction and Reverse Transcription-PCR

Total cellular RNA was isolated by using RNeasy micro kit (for homogenized frozen human bronchial biopsy tissues and LCM epithelial cells) or mini kit (for purified peripheral blood neutrophils) extraction columns (Qiagen) following the manufacturer’s instructions. RNA was eluted in 35 µl nuclease-free water, and cDNA was generated by reverse transcription in a 40 µl reaction, using 0.5 µg of total RNA as template, oligo(dT)12–18 primers, and Superscript II reverse transcriptase, in the presence of Rnase inhibitor (all from Invitrogen Life Technologies).
2.4 Real Time-Polymerase Chain Reaction

Quantification of mRNA expression for IL-17A, IL-17F, IL-8 and Neutrophil (NGAL- Neutrophil gelatinase-associated lipocalin) in bronchial biopsies was performed by real-time polymerase chain reaction (RT-PCR) using the LightCycler (Roche Diagnostics). Quantification of mRNA expression for IL-17A and F in purified peripheral blood neutrophils was performed using the 7500 Fast RT-PCR system (AB Applied Biosystems). All primers were designed using the Primer3 software (Whitehead Institute for Biomedical Research) and synthesized by Invitrogen Life Technologies.

IL-17A - forward, 5’CAT CCA TAA CCG GAA TAC CAA TA;

IL-17A – reverse, 5’TAG TCC ACG TTC CCA TCA GC.

IL-17F – forward, 5’GTG CCA GGA GGT AGT ATG AAG C;

IL-17F – reverse, 5’ATG TCT TCC TTT CCT TGA GCA TT.

IL-8 – forward, 5’GCC AGA TGC AAT ACA AGA TTC C;

IL-8 – reverse, 5’ATT CTC CCG TGC AAT ATC TAG G.

NGAL – forward, 5’GTC ACC TCC GTC CTG TTT AGG;

NGAL – reverse, 5’ATG CTG GTT GTA GTT GGT GCT.

GAPDH – forward, 5’AGT CAA CGG ATT TGG TCG TAT T;

GAPDH – reverse, 5’ATG GGT GGA ATC ATA TTG GAA C.
The sizes of the RT-PCR products for IL-17A and F were 174 and 200 base pairs respectively, which were confirmed visually by 1% agarose gel containing 0.2 µg/ml ethidium bromide. The PCR protocol consisted of three programs: denaturation, amplification, and melting curve analysis for product identification. The denaturation and amplification conditions for both IL-17A and F were 95°C for 15 min followed by 50 cycles of PCR. Each cycle included denaturation at 95°C for 10 s, annealing of 30 s at 60°C and extension of 20 s at 72°C. Fluorescence was measured at the end of every cycle to allow quantification of cDNA.

2.5 Blood Processing & Neutrophil isolation

Venous peripheral blood was used to isolate polymorphonuclear leukocytes (PMNL) using Polymorphprep separation media (AXIS-SHIELD PoC AS). Briefly, 5.0 ml of blood was carefully layered over 5.0 ml of separation media and centrifuged at 500 relative centrifugal force (RCF) for 35 min with the centrifuge brake disabled. This allowed the blood to separate out into 6 distinct bands: plasma, monocytes, isolation media, PMNL, more isolation media, and the red blood cells. The top three layers were discarded and the layer of PMNL and all of the isolation media beneath were transferred into a clean centrifuge tube. The PMNL fraction was then washed twice with 1% bovine fetal serum in PBS and centrifuged at 250 RCF for 5 min. Red blood cell (RBC) lysis buffer (Roche Diagnostics) was then added to the pellet and incubated for 5 min before another wash and centrifugation was performed. Following this an additional wash was performed to insure all the residual RBCs were removed and that the pellet only contained PMNL. The cells were then purified using MACS separation LS columns using human CD16 MicroBeads (Miltenyi Biotec) according to the manufacturer’s protocol to
obtain a pure neutrophil population. Cells were then washed twice previous to fixation and permeabilization using BD Cytofix/Cytoperm (BD Biosciences) performed for 15 min on ice. The cells were then washed once with 1X Perm/Wash solution (BD Biosciences) followed by a wash with PBS. Based on the differential count, the purity of our samples were above 95%. To prepare for immunocytochemistry experiments, purified neutrophils were cytopun on slides using Shandon cytopsin 3 centrifuge (Thermo Scientific) by adding 65 µl of 0.5 million neutrophils per ml for each slide. For RNA extraction experiments, aliquot tubes of 5 and 10 million neutrophils were prepared by adding 350 µl of RLT lysis buffer (Qiagen) and stored at -80°C until later usage.

