Expression and function of chemokine receptors on airway smooth muscle cells

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Abstract

Asthma is a respiratory disease that affects 2.5-3 million Canadians. This condition is characterized by a Th2-driven immune response that implicates the infiltration of eosinophils and remodelling of the airways. In the last decade, airway smooth muscle cells (ASMC) have became the subject of intense research in the field of inflammatory lung diseases including asthma. It is known that ASMC respond to a wide variety of inflammatory mediators such as cytokines and chemokines. Function of ASMC in the context of asthma has extended beyond its traditional role of a structural cell. Indeed, it is believed that they can participate in the initiation and the perpetuation of the inflammatory response that takes place in the airway of asthmatic subjects. The general aim of this work was to investigate the role of ASMC in the pathogenesis of asthma. More specifically, we studied the expression of two C-C chemokine receptors, CCR3 and CCR1 in the context of asthma.

For the first time, this work describes the expression of chemokine receptors by ASMC. We have shown that eotaxin, an important chemokine in asthma, induces migration of ASMC through the activation of CCR3. Although CCR3 expression is not regulated by Th2 cytokines *in vitro*, ASMC isolated from asthmatic patients expressed intrinsically higher levels of the surface receptor when compared to controls. We also describe the expression of CCR1 by ASMC, a receptor involved in airway remodelling in an animal model of asthma. We reported the expression of CCR1 mRNA in biopsies obtained from mild, moderate and severe asthmatics and showed that mild group express the highest level of CCR1. We also

confirmed that ASMC express the receptor *in vivo* and showed that stimulation of this receptor with its ligands induces intra-cellular calcium mobilization, which confirms its functionality. Regulation of CCR1 on ASMC was also assessed using proinflammatory, Th1 and Th2 cytokines. We found that TNF-α and to a lesser extent, IFN-γ, upregulated CCR1 mRNA and protein expression, while Th2 cytokines had no effect. The effect of these two cytokines was totally suppressed by either dexamethasone or mithramycin.

Collectively, our results demonstrate the expression of functional C-C chemokine receptors by ASMC. Interestingly, we have shown that CCR3 activation mediates ASMC migration and provides a new possible mechanism for the increased smooth muscle mass observed in asthmatic patients. Although the exact function of the CCR1 expressed by ASMC is unknown, our results suggest an involvement in asthma pathogenesis, possibly through airway remodelling.

Sommaire

L'asthme est une maladie respiratoire qui affecte 2.5-3 millions de canadiens et qui semble en constante progression. Elle est caractérisée par une réponse immunitaire à profil Th2, associée à un remodellage des voies respiratoires et une infiltration d'éosinophiles. Au cours de la dernière décennie, un intérêt grandissant s'est manifesté envers les cellules musculaires lisses respiratoires (CMLR), particulièrement dans le contexte de certaines maladies inflammatoires, dont l'asthme. Il est maintenu reconnu que ces cellules peuvent produire une grande variété de médiateurs inflammatoires, incluant des cytokines et des chimiokines, ainsi qu'une vaste panoplie de récepteurs. La perception du rôle des CMLR dans la pathologénie de l'asthme a évolué considérablement au cours des dernières années. La fonction de cette cellule est davantage que la stricte cellule structurale chez qui l'on se limitait à étudier les propriétés contractiles. Il semble maintenant évident qu'elle participe à l'initiation et au maintien de la réaction inflammatoire qui survient dans les voies respiratoires des patients lors de la survenu d'une crise d'asthme. L'objectif général de cette thèse consistait à investiguer le rôle que joue la CMLR dans la réaction inflammatoire asthmatique. Plus spécifiquement, nous avons étudié l'expression des récepteurs aux chimiokines CCR1 et CCR3 par cette cellule, dans le contexte de l'asthme.

Cette thèse rapporte pour la première fois l'expression de récepteurs aux chimiokines chez les CMLR. Nous avons démontré que l'éotaxin, une chimiokine de type C-C très importante dans la pathogénie de l'asthme, induit la migration des CMLR par l'activation du CCR3. Bien que nos travaux ont démontré que les cytokines de type Th2 n'avaient aucune effet sur l'expression du récepteur *in*

vitro, nous avons observé que le TNF-α augmentait la présence du CCR3 à la surface des cellules. De plus, nous avons observé que les CMLR isolés à partir de biopsies obtenues chez des patients asthmatiques exprimaient davantage du récepteur que celles des patients non affectés par la maladie.

Dans le cadre de cette thèse, nous avons aussi démontré l'expression du CCR1 par les CMLR. Par le biais d'études réalisées chez les modèles expérimentaux d'asthma, il a été démontré que ce récepteur semblait impliqué dans le remodellage des voies respiratoires survenant chez les asthmatiques. Nous avons comparé l'expression de l'ARNm codant pour le CCR1 dans des biopsies obtenues chez des patients contrôles et chez des asthmatiques légers, modérés et sévères. Bien que tous les groupes d'asthmatiques démontraient des niveaux supérieurs d'ARNm que chez le groupe contrôle, le groupe exprimant le plus grand niveau de CCR1 était surprenamment le groupe d'asthmatiques légers. Afin de prouver l'expression du récepteur *in vivo,* nous avons confirmé sa présence dans les voies respiratoires humaines. La fonctionalité du récepteur a aussi été prouvé par l'induction de calcium intracellulaire suite à l'administration de deux ligands du CCR1. Nous avons aussi observé que le TNF-α et, à un niveau moindre, l'IFN-γ, augmentaient l'expression du CCR1. Par l'utilisation de dexamethasone et de mythramycine, il était possible d'inhibier totalement cet effet des deux cytokines, fournissant ainsi une explication possible aux résultats obtenus avec les biopsies d'asthmatiques.

En conclusion, nos résultats démontrent l'expression de deux récepteurs aux chimiokines fonctionels chez les CMLR, soit le CCR1 et le CCR3. Alors que rôle

du CCR1 chez les asthmatiques demeure incertain, celui du CCR3 pourrait être lié à l'augmentation de la masse musculaire lisse typiquement retrouvée chez gens souffrant de cette maladie.

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1. **Joubert P**, Cordeau ME, Boyer A, Silversides DW, Lavoie JP. Cytokine expression by peripheral blood neutrophils from heaves-affected horses before and after allergen challenge. The Veterinary Journal 2007; In press.

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Contributions of authors

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List of abbreviations

AC:	Adenylate cyclase
ADAM:	A disintegrin and metalloprotease
AHR:	Airway hyperresponsiveness
cAMP:	cyclic adenosine monophosphate
ASMC:	Airway smooth muscle cell
BAL:	Bronchoalveolar lavage
BSA:	Bovine serum albumin
B/TSMC:	Bronchial/tracheal smooth muscle cell
CCR:	C-C chemokine receptor
CD:	Cluster of differentiation
CK:	chemokinesis
CMLR:	Cellules musculaire lisses respiratoires
COPD:	Chronic obstructive pulmonary disease
CREB:	cAMP response element binding
CRTH2:	chemoattractant receptor-homologous molecule expressed on Th2
DMEM:	Dulbecco's modified Eagle's medium
DNA:	Desoxyribonucleic acid
ECM:	Extracellular matrix
ECMP:	Extracellular matrix protein
EDTA:	Ethylenediaminetetraacetic acid
EGF:	Epidermal growth factor
ERK:	Extracellular signal-regulated kinase
FACS:	Fluorescence activated cell sorter
FBS:	Fetal bovine serum
FEV1:	Forced expiratory volume in one second
FGF:	Fibroblast growth factor
FITC:	Fluorescein isothiocyanate
FSC:	Forward scattered
GPCR:	G-protein-coupled receptor
GM-CSF:	Granulocyte/Monocyte colony stimulating factor

HBSS:	Hank's buffered salt solution
IL:	Interleukin
IFN:	Interferon
IGF:	Insulin growth factor
JNK:	c-Jun kinase
LT:	Leukotriene
MAP:	Mitogen activated protein
MCP:	Monocyte chemoattractant protein
MMP:	Matrix metalloproteinase
NF:	Nuclear factor
OVA:	Ovalbumine
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction
PDGF:	Platelet-derived growth factor
PE:	Phycoerythrin
PK:	Protein kinase
QPCR:	Quantitative PCR
RANTES:	Regulated upon activation normal T cells expressed and secreted
RNA:	Ribonucleic acid
ROCK:	Rho-associated coil forming protein kinase
RT:	Room temperature
RT-PCR:	Reverse transcription PCR
Shh:	Sonic hedgehog
SSC:	Side scattered
STAT:	Signal transducers and activation of transcription
Th:	T helper
TNF:	Tumor necrosis factor
TGF:	Transforming growth factor
VEGF:	Vascular endothelial growth factor
VSMC:	Vascular smooth muscle cell

CHAPTER I: INTRODUCTION

1.0 Definition of asthma

Asthma is a disease of the respiratory system which generally appears during childhood, but can also develop later on in life. It was first described 3500 years ago in an Egyptian manuscript called Ebers Papyrus. The word is of Greek origin and literally means «to exhale with open mouth, to pant». It was first used to describe an illness 500 years later by Hippocrates[4]. In 1662 the Belgian physician Jean van Helmont, who suffered from the disease, provided a detailed account of the asthma phenotype and offered one of the first pathophysiologic mechanisms of asthma: "the lungs are contracted or drawn together"[5]. Since then, several definitions of asthma have been proposed in attempts to describe the disease at both the pathological and functional levels. However, the lack of understanding of the exact mechanisms underlying the disease has made the task difficult. In 1997, The National Heart, Lung and Blood Institute (NHLBI's) published a definition of asthma that considers the inflammatory components and their functional consequences[6]:

> " Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation causes an associated increase in airway hyperresponsiveness (AHR) that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment."

The current view is that there are three main defining features of asthma: AHR, airway obstruction -generally reversible- and airway inflammation. In earlier years, bronchospasm, oedema and hypersecretion were considered the basic characteristics of asthma. Later on evidence of bronchial inflammation came from studies using bronchoalveolar lavage, bronchial biopsies, induced sputum and post-mortem examination of asthmatic lungs. Another pathological hallmark of this disease is the series of structural changes that occur over time and is referred to as airway remodelling. Although the concept of airway remodelling has been described for sometime, its implication in the pathogenesis as a cause rather than a consequence of airway inflammation and/or AHR remains unclear. In recent years, it became clearer that the importance of airway remodelling in the development and the persistence of the disease extends beyond what was initially thought[7, 8]. The clinical manifestations of asthma consist of recurrent episodes of wheezing, shortness of breath, chest tightness, and coughing. This is mainly due to airway narrowing, AHR and airflow obstruction that occur in the airways of affected patients.

1.1 Epidemiology of asthma

Several epidemiological studies have reported an increase in the incidence of asthma in the past 30 years. In Canada and the United States, the prevalence of asthma symptoms and diagnosed asthma is among the highest in the world for both children and adults[9]. Based on the recent data published in 2004, the mean prevalence of clinical asthma in North America is 11.2%. According to the Canadian Institute for Health Information, over two million Canadians are

currently affected by asthma[10]. In the United States, the prevalence of the disease has increased by 25-75% per decade since 1960. Although no equivalent data are available, we can assume that a similar phenomenon has been observed in Canada.

Based on data obtained in 1993, the Canadian asthma mortality rate of 0.25 per 100000 people is farily low. However, asthma worldwide accounts for about 1 in every 250 (rate of 4) deaths. The economical burden associated with the disease is considerable. In United States alone, the direct and indirect costs associated with asthma were estimated to be over US $12 billion in 1998[11]. Moreover, this disease is one of the most common causes of disability among the workforce[12].

1.2 Etiology of asthma

Asthma is a heterogeneous disorder of unknown etiology. However, many risk factors have been identified and they may be classified into two main categories: 1) genetic and 2) environmental factors. While genetic factors are pre-determined by the host, the environmental factors influence the susceptibility to the development of asthma in predisposed individuals. Genetic factors include: gender, race, presence of atopy and airway responsiveness. Environmental factors include: exposure to allergens and air pollution, respiratory or parasitic infection, smoke, obesity and socioeconomic status. In the following paragraphs, we will focus on risk factors for asthma that have received the most attention in the recent years.

1.2.1 Genetic factors

Regardless of the specific nature of changing environmental influences, it is most probable that changes in environmental exposures have led to the expression of asthmatic phenotypes in genetically susceptible individuals. Family and twin studies have provided evidence that asthma has a strong heritable component. Studies have estimated the heritability of asthma to be 36-79%[13, 14]. The difficulty in defining inheritance patterns have led to the conclusion that asthma is a complex genetic disorder[15-17]. Using multiple genome-wide linkage studies, several loci located on different chromosomes have so far been linked to an increased susceptibility to asthma[18]. In particular, human chromosomes 5q23-31, 6p24-21, 11q13-21, 12q21-24, 13q12-14, 17p11-q11 and 20q13 have received the greatest attention because they contain a large number of genes relevant to asthma, including interleukin (IL)-4, IL-5, IL-9 and IL-12, IL-13, granulocyte/monocyte-colony stimulating factor (GM-CSF) cytokine cluster, CC chemokine cluster, CRTH2 (chemoattractant receptor-homologous molecule expressed on Th2)and STAT6 (signal transducer and activator of transcription-6)[18]. Because of the limited precision of the genetic tools used for these studies, the exact identification of the genes involved and their functional polymorphisms remain unknown. The recent availability of large scale genotype technology has led to the identification of novel loci related to asthma. In 2002, a disintegrin and metalloprotease (ADAM) family members, ADAM33, was reported by Van Eerdewegh *et al.*[19]. Although later studies have successfully replicated the original finding[20], they failed to identify a single-nucleotide polymorphism that was associated across all populations.

1.2.2 Atopy

Atopy is defined as the production of abnormal amounts of IgE antibodies in response to common environmental allergens. It is one of the most important host factors for the development of asthma[21]. The literature suggests that the prevalence of asthma in the atopic population is about 50%. Of note, this association between asthma and atopy seems to be age-dependent. Most children who become sensitized during the first three years of life will develop asthma, while children who become sensitized after the age of 8 to 10 years have a similar risk of developing asthma to children who do not become sensitized[22].

1.2.3 Airway hyperresponsiveness

AHR is a state of abnormal sensitivity of the airways to a wide range of stimuli, including cold air, irritants and smooth muscle agonists. Subjects with asymptomatic AHR more frequently develop asthma symptoms than normoresponsive subjects[23]. The condition has been shown to correlate with the levels of total serum IgE, suggesting a link between the presence of atopy and the development of AHR. Moreover, a gene governing the development of AHR is in close association with a major locus that regulates serum IgE levels on chromosome 5[24], also highlighting the genetic component of AHR.