2.6 Laser Capture Microdissection

To confirm the expression of IL-17F in epithelial cells, laser capture microdissection was performed on frozen bronchial biopsy slides. The slides were obtained from the Tissue Bank (Montreal Chest Institute/Meakins-Christie Tissue Bank, McGill University). The slides were stained with H&E, rinsed in an ethanol gradient, and dehydrated in a mixture of xylenes. The epithelial cells were carefully captured using the Pixcell laser capture microscope (Arcturus). Approximately 1200 to 2700 laser pulses were applied to capture these cells. During this process, cellular material was transferred to CapSure HS LCM Caps (Arcturus Bioscience) and digested in RLT lysis buffer (Qiagen). RNA was extracted using the RNeasy micro kit (Qiagen) following the manufacturer’s instruction. mRNA was eluted in 12 µl of water. Reverse transcription followed by RT-PCR was performed as described above to quantify the mRNA levels of IL-17F. The PCR products were then visualized on a 1% agarose gel containing 0.2 µg/ml ethidium bromide.
2.7 Statistics

All values are reported as means ± SEM. Single (1-way) ANOVA was used to analyze differences between the groups followed by either Bonferroni’s or Dunn’s multiple comparison tests. A difference of p less than or equal to 0.05 was considered to be statistically significant.
Chapter 3: Results

3.1 Expression of IL-17A & F Proteins in Airway Subepithelial Layer

To detect the protein expression of Th-17 related cytokines in lung tissue, immunocytochemistry was performed on 10 samples from each group of mild, moderate and severe asthma patients as well as 10 healthy control subjects. Staining was carried out using specific antibodies for IL-17A and F, and to ensure specific staining, negative control goat IgG isotype staining was also included. Both IL-17A and F staining were observed in the bronchial tissue, and were mostly present in inflammatory infiltrates in the subepithelial compartment (figure 3). Some immunoreactivity for IL-17F was also present in the epithelium (see section 3.2)

IL-17A and F positive cells infiltrating the mucosa were subsequently counted and expressed as the number of positive cells per mm$^2$ of tissue. IL-17A positive cells were significantly elevated in the severe asthma patients compared to all other groups. There was also an increase in these cells in moderate but not in mild asthmatics compared to healthy control subjects. In contrast, the IL-17F positive cells infiltrating the mucosa were shown to be only significantly increased in the severe asthmatics compared to the mild asthma and healthy control groups (figure 4).
Figure 3: Representative Immunocytochemistry staining for IL-17A and F in biopsies of asthmatic patients and control subjects.
IL-17A and F staining 200X magnification from patients with mild asthma are presented as B and E, respectively; severe asthma as C and F, respectively; and negative control Gt IgG isotype staining as A and D, respectively.
Figure 4: Increased IL-17A- and IL-17F- immunoreactivity in lung tissue sections (biopsies) from patients with severe asthma.

The numbers of IL-17A and IL-17F-immunoreactive cells are expressed as the no. of cells per mm2 of subepithelial surface (A and B, respectively). * indicate p<0.05 (ANOVA, Dunn’s multiple comparison tests). N=10 for each group.
3.2 Expression of IL-17F Protein in Airway Epithelial cells

In addition to being expressed in the bronchial mucosa subepithelium, immunocytochemistry results showed that IL-17F but not IL-17A was uniquely expressed in airway epithelium. Epithelial expression of IL-17F was assessed by blinded scorers, using a scoring technique based on the percentage of positive epithelium. The intensity of IL-17F immunostaining was evaluated and giving a grade that range from 0 to 8, with a grade of 0 corresponding to 0% of stained epithelium and a grade of 8 equivalent to a 100% stained epithelium. In this manner, IL-17F immunoreactivity in the epithelium was shown to be significantly higher in severe asthmatics compared to mild asthma patients and healthy control subjects. In addition, moderate asthmatics also had significantly elevated expression compared to healthy controls (figure 5).
Figure 5: IL-17F immunoreactivity in airway epithelium.
Intensity of IL-17F immunostaining was graded by blinded scorers and expressed as scores of stained epithelium ranging from 0 to 8 (0 = no staining; 8 = 100% staining) (A). Representative images of IL-17F immunoreactivity in bronchial epithelium from normal control (B) and from severe asthma patient (C). * indicate p<0.05 (ANOVA, Dunn’s multiple comparison tests). N=10 for each group.
To confirm the expression of IL-17F in airway epithelial cells, laser capture microdissection was performed on 3 severe asthmatics and 3 healthy controls. Using frozen bronchial biopsy sections, epithelial cells were laser captured and RNA content was extracted, reversed-transcribed and used for subsequent RT-PCR analysis. A specific signal for IL-17F was obtained by RT-PCR in all severe asthmatic and healthy control samples, which was absent in the negative control sample. Gel electrophoresis was also carried out to further check the specificity of the RT-PCR amplified products. Specific bands from amplified cDNA products corresponded with that of reported 200 base pair size of IL-17F (figure 6).
Figure 6: IL-17F expression in isolated epithelial layer.
Representative photomicrographs of laser capture microdissected (LCM) epithelial cells from frozen bronchial tissue section of a patient with severe asthma (A). RNA was extracted from excised cells and then converted into cDNA by reverse-transcription PCR. Specific amplification of IL-17F was obtained from all LCM epithelial cells and positive control cDNA samples (B). Agarose gel showing mRNA detection of IL-17F in bronchial epithelial cells (C). PCR product of IL-17F was 200 bp. Lane 1 = negative control; lane 2 = positive control peripheral blood mononuclear cells (PBMC); lane 3 = severe asthma patient (SAP) #1 epithelium; lane 4 = ladder; lanes 5 = severe asthma patient #2 epithelium; lane 6 and 7 = healthy subject (Subj) #1 and #2 epithelium, respectively. N = 3 for each severe asthma and healthy control group.
3.3 Expression of IL-17A and F, IL-8 and Neutrophil marker mRNA in Airway Biopsies

To confirm the immunocytochemistry results, RT-PCR was performed on RNA extracted from bronchial biopsies from 5 cases per group using specific primers for IL-17A and F. IL-17A mRNA level was significantly increased in the severe asthmatic patients compared to healthy control. Similarly, IL-17F mRNA was also significantly elevated in severe asthmatics compared to both mild asthmatics and healthy control subjects (figure 7).

In order to correlate IL-17A and F expression with that of IL-8 and neutrophils, RT-PCR analysis was also performed on the same samples as above using specific primers for both IL-8 and neutrophils. IL-8 mRNA expression was significantly increased in severe asthmatics compared to control healthy subjects. Neutrophil expression was determined using a neutrophil marker, i.e. neutrophil gelatinase-associated lipocalin (NGAL). Similar to IL-8, NGAL expression was only significantly elevated in severe asthmatics compared to control healthy subjects (figure 8). We did not have a direct correlation between IL-17A and F expression and IL-8 and NGAL; however we observed an expression trend between these four cytokines and the three groups. Both IL-17A and F were significantly increased in severe asthma group as well as IL-8 and NGAL. Moreover, they had the same expression tendency between mild asthma and control healthy groups.
Figure 7: Quantitative RT-PCR analysis of IL-17A and IL-17F mRNA extracted from airway biopsies obtained from mild and severe asthmatics and control subjects. Increased IL-17A and IL-17F mRNA expression in severe asthmatics compared to other groups (A and B, respectively). * indicate p<0.05 (ANOVA, Bonferroni’s multiple comparison tests). N=5 for each group.
Figure 8: Quantitative RT-PCR analysis of IL-8 and NGAL (neutrophil marker) mRNA extracted from airway biopsies obtained from mild and severe asthmatics and control subjects. Increased IL-8 and NGAL mRNA expression in severe asthmatics compared to control group (A and B, respectively). * indicate p<0.05 (ANOVA, Bonferroni’s multiple comparison tests). N=5 for each group. Neutrophil gelatinase-associated lipocalin (NGAL).
3.4 Expression of IL-17A & F Proteins & mRNA in Human Neutrophils

We suspected that polymorphonuclear leukocytes (PMNL) may be immunoreactive source for IL-17A and/or F as evident in positive staining of these cells in the capillaries within bronchial tissue sections. For this reason, we decided to investigate the potential expression of these cytokines in neutrophils at both the protein and mRNA level. PMNL were separated from venous blood from 5 severe asthmatics, 5 mild asthmatics and 5 control subjects. The purity of PMNL samples was determined by differential count to equal 95% or more (see figure 9).