1.2.4 Gender and race

The prevalence of childhood asthma is higher in males than in females. This phenomenon can be attributed to the higher bronchial muscle tone, narrower airways and higher levels of IgE present in boys compared to girls[25].

There is no evidence in the literature that ethnicity or race affects asthma prevalence. In fact, the socioeconomic status and environmental factors related to the geographic context most likely explain the differences documented by some authors when evaluating the prevalence of asthma in different countries and races[26, 27].

1.2.5 Environmental factors

Increasing evidence suggests that the interactions between genes and environment might play a critical role in the pathogenesis of asthma, which illustrates a heritable component but do not follow Mendelelian laws[16]. As previously mentioned, environmental factors are likely to explain the differences observed in asthma prevalence among people of the same ethnicity located in different parts of the world. The importance of environmental factors in the development of asthma has been emphasized in recent years with the emergence of new theories, to explain the increase in asthma in the last three decades. The hygiene hypothesis states that allergy is a consequence of reducing infectious stressors during early childhood. Mechanistic explanations for this hypothesis involve the Th1/Th2 balance and the production of anti-inflammatory cytokines such as IL-10[28]. One of the major influences on the Th1/Th2 balance is the exposure to infectious agents. The improvement in our lifestyle, including the

preparation of food and personal hygiene, has led to a diminished stimulation of our Th1 immune system with bacteria and viruses during childhood, hence favouring an unopposed Th2 development[29]. Antibiotic use during the first two years of life is also associated with a pronounced, dose-dependent increase in the risk of developing allergic diseases[30], since it decreases the infectious burden of young children. Recent studies have proposed that IL-10 might be a key cytokine regulating the stimulation/suppression phenomenon that occurs during a Th1/Th2 response[31]. This cytokine is released in large amounts, following infections with bacteria, viruses or parasites and seems to be expressed at lower levels in asthmatics compared to normal controls[32]. Although appealing, the hygiene hypothesis will require further experimental testing since the presence of conflicting results has prevented scientists from drawing any firm conclusions[33, 34].

The increase in prevalence of obesity in industrialized countries follows a similar pattern than the evolution of asthma. Most of the existing epidemiological studies show a consistently positive association of obesity with both the prevalence and incidence of asthma in children and adults[35]. The dose-response relationship is demonstrated by the finding that the greater the obesity, the greater the effect on asthma[36]. This topic is currently the focus of extensive research worldwide in an effort to identify molecules that could potentially link these two conditions and perhaps explain the phenomenon.

The relationship between asthma and respiratory infections is complex. It has been shown that respiratory infection early in life protects from asthma, whereas later in life respiratory infections herald the onset of exacerbations. Many

infectious agents have been linked with development of asthma, including *Chlamydia pneumoniae*, rhinoviruses and parainfluenza viruses[37]. However, the mechanisms underlying this association seem complex and still remain to be elucidated.

Several others environmental factors may be involved in the development of asthma, including pollution, exposure to allergens, tobacco smoke, etc. The implication of several of these factors is possibly responsible for the initiation and perpetuation of the disease and it emphasizes its multi-etiological character.

1.3 Pathogenesis of asthma

The pathogenesis of asthma can be regarded as a two-step process. The first step consists of sensitization to an aeroallergen involving the development of a specific subset of T cells, namely Th2 lymphocytes. The second step consists of targeting the Th2-driven allergic inflammation to the airways. This inflammatory response is orchestrated and regulated by a complex network of mutually interacting immune mediators, including cytokines, chemokines and growth factors that are produced by inflammatory and structural cells (see Figure 1). The resulting chronic inflammatory process is believed to lead to remodelling of the airways, resulting in specific structural alterations that consequently affect the mechanical properties of the lung and the respiratory function[38]. Moreover, there are reports suggesting that airway inflammation and remodelling are events that happen in parallel rather than sequentially[39]. The following paragraphs will focus on these two components of asthma, namely airway inflammation and airway remodelling.

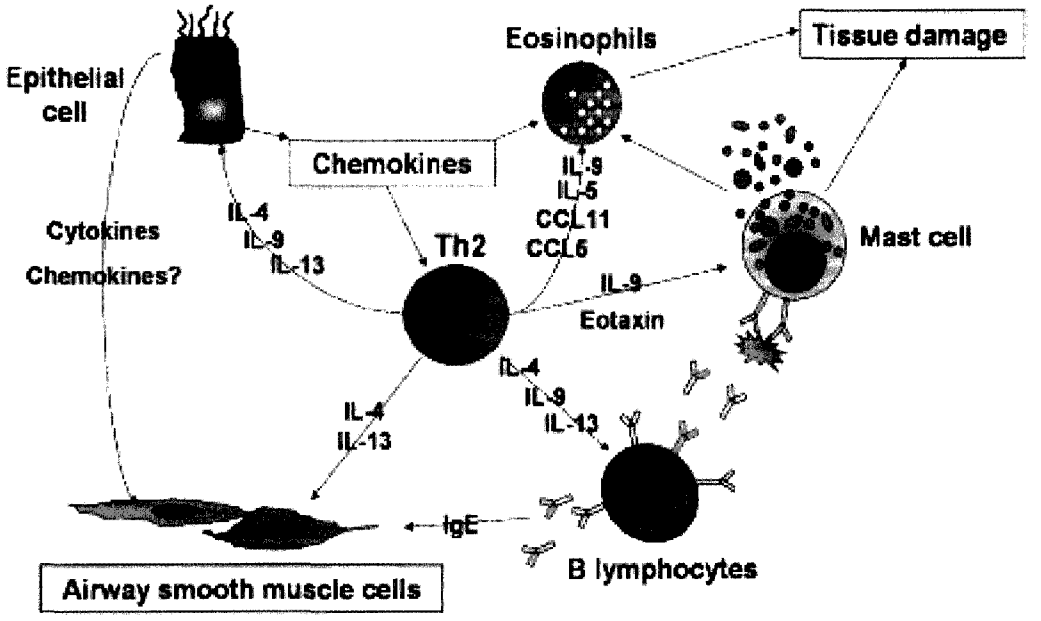

Figure 1. Figure representing the major cells involved in asthma pathogenesis. Th2 lymphocyte represents the cornerstone of the immune response characterizing the disease. Several mediators have been shown to be involved, including Th2 cytokines and C-C chemokines.

1.3.1 Airway inflammation

The earliest reports of the histopathology of asthma came from post-mortem evaluation of asthmatic individuals who died of *status asthmaticus*[40]. With the development of less invasive tools for the evaluation of the airways procedures, such as fiberoptic bronchoscopy, sputum evaluation and bronchial biopsies, it made it possible to characterize the airways of asthmatic subjects during periods of disease activity. It is clear that inflammation is an important feature of asthmatic airways[41]. Furthermore, through the development of immunocytochemical markers for inflammatory cells and electron microscopy, it has been possible to further describe the type and the intensity of the inflammatory process.

Typically, asthmatic airways are characterized by the presence of occlusive mucous plugs within the bronchi, infiltration of the airway wall with mostly eosinophils and CD4+ lymphocytes, increase in the amount of smooth muscle cells, and thickening of the reticular basement membrane[42]. The inflammatory reaction seen in asthma occurs in both acute and chronic phases.

The early-phase reaction usually follows the inhalation of an allergen and can be explained by the rapid activation of cells bearing allergen-specific IgE, more specifically mast cells and basophils[43]. The activated cells immediately release vasoactive and proinflammatory mediators, including histamine, eicosanoids and reactive oxygen species. This induces contraction of airway smooth muscle cells and vasodilatation, resulting in the airway narrowing and obstruction defining acute onset of asthma episode[44]. During the early-phase, inflammatory cells also release preformed chemotactic agents such as chemokines and cytokines that recruit inflammatory cells within the airways, a few hours after the inhalation of the allergen. This phase, which is also called the late-phase reaction, is characterized by the activation of Th2 lymphocytes, which are widely considered as the cornerstone of the immune response that takes place in the airways of asthmatic subjects[44].

As the primary orchestrator of the specific immune response, Th2 cells were implicated in the pathogenesis of human asthma in the early 90's[45, 46], a few years after the first description of their existence by Mosmann and Coffmann[47]. This landmark paper classified two CD4+ lymphocytes subsets in mice, based on their profile of expression of cytokines. While Th1 lymphocytes mainly express IFN-γ and IL-2, Th2 lymphocytes predominantly produce IL-4, IL-5, IL-9 and IL-

13[47-49]. Although the phenomenon of Th1/Th2 polarization is not as clear in humans as it is in mice, its existence has been confirmed *in vivo* in human in the context of diseases such as atopy, asthma, rheumatoid arthritis and autoimmune diseases[50]. At the functional level, Th1 lymphocytes appear to be critical in the development of cell-mediated immunity, whereas Th2 lymphocytes stimulate the production of IgE, mucosal mastocytosis, and eosinophilia and seem important in the humoral response as well as in protection against parasites[51]. Th1 and Th2 lymphocytes are derived from the same precursor cells (also called Th0 lymphocytes or Thp) and acquire their specific subset during the maturation process. Among the factors that influence the outcome of the lymphocyte during this process are the dose and nature of antigens, strength of signals through the T cell receptor, nature of the antigen presenting cells and the cytokine milieu[52]. The differentiation of uncommitted T cell precursors into Th2 cells is largely driven by IL-4 via Stat6, while Th1 maturation requires IL-12 and IFN-γ through the activation of Stat4[53].

A considerable body of literature has demonstrated that Th2 lymphocytes are major players in the initiation and the perpetuation of the inflammation found in asthmatic airways. However, it is important to mention that the Th1/Th2 cytokine imbalance is not necessarily pathognomonic for asthma. Several studies have shown that IFN-γ positive T cells are increased in asthmatic blood and airways while Birkisson suggests that a decrease in Th1 type cytokines is unlikely in asthma [54-59]. While studies in animal models show that overexpression of specific Th2-type cytokines reproduce some of the features of human asthma,

studies in which IFN-γ is overproduced has led to contradictory results[60]. However, it is clear that the increased production of Th2 cytokines such as IL-4, IL-5 and IL-13 drive the immune response that take place in the asthmatic airways. This occurs by affecting various population of cells, including leucocytes but also structural cells. The following section will focus on the most important population of cells involved in asthma pathogenesis.

1.3.2 Eosinophils

Eosinophils are derived from the CD34+ stem cells in the bone-marrow. They mature and migrate towards tissues under the influence of GM-CSF, IL-3 and IL-5 and many C-C chemokines[61]. Under normal circumstances, eosinophils are generally found in low numbers in both peripheral blood and tissues, accounting for less than 1% of the total leucocytes. However, following allergen challenge or acute exacerbation of asthma, they rapidly migrate within the airways in high numbers, in both airway tissues and lumen. The mature eosinophil possesses intra-cellular granules that contain inflammatory proteins, including major basic protein, peroxidase and cationic protein. Eosinophils also have the capability to produce a wide variety of mediators, including pro-fibrotic cytokines and growth factors such as transforming growth factor (TGF)-β, IL-11, IL-17A, fibroblast growth factor (FGF)-2, vascular endothelial growth factor (VEGF) and angiogenin[62], and bronchoactive mediators such as leukotrienes[63]. Although the presence of eosinophils at sites of allergic inflammation has been recognized for more than two centuries, the involvement of these cells in the pathogenesis of asthma has been a matter of debate for many years[64]. Recent studies using mice in

which the eosinophil lineage was ablated have addressed the contribution of eosinophils to the pathogenesis of asthma. One group showed that eosinophils were necessary for AHR and mucus accumulation, while the other group showed that these cells were required for airway remodelling[65, 66]. Although both papers had contradictory results, the overall conclusion strengthened by previous studies, suggests that eosinophils are important in the pathophysiology of asthma[67, 68]. In humans, the administration of a monoclonal antibody directed against IL-5 markedly reduced the presence of eosinophils in both the blood and the sputum of asthmatics[69]. Despite this significant effect, there was no reduction in the allergen-induced late asthmatic response or postallergen AHR, which suggested the involvement of other populations of cells in these responses[69].

The finding that eosinophils can produce growth factors and pro-fibrotic cytokines in addition to the findings of Humbles *et al.*, directed attention to their potential role in airway remodelling[66]. Furthermore, a paper published by Flood-Page *et al.* revealed that the use of an antibody directed against IL-5 in human not only reduced the amount of eosinophils within the airways of asthmatic subjects, but also diminished some features of airway remodelling such as the deposition of extra-cellular matrix proteins, apparently through the decreased production of TGF-β1[70].

1.3.3 Mast cells

Mast cells arise in the bone marrow, enter the circulation as CD34+ mononuclear cells and acquire their final phenotype only after migration into tissue, under the influence of locally derived growth factors and cytokines[71]. Mast

cells are widely distributed throughout the body in both connective tissue and mucosal surfaces. In the airways, mast cells are located in the lumen, bronchial epithelium, submucosa and lung parenchyma. They have the ability to produce several cytokines, including Th2-type cytokines such as IL-4 and IL-5[72]. Mast cells are implicated in the early-phase of asthma through the release of vasoactive mediators such as histamine and leukotrienes following the cross-binding of IgE to their high-affinity receptors. They also produce a wide variety of chemotactic factors that attract the inflammatory cells responsible for the prolonged late-phase reaction occurring 6 to 8 hours after inhalation of the allergen[73]. A recent study published by Wardlaw *et al.* has put the mast cells firmly back on the asthma stage[74]. By analyzing eosinophilic bronchitis, a condition that shares many similarities with asthma -except for the presence of AHR-, the authors found that the only striking difference between the two conditions was the presence of mast cells infiltrating the smooth muscle layer in asthma. They concluded that the asthma phenotype is caused by an abnormality of airway smooth muscle cell physiology, in part, because of mast cell myositis. Whether or not this phenomenon really plays a role in the development of AHR in human asthma has yet to be determined and requires further examination.

1.3.4 Lymphocytes

As we previously mentioned in this chapter, Th2 lymphocytes are the cornerstone of the immune response that takes place in asthmatic airways. They are roughly classified into two distinct families according to the presence of specific markers; those expressing the CD4 antigen and mainly involved in the

humoral immunity and those expressing the CD8 antigen and responsible for the cell-mediated response[75]. Evidence from literature indicates that lymphocytes are critical for the development of asthma and are found in the airways of asthmatics in proportion with the severity of the disease[76, 77]. Furthermore, the lymphocytes found within the airways are activated according to the expression of the IL-2R (CD25)[78]. The function and contribution of lymphocytes to asthma is multifactorial and mainly centers on their capacity to release cytokines.