Immunocytochemistry results shows that a small percentage (approximately 5%) of neutrophils expresses IL-17A and/or F proteins (figure 9). The percentage of neutrophils positive for IL-17A and/or F proteins was not significantly different between groups. Results from RT-PCR confirm the expression of both IL-17A and F by PMNL. More specifically, IL-17A mRNA expression in PMNL of severe asthmatics was elevated, however did not reach statistical significance compared to mild asthma patients and control subjects. In contrast, IL-17F mRNA expression was significantly increased in severe asthmatic’s PMNL compared to control subjects (figure 10).
Figure 9: Expression of IL-17A and F in blood neutrophils of mild and severe asthmatics and control subjects.
H&E staining on purified blood neutrophils, with calculated sample purity ≥ 95% (A). Representative immunocytochemistry stainings for IL-17A and F in severe asthma neutrophil samples (B). IL-17A and F expressed in a proportion of stained neutrophils 200X magnification are presented as I and III, and 400X magnification as II and IV, respectively. Hematoxylin and eosin (H&E)
Figure 10: Quantitative RT-PCR analysis of IL-17A and IL-17F mRNA extracted from isolated purified neutrophil blood obtained from mild and severe asthma patients as well as control subjects. IL-17A mRNA expression is elevated in severe asthma group but not significantly different compared to mild asthma and control groups (A). IL-17F mRNA expression is significantly increased in severe asthmatics compared to control subjects (B). * indicate p<0.05 (ANOVA, Bonferroni’s multiple comparison tests). N=5 for each group.
Chapter 4: Discussion

The aim of this study was to investigate the expression of Th-17 associated cytokines (IL-17A and F) in airways of severe asthma patients. This thesis describes for the first time the expression of both IL-17A and F cytokines in airway biopsies of patients with asthma of different severity and healthy individuals. Both IL-17A and F were expressed in the mucosa and submucosa of the airway walls. IL-17A and F are critical cytokines involved in inflammatory and autoimmune diseases. Both IL-17A and F possess proinflammatory and profibrotic properties that can induce tissue damage and airway remodelling.

The immunoreactivity for both IL-17A and F was observed in the subepithelium compartment of the airway wall, within clusters of inflammatory cells. Morphologically, most of the inflammatory infiltrates positive for these cytokines appear to be of mononuclear cell origin. This finding is in agreement with others describing T cells and monocytes to be the major source of IL-17A and F (Starnes 2001; Lockhart 2006; Rachitskaya 2008). IL-17F but not IL-17A was also expressed by epithelial cells. This epithelial expression of IL-17F is novel, however not surprising as the epithelium is a major source of many mediators and is an important component of asthma pathology.

We have also observed in some of our ICC staining, IL-17A and F immunoreactivity in smooth muscle bundles. Other members of our laboratory have recently confirmed this phenomenon and are currently investigating the effects of these cytokines on SMC function. The potential effects of IL-17A and F on SMC migration could be an important component in airway remodelling, especially for severe asthma
(personal communication). These cytokines can promote directly or indirectly airway remodelling as they possess remodelling properties, or they can induce other cells to express potent remodelling mediators. Importantly, IL-17A and F may be involved in airflow obstruction observed in asthmatics. A recent study shows the important migratory effect of IL-17 on vascular SMC and its implication in post-angioplastics restenosis (Cheng 2009).