1.3.5 Structural cells

Structural cells in asthmatic airways have long been regarded as having a limited functional role. For example, epithelial cells serving as a protective barrier, airway smooth muscle as the contractile element of the airways and fibroblasts as the major source of extracellular matrix (ECM) components. However, in the last decade, these cells have drawn the attention to their many other functions in physiology and pathology. In asthma, airway smooth muscle cells, fibroblasts and epithelial cells are of particular interest since all exhibit potent immune and inflammatory functions. Epithelial cells for instance, express leukocyte-associated antigen and present antigen[44]. They also have the capability to produce a wide variety of inflammatory cytokines and chemokines as well as their receptors[79-81]. Similarly, fibroblasts and smooth muscle cells have been implicated in many aspects of the pathogenesis of asthma, including immune regulation, AHR and airway remodelling[82, 83]. Fibroblasts are a potent source of extra-cellular matrix protein and pro-fibrotic cytokines[84]. In animals, they have been shown to differentiate into myofibroblasts following an allergic challenge[85].

Furthermore, they increase their production of procollagen-αI in response to IL-4[86]. The role of ASMC and their potential effect on airway remodelling through the release of inflammatory mediators will be discussed in later sections.

1.4 Airway remodelling

Airway remodelling is a process describing tissue repair and the subsequent structural changes occurring in the airways in response to acute injury or inflammation[87]. These structural changes in asthmatic airways were extensively reviewed [39, 88] and include thickening of the lamina reticularis, hypertrophy and hyperplasia of goblet cells and mucous glands, and increased amount of airway smooth muscle (see figure 2).

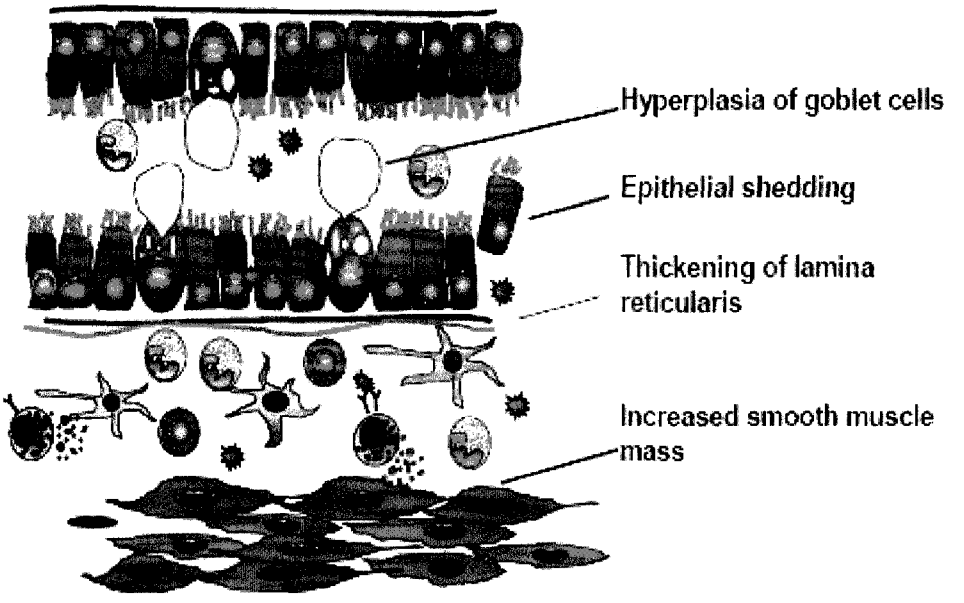

Figure 2. Features of airway remodelling. Asthmatic airways are characterized by: 1) Hyperplasia of goblet cells 2)Epithelial shedding 3) Thickening of lamina reticularis and 4) Increased smooth muscle mass. All these structural changes contribute to airway narrowing and typical asthmatic symptoms.

Although some have found no significant differences between the integrity of the epithelium obtained in control subjects versus severe asthmatic subjects, epithelial

shedding has been included for several years as a prominent feature of airway remodelling[89]. These structural modifications in the structure of the airways may be in part responsible for the narrowing characterizing the asthmatic airways as well as the alteration of the pulmonary mechanics of these patients[87].

For decades, asthma has been considered a condition of reversible airway obstruction. However, it has been recently pointed out that in some individuals, even after optimal treatment with corticosteroids, residual obstruction remains, possibly as a consequence of airway remodelling, and resulting in permanent airflow obstruction[90, 91]. Other evidence indicating the involvement of airway remodelling in changing the biophysical properties of the airways, comes from elderly individuals who have had persistent asthma for decades. When their respiratory function is compared with those with asthma of short duration, they have a significantly lower forced expiratory volume in one second (FEV1), suggesting that long-standing asthma is characterized by a greater degree of decline lung functions[92].

Different cellular and molecular mechanisms are potentially involved in the pathogenesis of airway remodelling. It is possible that the inflammation process characterizing asthmatic airways, both through the humoral and cellular effectors, plays a major role in the initiation and the perpetuation of the different features of airway remodelling. It is also likely that inflammation and remodelling occur in parallel rather than cause and effect[39]. There is little known about the individual functions of inflammatory cells and mediators in the overall picture of the structural changes occurring in the airways. In the next paragraphs, we will

review what is known about the most important characteristics of airway remodelling.

1.4.1 Thickening of the recticular basement membrane

One of the most characteristic features of asthma in pathology is the deposition of excess connective tissue beneath the airway epithelium[93-95]. Although thickening of the reticular basement membrane is found in the airways of most asthmatics, it is also associated with other respiratory diseases such as chronic obstructive pulmonary disease (COPD). Increased deposition of several proteins including collagen I and III, fibronectin and proteoglycans such as lumican and versican has been reported by different groups[93, 96-98]. Furthermore, Huang *et al.* reported that there was a close relation between the amount of proteoglycans immunoreactivity and the degree of airway responsiveness, highlighting another possible link between airway remodelling and the development of symptoms in asthmatic individuals[93]. These proteins are produced by activated myofibroblasts that migrate from peripheral circulation and that lay underneath the membrane and resident fibroblasts[85, 99].

1.4.2 Increased number of goblet cells and mucous glands

The increase in the number of goblet cells in the airway epithelium as well as the number of submucosal glands is another hallmark of asthma pathology. Widespread plugging of the airways lumen with mucus is observed at necropsy in cases of fatal asthma[100]. Studies have shown an increased number of goblet cells in subjects with asthma and submucosal glands[100-102]. These results suggest that

the overproduction of mucus is an important aspect of asthma pathogenesis, particularly in cases of fatal asthma.

Previous studies have shown that the production of mucus by goblet cells and submucosal glands might be influenced by inflammatory mediators[103, 104]. Cytokines such as IL-4, IL-6, IL-9 and TNF-α have been shown to induce the release of mucous, mainly through their effects on goblet cells[105-107]. The functional relevance of mucus overproduction comes from its implication at two levels in the pathogenesis of asthma: airway obstruction and AHR. The airway obstruction is associated with the formation of mucus plugs within the airways, particularly in the small airways, which are more easily clogged by mucus hypersecretion. Studies have also shown that an overproduction of mucus can lead to hyperresponsiveness of the airways. However, the mechanisms underlying this phenomenon are still unknown[108].

1.4.3 Increased airway smooth muscle mass

Increase in airway smooth muscle mass has been recognized as a central feature of asthma for many years, although the initial studies were thought to have overestimated the amount of airway smooth muscle due to a lack of a reliable methods of measurement[109, 110]. However, later data obtained with more precise methods confirmed the initial observations[89, 111]. As previously mentioned, the remodelling process can modify the behaviour of the ASMC through a combination of different alterations of airway mechanics[112]. An increase in the amount of smooth muscle, with normal contractile properties, might be enough by

itself to induce AHR. It is still unclear whether the phenomenon is due to fundamental changes in the phenotype of the smooth muscle, or caused by alterations in the relationship of the airway wall with the surrounding lung parenchyma.

Different hypotheses have been postulated in order to explain the increase in smooth muscle mass in asthma. Both hypertrophy and hyperplasia could contribute to this increase, although conflicting results do not allow any definitive conclusion[89, 113]. In atherosclerosis, an inflammatory disease of the blood vessels, migration of vascular smooth muscle cells from peripheral circulation has been described in order to explain the increase of these cells in the atherosclerosis plaques[114]. A similar phenomenon has never been reported in asthma, although recent evidence suggests that such events could be possible[115-117]. Mediators such as cytokines, chemokines, leukotrienes and growth factors could modify smooth muscle cells behaviour and/or phenotype[118-120], and thus induce changes in proliferation and apoptosis, cytokine release, adhesion molecules and receptors expression, production of extracellular matrix proteins (ECMP), and contractility. Several mediators have been shown to be upregulated in asthmatic airways and could affect ASMC growth. Among them, are epidermal growth factor (EGF), histamine, platelet-derived growth factor (PDGF) and leukotrienes D4 (LTD4) which are all mitogens for ASMC *in vitro*[121-123]. Intrinsic alteration of ASMC phenotype has also been postulated as a potential explanation for the increase in smooth muscle mass since Johnson and co-worker have observed that ASMC obtained from asthmatic subjects grow faster than cells obtained from normal subjects[124]. These results suggest that the cells are capable of growing faster

throughout multiple passages *in vitro,* even when removed from their *in vivo* environment. Whether one or, more likely, a combination of factors are responsible for the increase of smooth muscle mass still requires further investigations.

1.5 Airway smooth muscle cells

1.5.1 Origin of airway smooth muscle

Appearance of ASMC in the lung occurs shortly after the maturation of the epithelium, following initiation of branching of the lung from the laryngotracheal bud[125]. The ASMC differentiation seems to be initiated by stimuli coming from the epithelium and the basement membrane. Work carried out in mice has demonstrated that ASMC is derived from undifferentiated mesenchymal cells that undergo further maturation following stimulation with sonic hedgehog (Shh), produced by the lung epithelial layer[126]. Furthermore, exposure to the basement membrane laminins as well as to the positive pressure applied in the airway lumen also seems to trigger maturation of ASMC[127].

1.5.2 Phenotype of ASMC

Heterogeneity in function and responsiveness of ASMC is emerging as a relevant topic in the context of asthma. It describes the presence of different types of ASMC occurring at the same or different sites in the lung. This was described in vascular smooth muscle cells (VSMC) and seems to exist in ASMC[128, 129]. For example, in the wall of the pulmonary artery, the media contains at least four phenotypically heterogenous populations of smooth muscle cells[128]. In human

airways, the functional consequences of the presence of different ASMC phenotypes have not yet been elucidated. Some authors hypothesized that in asthma, an imbalance between the different phenotypes may alter the contractile and proliferative properties of the tissue, thus favouring the development of hypercontractile airways[130, 131].

In culture, two main phenotypes of ASMCs have been identified: contractile and synthetic-proliferative. As suggested by its name, cultured ASMCs of contractile phenotype have the ability to respond to specific contractile agonists [132]. Maintenance of the specific phenotype in cultured ASMC is dependent upon a number of factors, including cell density, absence of fetal bovine serum and presence of heparin[133, 134]. Following the exposure to a mitogenic stimulus such as fetal bovine serum, the ASMC undergoes a reversible modification of its phenotype from contractile to synthetic, which is characterized by a loss of the contractile apparatus, such as myosin heavy chain and α-smooth muscle actin, and the expression of other proteins such as protein kinase C (PKC) and CD44. Contractile cells are characterized by a high density of contractile proteins and few biosynthetic intracellular organelles and do not divide in their resting state. Synthetic ASMCs have a low density of contractile proteins and high fraction of biosynthetic organelles. They actively divide but they lose their ability to contract in response to spasmogens[135, 136]. Although it is accepted that in the airway wall the function of the contractile smooth muscle phenotype is dedicated to the regulation of airway calibre, the existence and function of the synthetic-proliferative phenotype is unclear. In atherosclerosis, this phenotype is believed to

be central to the pathogenesis of the disease through recruitment and activation of inflammatory cells, production of ECMP and vascular calcification[137]. Data obtained from Leguillette *et al.* suggests that an increased presence of regulatory proteins involved in the contraction of human ASMC might explain the hyperreactive airways found in asthmatic subjects[130]. The role played by the synthetic-proliferative phenotype in the pathogenesis of asthma is still unclear, although several studies have provided clear evidence that ASMC can produce a wide variety of mediators. In the next section of this chapter, we will review the potential of ASMC to release cytokines, chemokines and other mediators found in asthmatic airways in greater detail.

1.5.3 Synthetic functions of ASMC

As we previously mentioned, ASMC plays a pivotal role in the pathogenesis of asthma in different ways. The shortening of these cells is at the root of the symptoms of asthma. The width/area of the smooth muscle bundles examined on transverse sections of airways is increased by 50-200% in fatal and 25-55% in non-fatal asthma, compared with control cases[138]. The increase in smooth muscle mass is also one of the major contributors to airway narrowing and AHR[84, 139]. However, early work in asthma provided evidence that the functions of ASMC could extend beyond their structural and contractile properties. It has been recently recognised that the synthetic function of ASMC may be related to the perpetuation and intensity of airway wall inflammation. A number of studies, the majority being *in vitro*, demonstrated that ASMC is also a rich source of biologically active cytokines, chemokines and growth factors (see figure 3),

which can regulate airway inflammation through chemotactic, autocrine and paracrine effects.

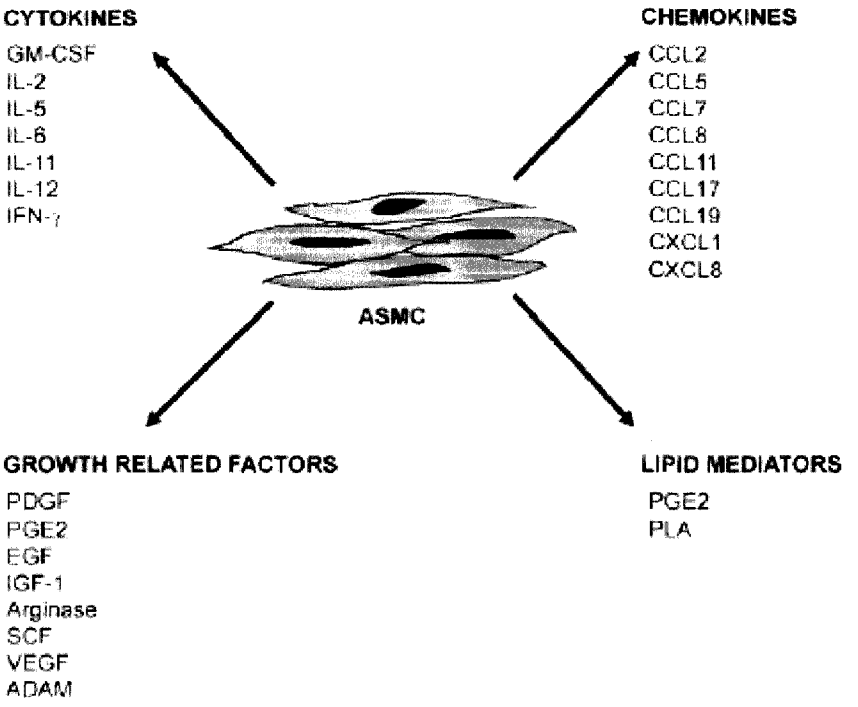

Figure 3. Synthetic potential of ASMC. Chemokines, cytokines, growth factors and lipid mediators release by ASMC. GM-CSF: granulocyte-macrophage colony stimulating factor, PDGF: platelet-derived growth factor, PGE2: prostaglandin E2, EGF: Epidermal growth factor, SCF: stem cell factor, VEGF: vascular endothelial growth factor, ADAM: A disintegrin and metalloproteinase domain, PLA: phospholipase A.[1-3].

1.5.4 Cytokine production

As discussed in section 1.3.1, asthmatic airway inflammation is orchestrated and regulated by a complex network of mediators, including cytokines which display a wide array of effects (see figure 1). In 1996, a report by Berkman *et al.* reported positive immunohistochemical staining for CCL5 (see table I for chemokine nomenclature) in the smooth muscle layer present in

bronchial biopsies from both normal and asthmatic subjects[140]. These results were rapidly confirmed when a second paper was published, demonstrating the release of GM-CSF and its regulation by dexamethasone in the same population of cells[141, 142]. Since then, ASMC has been shown to produce a wide variety of cytokines and chemokines, including pro-inflammatory mediators such as IL-1β and IL-6[143, 144]. These two cytokines exert several pro-inflammatory effects such as the release of ECMP, the activation of leukocytes and other structural cells, B cell maturation, the upregulation of IL-4 dependent IgE production and mucus hypersecretion[145].

ASMC have also been shown to produce Th1 and Th2 cytokines, which in turn have the potential to influence airway inflammation and the development of airway remodelling. IFN-γ, IL-2 and IL-12 are the three key-cytokines of the Th1 response that are produced by ASMCs[146, 147]. According to Hakonarson and co-workers, these cytokines may play a protective role in the asthmatic airways[147]. Production of Th2-related cytokines by ASMC has also been reported, as ASMC release GM-CSF and low levels of IL-5[142, 147]. GM-CSF is an important factor required for the maturation, the activation and the survival of eosinophils, while IL-5 is also involved in the maturation and the recruitment of eosinophils, as well as in diverse aspects of the Th2 response[145, 148].

Systematic Name	Human Ligand	Human Aliases	Receptor
C Family			
XCL1	Lptn	SCM-1α, ATAC	XCR1
XCL2	SCM-1ß		XCR1
CX_3C Family			
CX_3CL1	Fractalkine	ABCD-3	CX_3CR1
CC Family			
CCL1	I-309		CCR8
CCL2	MCP-1	MCAF, HC11	CCR2
CCL3	MIP-1α	LD78α, LD78ß, GOS19, Pat464	CCR1, CCR5
CCL4	MIP-1ß	pAT744, ACT-2, G-26, HC21	CCR5
CCL5	RANTES		CCR1, CCR3, CCR5
CCL6	MIP-1β		CCR5
CCL7	MCP-3		CCR1, CCR2, CCR3
CCL8	MCP-2	HC14	CCR1, CCR2, CCR3, CCR5
CCL9/10			CCR1
CCL11	Eotaxin		CCR3
CCL12			CCR2
CCL13	MCP-4	Ckß10, NCC-1	CCR1, CCR2, CCR3
CCL15	HCC-1	MCIF, Ckß1, NCC-2,CCL	CCR1
CCL15	MIP-1δ	CC-2, MIP-5, HCC-2, NCC-3	CCR1, CCR3
CCL16	HCC-4	LEC, ILINK, NCC-4, LEC, LMC	CCR1
CCL17	TARC	Dendrokine	CCR4, CCR8
CCL18	PARC	DC-CK1, AMAC-1, Ckß7, MIP-4	
CCL19	MIP-3ß	ELC, Exodus-3, Ckß11	CCR7
CCL20	MIP-3α	LARC, Exodus-1	CCR6
CCL21	6Ckine	Exodus-2, SLC, TCA-4, CKß9	CCR7
CCL22	MDC		CCR4
CCL23	MPIF	Ckß8, Ckß8-1, MIP-3, MPIF-1	CCR1
CCL24	Eotaxin-2,	MPIF-2, Ckß6	CCR3
CCL25	TECK	Ckß15	CCR9
CCL26	Eotaxin-3	MIP-4α, IMAC, TSC-1	CCR3
CCL27	CTACK	ILC, PESKY, ESkine, Skinkine	CCR10
CCL28	CCL28	MEC	CCR10
CC Family			
CXCL1	GROα	MGSA, GRO1, NAP-3	CXCR2

CXCL2	GROß	MIP-2α, GRO2	CXCR2
CXCL3	GRO?	MIP-2ß, GRO3	CXCR2
CXCL4	PF4		CXCR3
CXCL5	ENA-78		CXCR2
CXCL6	GCP-2		CXCR2
CXCL7	NAP-2	CTAPIII, ß-Tα, PEP	CXCR2
CXCL8	IL-8	NAP-1, MDNCF, GCP-1	CXCR1, CXCR2
CXCL9	MIG		CXCR3
CXCL10	IP-10		CXCR3
CXCL11	I-TAC	ß-R1, H174, IP-9	CXCR3
CXCL12	SDF-1α/ß	PBSF	CXCR4
CXCL13	BCA-1		CXCR5
CXCL14	BRAK		?
CXCL15	*In mouse only*		
CXCL16	CXCL16	SRPSOX	CXCR6

Table I. New and old nomenclature for chemokines and their receptors. BCA-1, B-cell-attracting chemokine 1; CTACK, cutaneous T-cell-attracting chemokine; DC-CK1, dendritic cell-derived CC chemokine 1; ELC, EBL-1-ligand chemokine; ENA-78, epithelial-cell-derived neutrophil attractant 78; GCP, granulocyte chemotactic protein; GRO, growth-related oncogene; HCC, haemofiltrateCC chemokine; IL, interleukin; IP-10, interferon-inducible protein 10; I-TAC, interferon-inducible T-cell alpha chemoattractant; LARC, liver- and activation-regulated chemokine; LEC, liver-expressed chemokine; LCC-1,liver-specific CC chemokine-1; Lkn-1, leukotactin; MCP, monocyte chemoattractant protein; MDC, macrophage-derived chemokine; MEC, mammary-enriched chemokine; Mig, monokine induced by interferon γ; MIP, macrophage inflammatory protein; MPIF, myeloid progenitor inhibitory factor; NAP,neutrophil-activating peptide; PF4, platelet factor 4; RANTES, 'regulated on activation, normally T-cell expressed and -secreted'; SCM-1α/β, single C motif-1 α/β; SDF, stromal-cell-derived factor; SLC, secondary lymphoid tissue chemokine; TARC, thymus- and activation-regulated chemokine; TECK, thymus-expressed chemokine.

Among the other relevant products released by ASMC, it is noteworthy to mention a few growth factors such as PDGF, VEGF and insulin-growth-factor (IGF). These mediators have been involved in the proliferation and the survival of structural cells and leukocytes in the context of an inflammatory response. VEGF has been implicated in the angiogenesis process that takes place in asthmatic airways[149, 150]. ASMC has also been shown to constitutively produce TGF-β[151], which is a potent pro-fibrotic cytokine possessing the ability to stimulate the

synthesis of collagen I, III and V, fibronectin, tenascin and several proteoglycans while

Cytokine	*Main target cells*	*Main effects*
GM-CSF	Eosinophils	Priming and increases survival of eosinophils Increases maturation of granulocyte lineage
IL-1	Inflammatory cells Structural cells	Activation of several cytokines and chemokines genes, AHR
IL-2	T cells	Proliferation of Th1 lymphocytes
IL-5	Eosinophils, Mast cells, T cells	Activation and priming of eosinophils, recruitment of eosinophils, increases survival of target cells, AHR
IL-6	Inflammatory cells Structural cells	Activation of structural cells (fibroblasts) Activation of pro-inflammatory genes Increase proliferation of ASMC
IL-11	Structural cells Plasma cells	Airway remodelling, AHR
IL-12	T cells	Promote Th1 cells Inhibits Th2 cells and IgE synthesis
IFN-γ	T cells Inflammatory and structural cells	Inhibits Th2 cells and IL-4, IL-13 and IgE production Activation of pro-inflammatory genes
Chemokine		
CCL2	Monocytes > Eosinophils	Recruitment Angiogenesis
CCL5	Eosinophils T cells Monocytes	Recruitment and activation
CCL7	Eosinophils Monocytes	Recruitment
CCL8	Eosinophils > Monocytes T cells	Recruitment
CCL11	Eosinophils Th2 cells Vascular smooth muscle cells	Recruitment within tissues and activation AHR
CCL17	Th2 cells	Recruitment and AHR
CCL19	T cells	Recruitment
CXCL1	Neutrophils	Recruitment
CXCL8	Neutrophils	Activation and recruitment

Table II. Main target cells and effects of cytokines and chemokines produced by ASMC in the context of asthma. [42, 152]

downregulating synthesis of matrix metalloproteinase (MMP). Although the relative importance of the release of these cytokines by ASMC remains to be evaluated, it raises the possibility that they contribute to the complex network of

cytokines characterizing the pathogenesis of asthma. Table II summarizes the different effects of the mediators released by ASMC in the context of asthma.

1.5.5 Chemokine production

Chemokine biology and their involvement in asthma will be discussed further as they represent a key component of this manuscript. Several papers reported the production of chemokines by ASMC[3]. Interestingly, many of the chemokines produced by ASMC are involved in the recruitment of inflammatory cells within the asthmatic airways, suggesting that the importance of ASMC in the inflammatory process might also be associated with the attraction and the activation of leukocytes. However, the exact contribution of ASMC to the recruitment of inflammatory cells in the context of asthma remains to be evaluated.

1.5.6 Production of extracellular matrix proteins

The term ECM includes both basement membrane connective and interstitial connective tissues. It provides support to the airways and its cellular components including ASMC. The ECM also influences distribution, activation status, survival and adhesion of inflammatory cells. The interstitial cellular matrix is composed of different proteins including collagen, laminin, lumican, fibronectin and versican. Furthermore, it interacts with ASMC in a bidirectional way: ASMC has the capacity to produce and modify the composition of the surrounding matrix, while the matrix itself influences ASMC proliferation, migration and synthetic capabilities[118, 153]. In asthma, it has been demonstrated

that the profile of ECM is altered. The deposition of collagen I,III,V, fibronectin, tenascin, hyaluronan, versican and laminin α2/β2 is increased, whereas the deposition of collagen IV and elastin is decreased[154-156]. This altered ECM composition in asthmatic airways could be due to increased synthesis of ECM proteins or decreased activity of its degrading enzyme, the MMP. Some have reported the effects of TGF-β and leukotriene D4 on the modulation of collagen I and IV, fibronectin, elastin and biglycan[157].

The influence of the ECM on ASMC behaviour has been documented at different levels. Hirst *et al.* reported that some of the components of the ECM such as collagen I, fibronectin and laminin can enhance the proliferation of ASMC in culture and modify the cell's phenotype, from a contractile phenotype towards a more proliferative phenotype[158]. More recently, Dekkers *et al.* showed that collagen I and fibronectin favours the synthetic-proliferative phenotype, while laminin can maintain the contractile phenotype [159]. Parameswaran *et al.* reported that migration of ASMC was facilitated by collagen III and V and fibronectin[116]. Lastly, Freyer *et al.* investigated the influence of ECM proteins on ASMC survival. They found that when cells were grown on elastin, they had a reduction in their apoptotic rate through the activation of integrins[160]. Taken all together, these data demonstrate the potential impacts of the ECM composition on ASMC biological behaviour, as they can modulate the synthetic and the proliferative responses of these cells. New insights into the mechanisms underlying increased smooth muscle mass and airway hypresponsiveness may result from the discovery that ECM composition is altered in asthmatic airways.

1.5.7 Expression of receptors by ASMC

The expression of different receptors by ASMC was studied in the last two decades in order to clarify the effects of specific mediators on cell functions. Several contractile agonists such as histamine, acetylcholine and leukotrienes, have all been widely used to study the contraction of smooth muscle, and act on G-protein-coupled-receptors present on the surface of ASMC. Expression of IL-1β in ASMC has also been reported. This potent pro-inflammatory molecule has been involved in the hyperresponsiveness of ASMC observed in asthmatic subjects[161, 162]. More recently, the expression of Th2-cytokine receptors such as IL-4, IL-5 and IL-13 was detected in cultured ASMC[146, 147, 163]. Data from the literature indicate that Th2-type cytokines such as IL-5 and IL-13 also increase the contractile response and/or decrease relaxant responses of ASMC[146, 163]. The existence of many of these receptors was inferred based on the effects of recombinant cytokines on ASMC functions. However, few groups have characterized the presence of the receptors themself [164]. The three isoforms of TGF-β were detected using immunocytochemistry and flow cytometry, while the two TNF-R were demonstrated using immunostaining and western blot.

The expression of chemokine receptors has never been shown in ASMC. However, expression of chemokine receptors has been reported by vascular smooth muscle cells in the context of atherosclerosis[165-168]. The different aspects of the expression of chemokine receptors by smooth muscle cells will be further elaborated in a subsequent section.

1.5.8 Increase in smooth muscle mass in asthma

In the section 1.4.3 of this chapter, we briefly introduced the different mechanisms potentially involved in the increased smooth muscle mass that is observed in asthma. To date, there are three commonly accepted theories: 1) hyperplasia of ASMC; 2) hypertrophy of ASMC and 3) migration of ASMC.

1.5.8.1 Hyperplasia

Several studies have described hyperplasia in the airways of both asthmatic subjects and animal models of asthma[113, 169-173]. The augmentation in the ASMC number could occur through the increased rate of proliferation of the cells and/or through a reduced rate of apoptosis. However, some groups have failed to detect any marker of proliferation in airway specimens obtained from asthmatic subjects. In a recent study, Benayoun *et al.* used Ki67 as a marker of proliferation to assess hyperplasia in biopsies obtained from severe, moderate, mild asthmatics and control patients[89]. They did not observe any significant differences in the proliferative state of ASMC between the groups. One explanation for this observation may come from the fact that the division of the cells occurs over a long period of time at a fairly low rate and it therefore becomes difficult to detect any changes at a specific time-point[174]. A study published by Johnson *et al.* also demonstrated that ASMC obtained from asthmatic lung specimens divides faster than specimens from control subjects when they are cultured *in vitro*. This finding suggests that asthmatic cells retain their ability to proliferate faster than normal cells, even after multiple passages, possibly due to an intrinsic property of the cells[124]. However, several research

groups have tried to identify a possible mediator that could promote ASMC proliferation in the context of asthma. Cell culture-based studies over the past decade have identified several potential mitogens of ASMC, some of which are present in increased amounts in BAL fluid or asthmatic airways, such as PDGF isoforms, EGF and FGF-2[175-177]. However, the relevance of any of these growth factors *in situ* remains to be elucidated. Although the presence of hyperplasia is likely to be involved in the increase in smooth muscle mass in asthma, thus far no studies have provided any conclusive data regarding this issue.