The expressions of both IL-17A and F in airway subepithelium were also compared between asthmatics and healthy subjects. We observed that severe asthma patients express at an elevated level both IL-17A and F compared to other groups. Our results are in parallel with other studies describing an increase level of Th-17 associated cytokines in lung tissues, BAL fluid, sputum and peripheral blood from asthmatics of different severity (Molet 2001; Barczyk 2003; Sun 2005; Bullens 2006; Zhao 2009). More specifically, IL-17A positive cells in airway subepithelium were significantly increased in severe asthma compared to mild asthma and healthy controls. In addition, moderate asthmatics also had a significantly elevated level of IL-17A positive cells compared to healthy controls. IL-17F positive cells were significantly increased only in severe asthma patients compared to mild asthma and healthy subjects. This is interesting as it suggests that IL-17A and F increases might be correlated to disease severity. The suggestion that both these cytokines are present in the more exacerbated disease form is supported by a recent study showing an increased presence of IL-17A and F in complicated, in contrast to intact, atherosclerotic plaques (de Boer 2010).

The expression of IL-17F in airway epithelium was assessed based on the intensity and percentage of stained epithelium. A blinded scorer assigned a grade between
0 representing 0% of stained epithelium, and 8 representing 100% of stained epithelium. In this way, we were able to semi-quantitatively compare the IL-17F epithelial expression between groups. Severe asthmatics had the most intense epithelial expression compared to mild asthmatics and healthy controls. Moderate asthmatics also showed a much higher IL-17F epithelial intensity staining compared to healthy subjects. This method of assessment has been considered to be valid among researchers (Giaid 1993; Hamid 1993; Prefontaine 2010). To confirm IL-17F epithelial expression, we performed laser capture microdissection. This technique was very useful in confirming our result. LCM provides a tool to isolate cells from sections and examine the mRNA specifically. Subsequent to epithelial cell excision, RNA is extracted and converted to cDNA which is later used as a template for amplification of housekeeping gene GAPDH and IL-17F specific mRNA by RT-PCR. We obtained a specific signal for IL-17F expression in mRNA isolated from both patients and healthy subjects.

We have also performed RT-PCR analysis on frozen bronchial biopsies obtained from mild and severe asthmatics as well as healthy controls. RT-PCR results confirmed the ICC results and demonstrated a significant increase in IL-17A and F mRNA expression in severe asthma patients. It was reported that airway neutrophilia is enhanced in a subset of asthma patients and that the degree of neutrophil infiltration correlates with asthma severity (Wenzel 1997; Louis 2000). We therefore assessed the mRNA expression for both IL-8 and neutrophils in the same samples. Indeed, we have a significant increase in IL-8 and that of the neutrophil marker (NGAL) mRNA expression in severe asthmatics compared to healthy controls. IL-17A and F mRNA expression correlates with that of IL-8 and neutrophil mRNA expression in all three groups. These findings are in agreement
with those of Bullens et al. demonstrating increased IL-17 mRNA expression in airway cells from asthmatics and their correlation with IL-8 expression and the number of neutrophils (Bullens 2006). IL-17A and F seem to contribute to neutrophilic accumulation, which is in accordance with their biological activity regulating growth factors and chemokines important for the recruitment and survival of neutrophils (Sun 2005). Perhaps these cytokines are involved in either a direct or indirect chemoattraction of neutrophils in these patients via regulation of IL-8.

Despite being a semi-quantitative technique, immunocytochemistry is considered to be a valuable detection method that can identify the exact location of a given protein within an examined tissue. Although the specificity of antibody is guaranteed by the manufacturer, cross-reaction with other proteins may occur, and thus can lead to false results. To further ensure the specificity of the signal, control experiments are performed including absorption staining test that we performed for each antibody used. Real-Time PCR is a valuable tool that allows for the specific quantification of mRNA levels. Although, mRNA levels do not always necessarily correlate with protein levels, it can nevertheless provide useful information about the amounts of these proteins. Furthermore, RT-PCR can quantify mRNA levels from a relatively small number of cells. The sensitivity of this system increases the possibility sample contamination and therefore can also lead to false results. For this reason, we took special caution during sample handling and experiment preparation. LCM is a simple one step transfer procedure technique that allows for the precise acquirement of a large number of specific cells from a given sample, once preparation conditions are optimized. Its major advantage includes the preservation of the morphology and the chemistry of the sample collected. LCM system
provides additional benefits, including the immediate viewing and storage of images on the computer and the visualization of the transfer film after capture. The main disadvantage of LCM is that optical resolution is reduced due to the use of air-dried tissue sections, thus rendering cell identification difficult. We had an expert in the LCM technique that supervised during our experiments as well as a senior pathologist for consultation.