1.5.8.2 Hypertrophy

Little is known about the involvement of hypertrophy in the increase in ASM mass. Conflicting results report an increase in ASMC size in asthmatic airways. While Benayoun described the phenomenon in both moderate and severe asthmatics, Woodruff *et al.* suggested that mild and moderate asthmatic ASMC do not display hypertrophy[89, 113]. Mediators such as TGF-β, cardiotrophin and IL-1β have been identified as potential inducers of ASMC hypertrophy in vitro[178]. Ebani et al. also suggested the involvement of hypertrophy in the increased smooth muscle mass. Interestingly, using 3-D morphometry, they found that hypertrophy is mainly localized in large airways in asthmatic patients, when compared to COPD and control patients[170, 179].

1.5.8.3 Migration of ASMC

Migration of ASMC in airways might be involved in asthma pathogenesis in two ways: 1) Migration of mature ASMC or progenitor cells from surrounding bundles or from peripheral circulation and 2) Migration of the smooth muscle layer towards the epithelium. The theory behind the first concept is very recent and seems plausible since a similar phenomenon has been extensively described in atherosclerosis[114]. However, there is no direct report that migration of ASMC occurs in asthma, although a recent paper demonstrated that precursor cells of myofibroblasts can migrate within the airways from the peripheral circulation following antigen challenge[85]. Whether or not myofibroblasts can differentiate and become ASMC still remains undetermined. The ability of ASMC to migrate was initially described by Hedges *et al.* in 1999[180] Since then a few authors reported the ASMC migration in response to different stimuli, including PDGF, leukotrienes, IL-1β, TGF-β and mechanical strain[115, 181-183]. In the context of this thesis, it is noteworthy to mention that the induction of migration of ASMC by chemokines has never been described.

The decreased distance between the airway smooth muscle layer and the epithelium is a common feature of asthmatic airways. However, the mechanisms underlying this observation have never been elucidated. Some may argue that the increase in smooth muscle mass pushes the bundle towards the epithelium, reducing the distance between the two structures. Others suggest that this phenomenon is the consequence of the migration of ASMC towards the

epithelium, leading to modifications of the mechanical properties of the airways and subsequently to AHR[89].

1.5.9 Signalling pathways involved in the migration of ASMC

Although little is known concerning the migration of ASMC, few groups have examined the intracellular pathways underlying the process. Similarly with migration of other cell populations, movement of ASMC involves members of the mitogen activated protein kinase (MAPK) family. So far, p38 and extracellular signal-regulated kinase (ERK) have been implicated in the response to PDGF and urokinase[180, 184]. When pharmacologically blocking the p38 pathway, the migration of ASMC towards a gradient of PDGF or urokinase is totally inhibited. ERK seems to be involved in the regulation of the migration, especially in response to urokinase, while cyclic AMP (camp), rho-kinases and protein kinase A (PKA) inhibit ASMC migration[183].

1.6 Chemokines

In order for the immune system to be effective against infections or during an inflammatory response, leukocytes must be able to migrate from the central circulation towards tissues and organs. Their movements are under the control of specific mediators called chemokines. The term chemokine, a short term for *chemo*tactic cyto*kines*, was coined in 1992[185]. The past decade has witnessed an explosion in research directed at understanding the contribution of these molecules in acute and chronic inflammation and their roles in other physiological responses. The importance of chemokines in dictating the migration and the activation of specific subpopulation of leukocytes to sites of inflammation has led to the development of promising therapeutic applications to regulate chemokine activity in the context of diseases.

Beside their ability to induce migration, chemokines can induce angiogenesis, organogenesis and cell activation. They can also promote the migration of structural cells such as fibroblasts, keratinocytes and vascular smooth muscle cells[168, 186-189]. In the coming sections, we will briefly review the various aspects of chemokine biology and we will focus of the diverse functions of these mediators in the context of asthma, particularly in relation with ASMC.

1.6.1 Chemokine nomenclature

A systematic nomenclature for chemokines and their receptors was adopted a few years ago in order to simplify the description and characterization of a constantly increasing number of them (see table I and table II)[190]. The receptors are named as CXC, CC, XC and CX3C followed by R and a number,

while the chemokines are defined by the same structure related acronyms, followed by L for ligand. The systematic nomenclature has been generally adopted for the receptors; however, chemokines are still mostly designated by their traditional names. Although this new classification system increases the difficulty to memorize the name of the mediators, it helps to reduce the complexity associated with the multiple names that have been given to a given chemokines (ex. CCL15 is also called leukotactin-1, MIP-1δ, HCC-2 or MIP-5). For the purpose of this thesis, we will designate the chemokines under the new nomenclature (see table I).

1.6.1.1 Chemokine classification and structure

Chemokines belong to a large family of structurally related proteins containing 50 members and about 30 receptors. As we previously mentioned, they are classified in 4 categories based on the position of the first two cysteine residues (N terminal) and the chromosomal location of the corresponding genes. Two main subfamilies, CXC and CC chemokines (also called α and β chemokines, respectively) contain most of the chemokines identified to date. The cysteines form two disulphide bonds which confer to the chemokine's three-dimensional folding (see figure 4). The disulphides keep two amino-terminal regions together, which is a primary requirement for receptor recognition and any biological activity of chemokines.

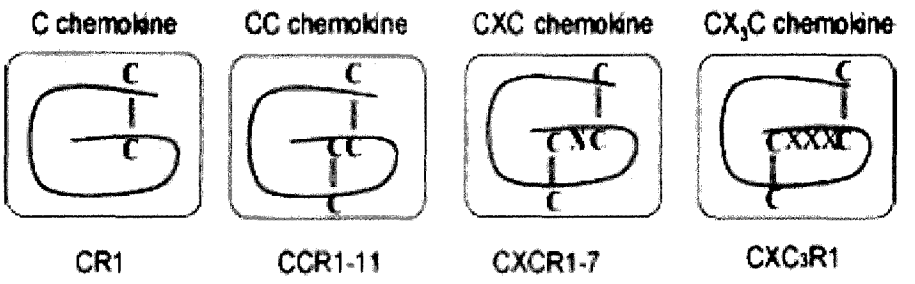

Figure 4. Schematic representation of chemokine receptors. The pink line represents the disulphide bridge, while black line represents the peptide chain. The chemokine receptors are divided into four categories, based on the position of the cysteine residue, as illustrated above.

A chemokine is composed of approximately 70-130 amino acids and usually weights between 8-12 kDa. Since most chemokines are secreted proteins, they are synthesized with a leader sequence of 20-25 amino acids, which is cleaved off before release. The three-dimensional structure of few chemokines have been determined using nuclear magnetic resonance[191]. The similarity in three-dimensional structure may explain the functional overlap among the different classes. Studies looking at the identification of chemokine domains that bind and activate the receptor have revealed the existence of a ELR motif (Glu-Leu-Arg), immediately preceding the first cysteine in the CXC group[192]. The deletion of this motif results in the loss of some chemokine activities such as angiogenesis and recruitment of neutrophils. CC chemokines which do not display this motif are usually associated with recruitment of mononuclear cells, such as monocytes and lymphocytes. Few members of this family, including CCL15, are potent attractant for eosinophils[193].

The majority of the genes for the CXC and CC chemokines are clustered on chromosomes 4q12-21 and 17q11.2-12 respectively, except for CCL19, whose

gene maps to chromosome 9 and CCL15 which maps to chromosome 2 [194, 195]. There is 20 to 50% homology among the CXC chemokines and 28 to 45% homology among the CC chemokines at the amino acid level. The significant structural homology and functional overlap suggest that the chemokine gene might have been created by duplication of a single ancestral gene[196].

1.6.2 Chemokine receptors

So far, seven CXC chemokine receptors (CXCR1 to CXCR7) and eleven CC receptors (CCR1 to CCR11) have been cloned and characterized (see table II)[197, 198]. All chemokines receptors belong to the seven-transmembrane-spanning G-protein-coupled receptor (GPCR) superfamily.

Receptor	***Agonists***
XCR1	CXCL1
XCR2	CXCL2
CX_3CR	CX_3CL1
CCR1	CCL3,CCL4,CCL5,CCL7,CCL14,CCL15,CCL16, CCL23
CCR2	CCL2,CCL6,CCL7,CCL13,CCL16
CCR3	CCL 5,CCL7,CCL8,CCL11,CCL13,CCL15, CCL24, CCL26,CCL28
CCR4	CCL17,CCL22
CCR5	CCL3,CCL4,CCL5,CCL8,CCL11,CCL14
CCR6	CCL20
CCR7	CCL19,CCL21
CCR8	CCL1,CCL16
CCR9	CCL25
CCR10	CCL27,CCL28
CCR11	CCL18
CXCR1	CXCL1,CXCL8,CXCL6
CXCR2	CXCL1,CXCL2,CXCL3,CXCL5,CXCL8
CXCR3	CXCL9,CXCL10,CXCL11
CXCR4	CXCL12
CXCR5	CXCL13
CXCR6	CXCL16
CXCR7	CXCL12

Table III. Structural classification of the chemokine families. This table shows the lack of selectivity in ligand binding, since many chemokines can bind to different receptors, and vice-versa.

A remarkable feature of the chemokine receptors is their relative lack of selectivity in ligand binding (see table III). With many chemokine receptors binding more than one chemokine with high affinity, one single chemokine can bind different receptors, as illustrated by CCL5 which binds to CCR1, CCR3 and CCR5. This phenomenon might explain the overlapping function of many chemokines.

A general model for plasma membrane insertion of the chemokine receptors has been suggested based on the analogy with the rhodopsin receptor, which is also a seven-transmembrane GPCR. Basically, the N-terminus part (containing the cysteine residues) of the receptor is located extracellularly, while the C-terminus is intracellular and initiates the intracellular cascade following the binding of the chemokine to its receptor. Each of the seven hydrophobic domains passes through the membrane, allowing three extracellular and intracellular loops[199]. Chemokine receptors are expressed on cells either constitutively or following stimulation. While most chemokine receptors are expressed on cells at a relative low number (1000 to 20000 per cell), CCR3 is highly expressed by eosinophils (40000 to 50000 per cell)[200].

Chemokine binding to its receptor initiates a conformational change that leads to a dissociation of the receptor associated hetero-trimeric G proteins into α and $\beta\gamma$ subunits. These proteins can then further activate various effector enzymes such as phospholipases, which induce inositol phosphate production, increase in intracellular Ca2+ and protein kinases. This activation of intracellular pathways culminates in the initiation of chemotaxis, phagocytosis, expression of adhesion

molecules, and so on. Other pathways have been shown to be triggered following activation of the receptor. One of them involves the activation of NF-κB through the activation of protein kinase C by the Gα subunit. This transcription factor is generally associated with the activation of proinflammatory genes such as TNF-α and IL-1β [201, 202]. The MAPK pathways are also involved in the signalling of chemokines. There are three subtypes of MAPK, the ERK pathway is usually associated with proliferation and growth factors, while the c-Jun NH2-terminal kinase (JNK) and p38 pathways are more responsive to cellular stress[203]. These pathways have been associated with chemokine synthesis[204, 205]. ERKs and p38, but not JNK, are also involved in the chemotaxis of eosinophils, through the activation of CCR3. Lastly, the regulation of adenylyl cyclase (AC) is another pathway, which can be either stimulated or inhibited depending on the chemokine, the receptor and the G protein subunit involved[206]. One of the direct effects of activation of AC is the increase of cAMP. CCL15 is a potent ligand for CCR1, CCR3 and CCR5 and has been shown to increase cAMP levels and subsequently the PKA/CREB (cAMP response element binding) pathway[207]. To simplify a very complex picture, we can state that the βγ subunit activates AC, while Gα inhibits it[206].

Another interesting aspect of chemokine receptor biology is the phenomenon of homo and heterodimerization following the binding of the ligand to its receptor. Depending on the chemokine concentration and the implicated receptor, chemokine receptors will either homo or heterodimerize, triggering different signalling pathways[208]. Homodimerization has been reported for

different receptors of the CC and CXC families, including CCR2, CCR5 and CXCR4[209]. These receptors, when stimulated, induce activation of different Janus activated kinase (JAK)/STAT family members. CCL5 through the stimulation of CCR5 promotes JAK1 and subsequently STAT5 transcriptional factor activation, while stimulation of CCR1 with CCL3 and CCL5 leads to the activation of STAT1 and STAT3 in T cells. On the other hand, heterodimerization of CCR2 and CCR5 has the capacity to activate cells at a lower concentration of the ligand (10 to 100 fold lower) and to trigger cell adhesion through an alternative pathway which has not been clearly identified but seems to involve Gq11, a subunit of Gq family proteins[208].

An interesting aspect of the chemokine receptor comes from its dynamic expression by leukocytes depending on the environmental milieu. Once the cells have been primed by the adhesion to the endothelium or by cytokines, they selectively upregulate or downregulate chemokine receptors. An example of this phenomenon is in the development and migration of eosinophils. Developed eosinophils usually express CCR1, CCR3 and, to a lesser extent, CXCR4. However, cytokine-primed eosinophils that have migrated towards inflammatory sites, express additional chemokine receptors such as CXCR1, CCR2 and CCR5. Basophils, neutrophils and monocytes also undergo a similar process.

1.6.3 Functions of chemokines

When added to *in vitro* cultures, chemokines can activate different leukocyte functions including chemotaxis, cytoskeletal reorganization, upregulation and activation of adhesion molecules and granule enzyme release by

myeloid cells such as neutrophils and eosinophils[188, 190, 210]. CXCL8 for instance, is a potent chemoattractant and activator of neutrophils. It induces the secretion of granular enzymes such as myeloperoxidase, β-glucuronidase, elastase, and gelatinase[211]. CXCL8 also induces the production and secretion of leukotriene B4 and oxygen radicals. Most of these functions are associated with an increase in the level of intracellular Ca2+, which is commonly used as an indicator of receptor activation. In addition to the previous functions, chemokines can also regulate cell proliferation and differentiation. Indeed, they have been implicated in myelopoiesis (CC chemokines mainly), angiogenesis (CXC ELR+ promote while CXC ELR- inhibit angiogenesis), and regulation of tumor cell growth[212-215]. Appropriate migration and homing of immune cells plays a primary role in T and B cells development. Chemokines become crucial in that process since they drive and direct the movement of maturing cells towards the secondary lymphoid organs, sites of further maturation for both families of lymphocytes. For example, in CXCR4 knockout mice, the number of B cells is dramatically reduced as well as the number of myeloid cell progenitors in bone marrow[216].

More importantly for us, chemokines have also been involved in T helper cell development. Differentiated Th1 and Th2 cells display a specific set of chemokine receptors. Th1 cells express CCR5, CXCR3 and CXCR6, whereas Th2 cells express CCR3, CCR4 and CCR8[217]. It is noteworthy to mention that this selective expression is not absolute as there are reports which indicate overlap between the two lymphocyte subsets[218].

1.6.4 Chemokine and chemokine receptors in asthma

First evidence of the implication of chemokines in asthma pathogenesis comes from the description of a novel eosinophil chemotactic agent found in bronchoalveolar lavage (BAL) fluid of an animal model of asthma[219]. This observation was later corroborated in human asthmatics in our lab when we demonstrated that CCL11 was increased in the BAL fluid of allergic patients[220]. Since then, the expression of several other chemokines during asthmatic disease has been well established[221]. CC chemokines such as CCL2, CCL3, CCL5, CCL7 and CCL8 have been shown to be upregulated in asthmatic airways, thereby highlighting the importance of these molecules to the pathogenesis of asthma[222-224]. Because eosinophil infiltration within asthmatic airways is a hallmark of the disease, a lot of the initial work on chemokines has focused on those that have chemotactic activity for these cells. In addition to CCL11, which is one of the most potent chemokines for eosinophils[225], CCL3, CCL5, CCL7 and CCL13 also elicit recruitment and degranulation of these cells[226]. CCL11, CCL7, CCL8, CCL2 and CCL5 are all ligands for CCR3. This receptor is expressed at high levels by eosinophils, but also by Th2 lymphocytes, mast cells and structural cell such as epithelial cells[80]. In addition to their effects on eosinophils migration in asthmatic airways, chemokines also recruit lymphocytes from the peripheral circulation. CCL1, CCL11, CCL17 and CCL22 have been shown to selectively attract Th2 lymphocytes, through the activation of CCR8, CCR3 and CCR4[227]. As previously mentioned, Th2 cells are the cornerstone of asthma pathogenesis and the importance of chemokines in the inflammatory process mediated by lymphocytes is likely to be crucial for the initiation and the perpetuation of the

disease. Several studies found that CCL11 neutralization reduces both airway inflammation and AHR[228-230]. More specifically, it has been shown that inhibition of CCL11 decreases trafficking of eosinophils and Th2 cells. However, in CCL11-/- knockout mouse, eosinophilic infiltration and allergic airway inflammation are not totally suppressed, highlighting the phenomenon of redundancy between chemokines and chemokine receptors[231].

The expression of specific chemokines at different time point of an allergic exacerbation suggests that there might be profiles of chemokines that mediate various stages of the disease. For example, the expression of CC chemokines such as CCL3, CCL2 and CCL5 in BAL fluid is upregulated 4 to 6 hours following endobronchial allergen challenge, while the levels of CCL11 are increased as quickly as 2 hours following a similar challgen, coinciding with the peak of recruitment of eosinophils within the airways[222, 232, 233]. The upregulation of chemokines during an active inflammatory process is controlled by several chemokines. Inflammatory cytokines, such as TNF-α and IL-1β have been shown to induce the expression of several chemokines, including CCL11, CCL5 and CCL13[234]. Synergism between IFN-γ and TNF-α has been reported regarding the induction of CCL11, CCL5, CXCL10 and CXCL9[235, 236]. IL-4 and IL-13, two Th2 cytokines, can also regulate the production of chemokines[237-243]. More recently, the effect of CCL11 on the regulation of CXCL8 production was also demonstrated. Collectively, these data open new horizons on the multiple roles of chemokines in the regulation of the immune response and the influence of the cytokine milieu on the expression of these chemokines[244].

The contribution of chemokine receptors has been thoroughly studied in asthma using gene-deficient mice. Of particular interest for this thesis, CCR1-deficient mice in a model of allergic airway disease showed a decreased number of goblet cells and diminished subepithelial fibrosis, suggesting the involvement of this receptor in airway remodelling[245]. CCR3 knockout mice had a reduction in the number of eosinophils in the airways after allergen challenge [246]. Unexpectedly, these animals had increased AHR, highlighting the controversial association between eosinophils and AHR. However, it is important to point out that animal models of asthma might not reflect all the components involved in the human disease. CCR3 null animals sensitized epicutaneously, followed by airway challenges with ovalbumin (OVA), showed that neither infiltration of eosinophils or AHR could be inhibited, suggesting that the route of sensitization may modify the features of the disease[247]. Interestingly, when CCR3-/- animals are sensitized systemically, the eosinophils are trapped in the subendothelial space and fail to reach the tissue. This suggests that CCR3-ligands are more involved in the recruitment of these cells within the tissues rather than out of the circulation. This observation implies an association between CCR3 and other chemokines or cytokine receptors in the whole process of eosinophil migration towards peripheral tissues.

Among the other chemokine receptors that have been studied in animals, CCR4, CCR5 and CCR6 were revealed to be potentially involved in asthma pathogenesis, since the knockout of their gene leads to a decreased number of eosinophils and reduced AHR[248]. All of these results indicate the involvement of several chemokine receptors in the recruitment of one single population of cells in

a specific disease, emphasizing the complexity of the chemokine network and the overlap of chemokine and chemokine receptor functions.

1.6.5 Cytokines and chemokine in airway hyperreactivity

As we highlighted in section 1.2.3, AHR is an important feature of asthma pathogenesis. In the last decades, researchers have attempted to identify the mediator(s) that could either induce bronchoconstriction of ASMC or render the cells more responsive to bronchoconstrictors. Several cytokines have been identified in animals as being potentially linked to the development of AHR in asthma[226]. When exogenously administered, IL-4, IL-5, IL-9, IL-11, IL-13 and GM-CSF increased the response of the airways to constrictor agonist. Two of the most important cytokines involved in this process seems to be IL-4 and IL-13 since the disruption of these two genes in mice totally abrogates AHR following antigen challenge[249-251]. However, it has also been shown in different experimental models, that AHR can be induced in a IL-4-independent mechanisms[252, 253]. Implication of IL-5 and IL-13 in development of AHR has also been shown in animal models of allergic airway disease, although the mechanisms underlying this observation might involve IL-4[254, 255].

Chemokine implication in the initiation of AHR has been supported by few studies. Campbell *et al.* showed that administration of CCL2 could induce AHR to metacholine in a murine model of cockroach allergen-induced airway disease[256]. The effects of CCL2 on AHR in this model are possibly mediated by the activation of CCR2 on mast cells, as suggested by Campbell et al[256]. The same author also showed that neither CCL11 nor CCL3, when injected alone, could

induce AHR, although these two chemokines have been involved in AHR in other studies[229, 257]. Mattes *et al.* demonstrated that CCL11-deficient mice show a partial reduction of eosinophil numbers in the airways, while the AHR was not modified. However, when animals deficient for both IL-5 and CCL11 were used, both airway eosinophilia and AHR were totally inhibited suggesting the requirement of these two mediators in the induction of asthma symptoms in this model[68]. Whether the effects of chemokines on AHR are mediated through a direct action on ASMC or through the recruitment of inflammatory cells is still not clear. Evidence from literature indicates that the presence of inflammatory cells such as eosinophils, Th2 lymphocytes, macrophages and mast cells strongly correlate with AHR, while other studies have failed to establish such a relationship[77, 258-262]. Further studies will be required in order to assess the *in vitro* effects of chemokines on ASMC contractility.

1.6.6 Cellular source of chemokines

Cellular sources of chemokines within the airways of asthmatic individuals include inflammatory cells but also structural resident cells. Activated macrophages and lymphocytes were believed to be the major source of chemokines in the airways of asthmatic subjects; however, the concept has evolved in the last few years, while researchers have demonstrated that epithelial cells, fibroblasts and airway smooth muscle also have the capacity to produce chemokines. For example, CCL3, CCL7 and CCL22 are released in high amounts by macrophages whereas CCL5, CCL11 and CCL13 seem to be predominantly produced by structural cells.

1.6.7 Chemokines and chemokine receptors in ASMC

It has been postulated that chemokines released by ASMC might significantly contribute to the chemokine signal generated by inflammatory cells[263]. In asthma, ASMC could quickly release CC chemokines following allergen inhalation and then contribute to the rapid mobilization of inflammatory cells such as eosinophils and Th2 lymphocytes[3]. Expression of a wide variety of chemokines by ASMC has been shown in the last decade (see figure 3). They include CC chemokines such as CCL11, CCL5, CCL2, CCL7 and CCL8, and CXC chemokines such as CXCL8 [264-267]. These chemokines have been implicated in the pathogenesis of asthma, mainly through the recruitment of inflammatory cells such as eosinophils (CCL11, CCL5, CCL2, CCL7, CCL8 and CCL13), lymphocytes (CCL11, CCL5, CCL17), neutrophils (CXCL8) and monocytes (CCL2, CCL5, CCL7 and CCL13). Because of the increased presence of smooth muscle in asthma and the release of cytokines such as TNF-α, IL1-β, IL-4 and IL-13, which have been shown to augment the release of CCL11, CCL5, CCL17 CCL2, CCL7, CCL8 and CCL13, the production of chemokines by ASMC is likely to play an important role in the initiation and the perpetuation of the asthmatic inflammatory response[268].

Studies of chemokine receptor expression by structural cells are a recent undertaking. Among the different structural cell populations found in the lungs, only endothelial and epithelial cells have been shown to express chemokine receptors in humans. Bronchial epithelial cells have been shown to express CCR3, CXCR3 and CXCR4[80, 269, 270]. While CCR3 binds mainly to CCL11,

CCL5, CCL7, CCL8 and CCL13, CXCR3 binds to CXCL9, CXCL10 and CXCL11, and CXCR4 binds to CXCL12. Although these receptors were shown induce an intracellular signalling on epithelial cells, their functions remain to be clarified. However, according to Kelsen *et al.*, the presence of chemokine receptors at the surface of epithelial cells could be associated with regulating the migration of these cells in response to certain chemokines as well as in mediating events associated with airway remodelling[269]. Endothelial cells have also been shown to express chemokine receptors, including CXCR3, CXCR4 and CCR1, CCR2, CCR3 and CCR8[271-277]. Proliferation, migration and cell activation are among the functions associated with the presence of these receptors.

Since the publication of our paper studying the expression of CCR3 by ASMC, another group showed the expression of CCR7 by these cells in the context of asthma[278, 279]. VSMC have been shown to express few CC and CXC chemokine receptors, including CXCR4, CCR3 and CCR5[165, 168, 272, 280]. The effects of chemokines on VSMC function comprise migration and regulation of cell activity[166, 189, 273].

CHAPTER II: CCR3 Expression and Function in Asthmatic Airway Smooth Muscle Cells

2.0 Prologue

Contribution of ASMC to asthma pathogenesis is not restricted to a contractile cell. In chapter I, we discussed the capacity of ASMC to produce cytokines and chemokines and to express receptors that make them potential targets for the inflammatory response characterizing asthma reaction. Chemokines are important proteins produced by inflammatory and structural cells and are mainly associated with recruitment of inflammatory cells. Recently, expression of a chemokine receptor has been described in epithelial cells, although the significance of this receptor on these cells remains unknown. In the following chapter, we study the expression of CCR3, a receptor for CCL5, CCL7 CCL13, CCL24 and CCL26. All these chemokines are believed to play a role in asthma pathogenesis and have been shown to be upregulated in the airways of asthmatic patients. We also assess the functional relevance of the expression of this receptor at the surface of ASMC.

CCR3 Expression and Function in Asthmatic Airway Smooth Muscle Cells[1]

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2.1 Abstract

Asthma is characterized by an increase in airway smooth muscle mass and a decreased distance between the smooth muscle layer and the epithelium. Furthermore, there is evidence to indicate that ASMC express a wide variety of receptors involved in the immune response. The aims of this study were to examine the expression of the CCR3 on ASMC, to compare this expression between asthmatic and non-asthmatic subjects, and to determine the implications of CCR3 expression in the migration of ASMC. We first demonstrated that ASMC constitutively express CCR3 at both mRNA and protein levels. Interestingly, TNF-alpha increases ASMC surface expression of CCR3 from 33 to 74%. Furthermore, using FACs analysis, we found that ASMC CCR3 is expressed to a greater degree in asthmatic versus control subjects (95% *vs* 75%). Functionality of the receptor was demonstrated by calcium assay; the addition of CCR3 ligand eotaxin to ASMC resulted in an increase in intracellular calcium production. Interestingly, ASMC was seen to demonstrate a positive chemotactic response to eotaxin. Indeed, ASMC significantly migrated towards 100 ng/ml of eotaxin (2.2 fold increase, compared to control). In conclusion, the expression of CCR3 by ASMC is increased in asthmatics, and our data shows that CCR3 ligand such as eotaxin induces migration of ASMC *in vitro*. These results may suggest that eotaxin could be involved in the increased smooth muscle mass observed in asthmatics through the activation of CCR3.

2.2 Introduction

Asthma is a common, chronic, inflammatory condition of the respiratory system, associated with paroxysmal bronchospasm, bronchial hyperresponsiveness and airway remodelling [281, 282]. Many factors, such as inflammatory mediators, have been implicated in the initiation and the perpetuation of airway remodelling [226, 282], with increases in airway smooth muscle mass being one of the major structural changes described in the airways of asthmatics [139, 170, 283]. Mechanisms responsible for this phenomenon are thought to be an increase in cell proliferation (hyperplasia) and/or an increase in the size of individual cells (hypertrophy) [124]. Recently, it has been proposed that migration of the ASMC toward the epithelium might also contribute to this phenomenon [182, 183]. These migrating cells could originate from deep smooth muscle bundles or from bone marrow cells, in a similar fashion to the migration of vascular smooth muscle cells in vascular diseases [284].

Airway smooth muscle was traditionally considered to be a structural cell involved primarily in bronchoconstriction. However, it has been recently shown that ASMC could play an important part in regulating local immune response. ASMC produce inflammatory cytokines and chemokines such as interleukin IL-1β, IL-6, IL-8, eotaxin and RANTES, that may act in both an autocrine and paracrine manner [285]. ASMC also express mRNA and immunoreactivity for a number of cytokine receptors [147]. To our knowledge, however, there has been no report on the expression of chemokine receptors in airway smooth muscle cells. CCR3 is a receptor for eotaxin-1 (CCL11), 2 (CCL24), 3 (CCL26), MCP-2

(CCL8), 3 (CCL7), 4 (CCL13) and RANTES (CCL5). Expression of CCR3 and eotaxin have been shown to be increased in asthmatic lungs and have been linked to the pathogenesis of asthma [286, 287]. We therefore hypothesized that ASMC express CCR3, and that eotaxin promotes the migration of ASMC through the activation of CCR3.