As described, most of the inflammatory immunoreactive cells for IL-17A and F observed in the airway subepithelial compartment were of mononuclear type. Nevertheless some polymorphonuclear cells in the capillaries and a few infiltrating the submucosa were also observed to be immuno-positive for these cytokines. For this reason, we decided to investigate the potential expression of IL-17A and F in neutrophils. It was previously demonstrated that IL-17A can be expressed in asthmatic eosinophils (Molet 2001), however no study so far has examined both IL-17A and F in neutrophils isolated from asthmatics. However, our ICC results demonstrate that not all neutrophils, but rather a subpopulation of neutrophils can express IL-17A and F. We did not obtain any statistical difference in the percentage of IL-17A or F immunoreactive neutrophils between the compared groups. This may be due to the small sample size in this experiment. Our RT-PCR results confirm the expression of IL-17A and F in neutrophils and revealed that IL-17F, but not IL-17A mRNA was significantly increased in neutrophils from patients with severe asthma compared to healthy controls. The possibility that a neutrophil can express IL-17A and IL-17F simultaneously is yet to be determined. Flow cytometry and confocal microscopy experiments examining the intracellular protein expression of neutrophils can be utilized to demonstrate co-
localization of IL-17A and F. Moreover, we hypothesize that the expression of these two proinflammatory pleiotropic cytokines by neutrophils may contribute to the steroid resistance phenomenon observed in severe asthma. It is possible that most asthmatic patients that are steroid resistant are of the highly IL-17A and/or F expressing neutrophil phenotype (Hamilos 2001).

Corticosteroids are potent anti-inflammatory mediators, widely used in the asthmatic population to control asthma symptoms. Studies from both human and animal models provides supporting evidence that steroid treatments can inhibit several cytokines, as well as their induced action on structural cells (Prause 2003; Herbert 2008; Hu 2009). However, not all patients may benefit from this type of treatment. Murine model experiments have provided evidence that steroids are not successful in limiting Th-17 cell-mediated airway inflammation and AHR (McKinley 2008). This is in accordance with a recent study which demonstrates that steroids are ineffective in human subjects to reduce IL-17 activity in contrast to immunosuppressive agents (Vanaudenaerde 2007). Various factors are implicated in the steroid resistance phenomenon observed in asthmatics, and these include 1) presence of fixed airway resistance (i.e. airway remodelling), 2) molecular mechanisms of steroid resistance, (Barnes 2004; Adecock 2008), 3) a steroid-insensitive form of inflammation (Wenzel 1997) or 4) distal lung inflammation not accessible to inhaled medications (Balzar 2005). Whether steroids exert their therapeuetic effect through regulating cytokine production in asthma or through other processes is important to investigate. It is possible that IL-17 might induce steroid hyporesponsiveness through its interaction with histone deacetylation or induction of
glucocorticosteroid receptor-β isotype in asthmatic lung. These mechanisms are being investigated by a number of groups in human subjects.

In conclusion, this thesis present a solid body of evidence for the expression of Th-17 associated cytokines (IL-17A and F) in severe asthma and suggests that this new subset of cytokines might be involved in the activation and accumulation of neutrophils as well as the severe remodelling process. It also opens a new option to study the effect of these cytokines in steroid responsiveness. If IL-17 proves to be critical, a strategy to target these cytokines might be very helpful in controlling the progression of the disease.
Chapter 5: Future Directions

Our detection of IL-17A and F protein and mRNA expression in severe asthma is novel. The increase of these cytokines in severe asthma patients compared to milder asthmatics and healthy controls indicates an important role for IL-17A and F in asthma pathogenesis. Interestingly, we also observed the expression of both IL-17A and F in neutrophils from asthmatics. An important interaction may be envisaged between IL-17A and F and neutrophils in severe asthma pathology. The signalling pathways by which these cytokines confer their action in asthma still require elucidation. Therefore, investigating the mechanism behind IL-17A and F action in asthma is important to the understanding of the disease as well as of therapeutic interest. The implication of these two cytokines to steroid resistance is also an important matter to explore. Understanding the pathways that these cytokines signal through and how they interact with other cytokines and cells as well as steroids is important to the understanding of the pathophysiology of severe asthma.
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APPENDIX 1.

Ethical approval for the use of human bronchial tissues and blood samples for this project. Documents attached.