The aims of this study were to demonstrate the expression of CCR3 by ASMC and to compare this expression between asthmatic and control subjects. In order to determine the presence and the functional relevance of this receptor on ASMC, we assessed the effect of eotaxin on ASMC function. Our results provide evidence for the expression of a functional CCR3 by ASMC. Furthermore, we have shown that ASMC migrate toward a gradient of eotaxin. This data suggests that the upregulation of CCR3 by ASMC in asthmatic subjects might be partly responsible for the increased smooth muscle mass and the airway hyperresponsiveness observed in such patients.

2.3 Materials and Methods

2.3.1 Cell culture

Human ASMC were obtained from two sources. B/TSMC were purchased from Clonetics (San Diego, CA, USA) and were positively stained for α-smooth muscle muscle actin, and negatively for factor VIII, CD45, and CD3, as indicated by the manufacturer. B/TSMC were grown in their optimal medium (SmGM-2; Clonetics) containing 5% FBS at 37°C in a humidified incubator with 5% CO_2, as recommended by the supplier. The second source of ASMC was from human bronchial biopsies. ASMC were isolated and purified from biopsies as described by Labonté *et al.* [288]. Briefly, bronchial biopsies obtained from mild steroid-naïve asthmatic subjects (mean age: 25 years; metacholine PC20 lower or equal 4.33 mg/ml; positive skin prick test for at least one inhaled allergen) and non-asthmatic non-allergic subjects (mean age: 29.3 years; metacholine PC20 higher than 24.4 mg/ml; negative skin prick test for the inhaled allergens) underwent four consecutive cycles of enzymatic digestion with collagenase (Roche, Manheim, Germany) and/or elastase (Sigma Chemical Co., St. Louis, MO) for 20 min at 37°C. Cells were then plated in flasks and cultured in complete DMEM-F12 media containing 10% FBS, 20 U/ml penicillin, 20 ug/ml streptomycin, 25 ng/ml fungizone, 5 mg/ml insulin, 10 ng/ml epidermal growth factor, 5 ug/ml transferrin, 10^{-10}M cholera toxin and 2 x 10^{-9}M T3. The media was replaced every 2-3 days and cells were passaged with 0.5% trypsin and 1 mM EDTA once confluence was reached. Cell identity was assessed by measuring the expression of α-smooth muscle actin, calponin, smooth muscle myosin heavy chain (SM-1

and SM-2), tropomyosin, desmin and vimentin [289]. ASMC demonstrated the characteristic hill and valley appearance, with an elongated and spindle shape, and possessing a central nucleus.

2.3.2 Cell stimulation

Confluent B/TSMC from Clonetics were used in passages 3-6 while ASMC from biopsies were used in passages 2-3. Cells were growth-arrested by FBS-deprivation for 24 hrs prior to stimulation with cytokines. After serum-deprivation, cells were stimulated in fresh, serum-free media, containing either TNF-α, IL-4, IL-13 or IFN-γ (R & D Systems, Minneapolis, MN) in a concentration and time-dependent manner. Cell viability was assessed by the Trypan blue dye exclusion test.

2.3.3 RNA extraction and RT-PCR

Total cellular RNA was isolated from B/TSMC, epithelial cells (calu-3), purified peripheral blood eosinophils and neutrophils, and CCR3-transfected Ghost-cells (National Institute of Health, AIDS reagent program, #3682). RNA was extracted using the RNeasy mini kit extraction columns (Qiagen, Mississauga, ON, Canada) as directed by the manufacturer. RNA was eluted in 30 μl nuclease-free water, and cDNA was generated in a 30 μl reaction, using 0.5 μg of total RNA, oligo(dT)12-18 primers (Amersham Pharmacia Biotech, Baie d'Urfé, QC, Canada) and Superscript II (Invitrogen, Burlington, ON, Canada), in the presence of RNAguard (Amersham Pharmacia Biotech, Baie d'Urfé, QC, Canada). Genes

of interest were amplified using conventional PCR. The PCR mixture consisted of 1.5 mM $MgCl_2$, 1X PCR buffer, 0.25 mM dNTP mixture, 2.5 units *Taq* Platinum polymerase (Invitrogen, Burlington, ON, Canada), 0.4 μM of each sense and antisense primers, and 1 μl of cDNA. Primers for the housekeeping gene ribosomal protein S9 and CCR3 were commercially generated (Invitrogen, Burlington, ON, Canada) using the following sequences: S9 sense 5'-TGC TGA CGC TTG ATG AGA AG-3'; antisense 5'-CGC AGA GAG AAG TCG ATG TG-3'; CCR3 sense 5'-TCC TTC TCT CTT CCT ATC AAT C-3'; antisense 5'-GGC AAT TTT CTG CAT CTG-3'. The samples were amplified in a thermal cycler (PTC-100, Programmable Thermal Controller, MJ Research Inc. Watertown, MA, USA) for 35 cycles, consisting of denaturation at 95°C, annealing at 57°C, and extension at 72°C. The PCR products were visualized on a 2% agarose gel containing 0.2 ug/mL ethidium bromide and correct band size was determined by comparison with a 100 bp DNA ladder (Invitrogen, Burlington, ON, Canada). Amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Mississauga, ON, Canada), ligated in pGEM®-T (Promega, Madison, WI, USA), and used for transformation into XL-1 blue bacteria. Plasmids representing each insert were purified using a HiSpeed Plasmid Maxi Kit (Qiagen, Mississauga, ON, Canada) and commercially sequenced to confirm integrity and identity (Pavillon de synthèse et d'analyse d'acides nucléiques, Université Laval, QC, Canada).

2.3.4 Immunohistochemistry

To determine whether ASMC have the capacity to produce CCR3 *in vivo*, immunohistochemistry was performed on sections of major airways (large bronchus) from 4 asthmatics and 4 normal subjects. The subjects were obtained from the Tissue Bank (MCI/Meakins-Christie Tissue Bank, McGill University). A clinical diagnosis of asthma was made based of the evaluation of the patient's medical file by a respiratory physician. Diagnostic criteria included prior diagnosis and treatment for asthma, documented evidence of variable airflow obstruction greater than 15%, and bronchial hyperresponsiveness. Immediately following biopsies, lung specimens were prepared for immunohistochemistry. Briefly, formalin-fixed tissues were paraffin-embedded and 5-μm-thick sections were prepared, deparaffinized in xylene, and hydrated through a graded alcohol series. Endogenous peroxidase was quenched by incubating the slides in 0.5% hydrogen peroxide in PBS for 30 min. After rinsing in PBS, sections were blocked with blocking solution (Dako Corporation, Carpinteria, CA) for 20 min at room temperature (RT). Primary Ab diluted in diluting buffer (Dako Corporation, Carpinteria, CA) was applied (5 μg/ml, goat polyclonal Ab anti-CCR3; Santa Cruz Biotechnology, Santa Cruz, CA) and sections were incubated overnight at 4°C. Control sections were incubated with isotype control (5 μg/ml, normal goat IgG; Caltag laboratories, Burlingame, CA). After rinsing in PBS, a biotinylated rabbit anti-goat Ab (1:100 dilution; Dako Corporation, Carpinteria, CA) was applied, and sections were incubated for 60 min at RT. Sections were thoroughly washed in PBS and incubated with the streptavidin-HRP conjugated for 60 min at

RT. After PBS washes, the reaction was revealed using 0.5 mg/ml diaminobenzidine tetrahydrochloride (DAB) in Tris buffer (pH 7.6) as the chromogen and 0.05% hydrogen peroxide as the substrate for 5 min. Sections were counterstained with hematoxylin and mounted.

2.3.5 Western blotting

Cells were rinsed twice in ice-cold PBS, and incubated on ice for 30 min with lysis buffer (150 mM NaCl; 10 mM Tris-HCl pH 7.4; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 0.5% NP40) containing a mini-complete protease inhibitor cocktail tablet (Roche Diagnostics, Laval, QC, Canada). Extracts were clarified at 14,000 x g at 4°C for 20 min, and protein concentrations were determined using the Bradford assay (Bio-Rad, Mississauga, ON, Canada). Using one-dimensional SDS-PAGE, 20 µg of protein extracts were resolved and electrophoretically transferred to Hybond polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech). Membrane blocking was achieved using 5% non-fat dry milk in TTBS (0.1% Tween-20, 10 mM Tris-buffered saline, pH 7.5) for 2 hr at RT, and then washing twice for 2 min with TTBS. Membranes were incubated for 1 hr with anti-CCR3 Ab (Santa Cruz, CA, USA; 1/2000) after which a 1 hr incubation at RT with an alkaline phosphatase conjugated anti-goat Ab (1/1000) was performed. The bound secondary Ab was detected using the CSPD chemiluminescence detection kit (Roche Diagnostics, Laval, QC, Canada). Following a double rinsing with PBS the signal was visualized on Kodak Bio-Max X-ray.

2.3.6 Flow cytometric analysis

FACS analysis was performed as follows: B/TSMC and ASMC derived from normal (n=4) and asthmatic subjects (n= 3) were detached from the flask by addition of a solution of PBS-EDTA (0.5 M) for 20 min at 37°C. Cells were resuspended at a concentration of 1 x 10^6 cells/ml, washed once with PBS, and then incubated with purified normal human IgG (Santa Cruz, CA, USA) at 4°C for 20 min to block any nonspecific binding. PE-conjugated anti-CCR3 (FAB155P, clone 61828.111, R&D Systems, Minneapolis, MN, USA) or control isotype (Rat IgG_{2A}; IC006P, clone 54447, R&D Systems, Minneapolis, MN, USA) Abs were incubated with the cells at 4°C for 30 min and after three washes with PBS-BSA 0.5%, cells were resuspended in PBS at 4°C. Cell-associated immunofluorescence was immediately analysed using FACS (Becton Dickinson, Mississauga, Canada) in order to determine the level of surface expression of CCR-3. At least 10000 cells were counted per analysis and ASMC were gated in order to include only viable cells. CCR3 was also identified using fluorochrome-labeled eotaxin (Fluorokine; R&D Systems, Minneapolis, MN, USA), and analyzed via flow cytometry. As described above, B/TSMC were prepared for labeling and cells were labeled with a Fluorokine Kit for Human CCR3 (Cytokine Flow Cytometry Reagent Biotin conjugate, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, 10 µl of biotinylated recombinant eotaxin reagent was added to 25 µl aliquots of washed cells (10^5) and incubated for 60 min on ice. Following the incubation period, 10 µl of streptavidin-FITC reagent was added, and cells were incubated for an additional 30 min at 4°C in the dark. Cells were then washed twice, using the buffer

provided in order to remove unreacted streptavidin-FITC, resuspended in 200 µl of PBS, and analyzed by flow cytometry. As a negative control, an identical sample of washed cells was incubated with 10 µl of biotinylated negative control reagent (supplied with the kit). The specificity of the reaction was assessed by mixing 20 µl of anti-human eotaxin blocking Ab with 10 µl of biotinylated eotaxin and incubating for 15 min at RT. The rest of the protocol is as described above.

2.3.7 Immunofluorescence detection of CCR3 in B/TSMC

B/TSMCs were cultured on glass cover-slips in a 6-wells plates until 40-50% confluency. Cells were then washed twice with PBS, and fixed with IntraPrep (Beckman-Coulter, ON, Canada) according to manufacturer direction. Cells were incubated overnight at 4^0C with either a rat mAb anti-human CCR3 (R&D Systems, Minneapolis, MN, USA; 5 µg/ml) or the control isotype Ab (Rat IgG_{2A}; R&D Systems, Minneapolis, USA). After washing with PBS, cells were incubated for 30 min at RT with biotinylated mouse mAb anti-rat IgG_{2A} (BD, Mississauga, ON, Canada; 2.5 µg/ml), followed by subsequent washings and incubation with streptavidin conjugated to Alexa594 (Molecular Probes, Burlington, ON, Canada). After a final wash, nuclei were counterstained with DAPI (Sigma, Oakville ON, Canada), slides were mounted, and cells were imaged on an Olympus inverted-phase microscope (IX-81) using a mounted digital camera (CoolSnapPro CF monochrome) equipped with a CRI filter. Images were

analyzed using ImagePro Plus software (Carsen Group Inc. Markham, ON, Canada).

2.3.8 Measurement of intracellular free Ca^{++}

For calcium measurements, glass cover-slips with confluent serum-deprived B/TSMC were rinsed twice with pre-warmed (37°C) HEPES-buffered (10mM, pH 7.4) HBSS/1% bovine serum albumin, and incubated for 1 hr at 37°C in the buffer containing calcium-sensitive dye, fura-2 AM (10 μM) as previously described [290]. Thereafter, the fura-2 loaded cultures were washed twice with HEPES-HBSS and then incubated in the dark at RT for 30 min prior to measurement of intracellular calcium. Glass slides were mounted on an inverted microscope (Olympus IX70) equipped with an OlymPix TE3/A/S digital camera controlled through a PC workstation. Cells were alternatively exited at 340 and 380 nm using a lambda 10 filter (Sutter Instruments; Novato, CA, USA). Emitted fluorescence (510 nm) was measured for 350 ms at each excitable wavelength and collected data were used to calculate calcium concentrations (in nM) at each pixel from an *in vitro* calibration curve of known free Ca^{2+} (0–1.35 μM) and pentapotassium fura 2 (50 μM). An Olympus UAPO/340 20x/0.75 objective was used in all studies and image size was set to 540 x 540 pixels. Calcium responses within individual cells were determined using UltraView v4.0 software (Olympus LSR, Markham, ON, Canada), by circumscribing single myocytes and spatially averaging fura 2 fluorescence within each cell. At the beginning of each experiment, each chamber contained 200 μl of HEPES-HBSS. Intracellular free

Ca^{2+} was first recorded for 30 sec to establish a baseline, then the cells were stimulated by adding an equal volume of HEPES-HBSS containing either recombinant human eotaxin-1 or MCP-4 (both at 50 ng/ml). Intracellular free Ca^{2+} was recorded for at least 200 sec to characterize peak and plateau responses and Acetylcholine (Ach) was used as a positive control. Studies were performed in duplicate using three cell lines, each acquired from a different donor.

2.3.9 Chemotaxis Assay

Migration of B/TSMCs in response to different concentrations of eotaxin (1, 10, 100 and 1000 ng/ml) was assessed in a 24-well microchemotaxis chamber (NeuroProbe, Cabin John, MD, USA) using a polycarbonate filter (8-μm pore size), as previously described [183]. Briefly, B/TSMCs were resuspended in Ham's F-12 media supplemented with 0.1% BSA, and 5 x 10^4 cells were then loaded into the upper chambers and tested for chemoattraction to media supplemented with either 0.1% BSA (negative control) or increasing doses of eotaxin. In some of the experiments, cells were incubated for one hour with 10 ug/ml of anti-CCR3 antibody (MAB155, clone 61828, R&D Systems, Minneapolis, MN, USA) prior to the loading of the cells into the chamber. The chambers were incubated at 37°C in 5% CO_2 for 4 hrs. Cells located on the upper surface of the filter were scraped off, and cells that migrated to the lower face of the membrane were stained with Diff-Quik. The number of migrated cells on the lower face of the filter was counted in five random fields using conventional microscopy.

2.3.10 Statistical analysis

Statistical significance was determined using a Student's *t* test. P values < 0.05 were considered statistically significant.

2.4 Results

2.4.1 ASMC constitutively express CCR3 at mRNA and protein levels.

In order to demonstrate the expression of CCR3 by ASMC at both mRNA and protein levels, we performed RT-PCR and western blot on RNA and proteins obtained from cultured ASMC, epithelial cells and CCR3-transfected cells, as well as from eosinophils and neutrophils purified from blood. Gel electrophoresis (Figure 1A) revealed bands corresponding to the expected size of the CCR3 cDNA product (313 bp). At the protein level, western blot revealed comparable expressions of CCR3 by ASMC, Calu-3 epithelial cells and CCR3-transfected cells, while eosinophils demonstrated greater signal intensity (Figure 1B). Surface expression of CCR3 by ASMC was confirmed using flow cytometry, and revealed a high percentage of unstarved serum cells expressing the receptor (see Figure 1C). In the left panel, a PE-labeled antibody against CCR3 was used. The specificity of the signal was confirmed by the addition of a CCR3 ligand: FITC-labeled eotaxin (see Figure 1C, right panel). This signal was completely suppressed when anti-eotaxin was added prior to eotaxin (see Figure 1C, right panel). Immunofluorescence was performed on unstimulated B/TSMC and demonstrated an increased signal compared to the isotype control. Also, a high percentage of cells stained positive for CCR3 (Figure 1D), confirming the previous results obtained by flow cytometry (Figure 1C).

2.4.2 CCR3 is expressed by ASMC in vivo.

To further investigate the protein expression of CCR3 in human airways, immunocytochemistry was performed. CCR3 immunoreactivity was detected in

ASMC in subjects with (Figure 2A) and without (Figure 2B) asthma. In asthmatic specimens, CCR3 protein was localized in the smooth muscle bundles, airway epithelium and infiltrating cells found within the submucosa, as previously demonstrated (data not shown) [80, 286].

2.4.3 TNF-α upregulates the expression of CCR3 by ASMC at the protein level.

Expression of chemokine receptors has been shown to be regulated by different cytokines [291]. In asthma in particular, the cytokine environment is characterized by increased TNF-α, IL-4 and IL-13 and decreased IFN-γ levels [60]. We investigated the effects of the addition of IL-4, IL-13, IFN-γ and TNF-α on B/TSM surface expression of the CCR3 receptor. Addition of IL-4, IL-13 and IFN-γ downregulated surface expression of CCR3 (33±5% to 23±8% of positive cells for IL-4 (p=0.14); 33±5% to 13±9% for IL-13 (p=0.06); 33±5% to 25±7% for IFN-γ (p=0.15) whereas TNF-α was shown to cause significant upregulation (33±5% to 74±8% of positive cells (p=0.001)) (Figure 3). The combination of TNF-α with either IL-4 or IL-13 did not modify the surface expression of CCR3, as seen with TNF-α, IL-4, IL-13 and IFN-γ alone (data not shown).

2.4.5 ASMC from asthmatics express more CCR3 than control ASMC.

Another objective was to compare the surface expression of CCR3 on ASMC between asthmatic and control patients using flow cytometry. Cells were used soon after initial passaging (P2-3) following isolation from asthmatic and non-asthmatic patients. From both groups, we examined the percentage of cells

expressing the CCR3 receptor as well as the intensity of this expression, *via* a mean assessment of their signal fluorescence. Our observations concluded a higher percentage of CCR3 expression (95±6% *vs* 75±2%, p<0,05), as well as a greater mean average fluorescence (21.5±4.3 *vs* 14.3±0.7, p<0,01) for ASMC isolated from asthmatics compared to cells isolated from non-asthmatics (Figure 4).

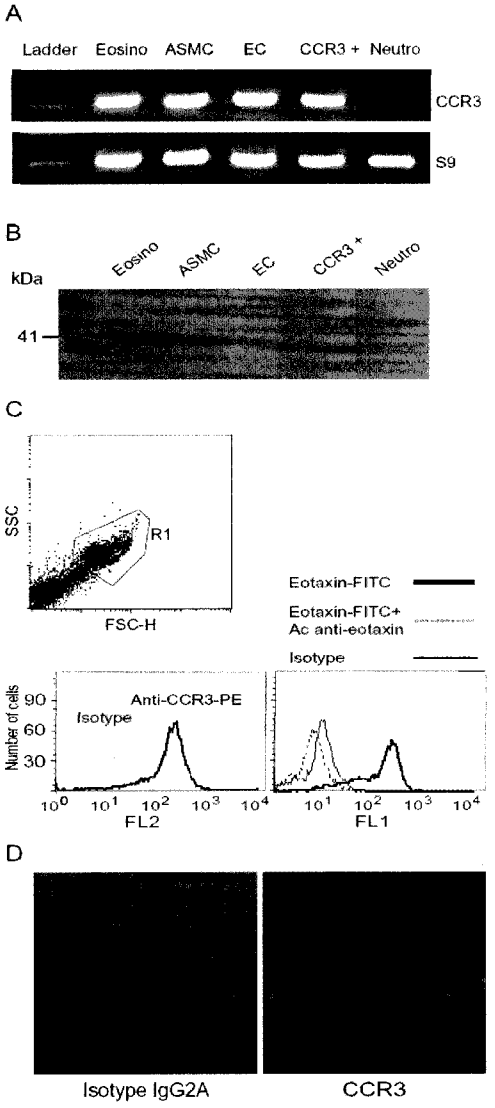
2.4.6 Addition of CCR3 ligands induces an increase in intracellular [Ca^{2+}].

In order to assess the functionality of CCR3, we stimulated B/TSMC with eotaxin and MCP-4. We observed a sharp increase in intracellular calcium[161] within smooth muscle cells following the addition of both eotaxin and MCP-4 (Figure 5). However, upon adding MCP-4 alone, we obtained a much weaker signal, presumably due to receptor desensitization. A similar desensitization phenomenon was observed when MCP-4 was used first to stimulate the cells, followed by addition of eotaxin, suggesting that the induction of intracellular [Ca^{2+}] is through the activation of CCR3 (results not shown).

2.4.7 Eotaxin induces migration of B/TSMC.

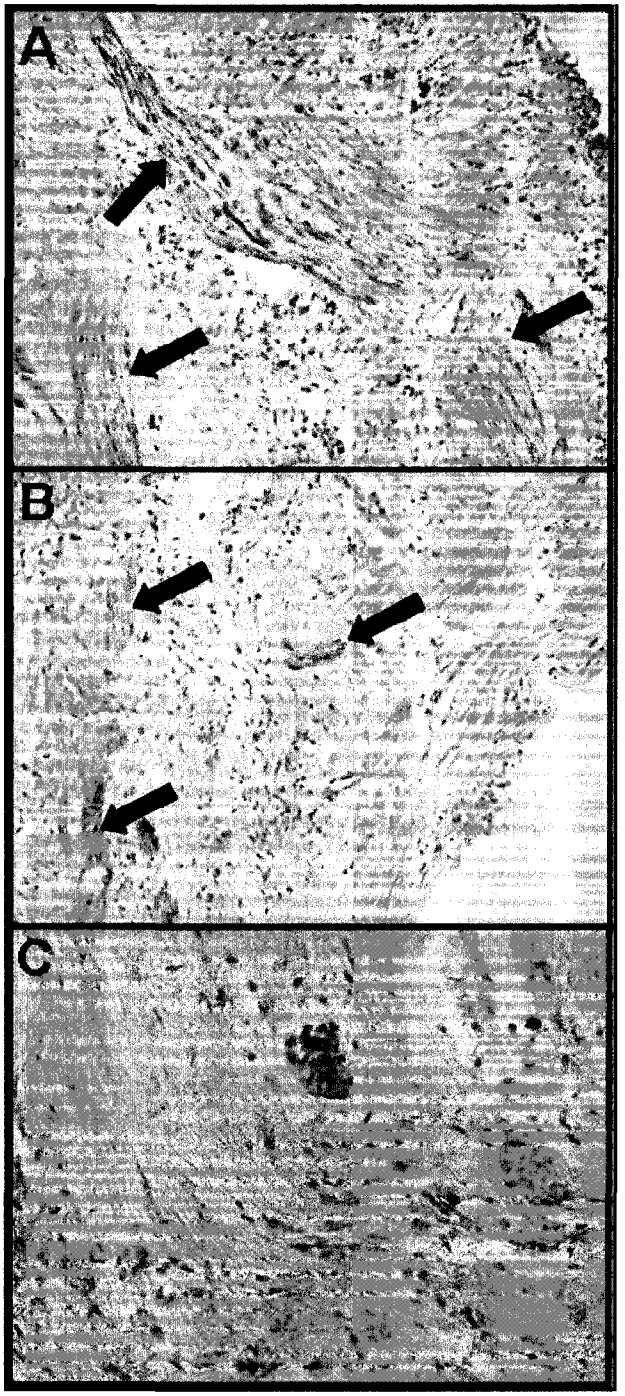
Characteristic of asthma, is the increase in smooth muscle mass, and the reduction in the distance between the smooth muscle layer and the epithelium, suggested to be associated with the migration of smooth muscle cells towards the smooth muscle layer. Consequently, we wanted to examine whether eotaxin could promote the migration of B/TSMC. As shown in Figure 6, eotaxin increased the migration of B/TSMC in a dose-dependent manner with a maximal response at

100 ng/ml (2.2±0.32 fold, compared to baseline). No significant differences between 100 ng/ml and 1000 ng/ml (2.06±0.48 fold) were observed when these two doses were compared, suggesting that 100 ng/ml is the peak dose for inducing migration of B/TSMC. PDGF was used as a positive control and increased cell migration by 2.5±0.30 fold, compared to the media. Migration of B/TSMC was totally abrogated (1.11±0.07) when the cells were preincubated with blocking anti-CCR3. Collectively, this data demonstrates the capacity of eotaxin to induce the migration of B/TSMC towards an increasing gradient of eotaxin, through the activation of CCR3. To address whether the obtained findings were the result of chemotaxis or chemokinesis, eotaxin was added to both upper and lower wells, and migration was examined after identical conditions of stimulation. As shown in Figure 6, the absence of a concentration gradient did not stimulate the migration of B/TSMC, confirming that eotaxin acts as a chemoattractant for B/TSMC. In order to assess the viability of the cells, ASMC were incubated with the same concentrations of cytokines or anti-CCR3 for 24 hours in a 6-well plate. No differences in cell viability were observed between the different conditions used.



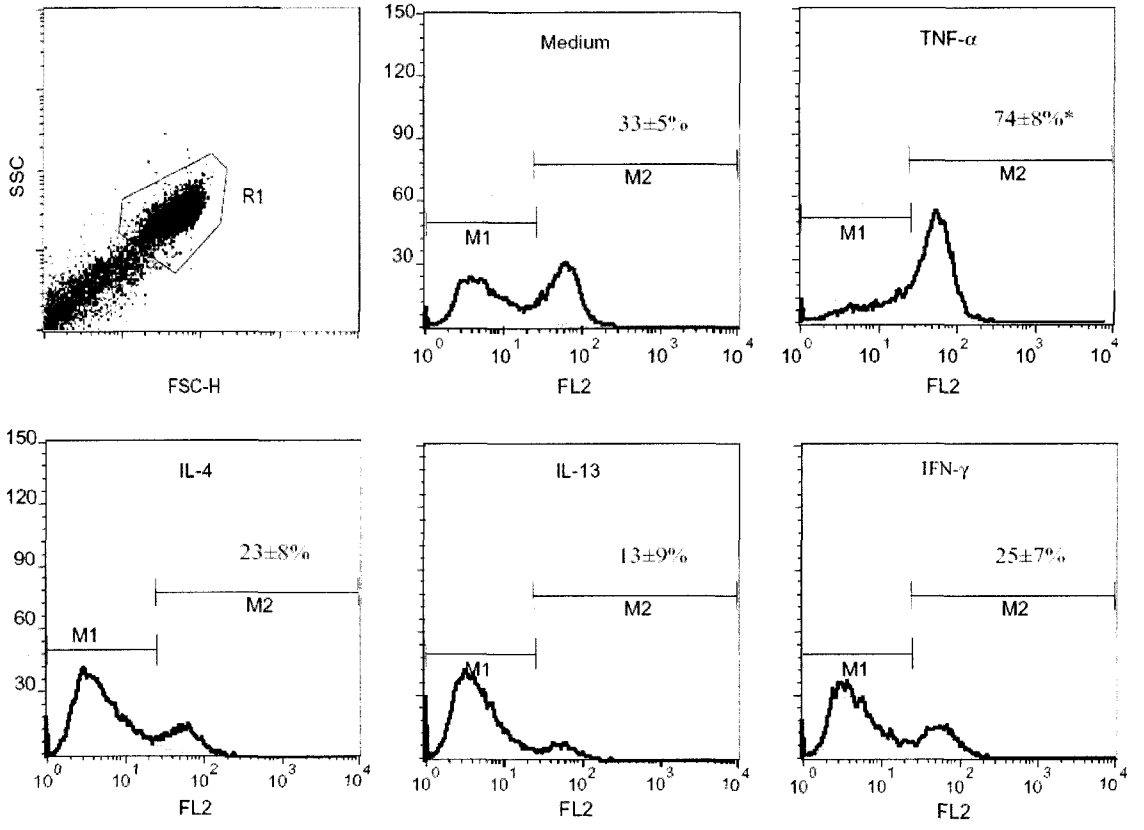
2.5 Figure 1

Detection of CCR3 in B/TSMC. *A,* RT-PCR and *B,* Western blot analysis of constitutive CCR3 expression by B/TSMC. Epithelial cells (EC), eosinophils (Eosino) and CCR3-transfected cells (CCR3 +) were used as positive controls, whereas purified neutrophils (Neutro) were used as a negative control (representative of n = 4). *C,* Determination of CCR3 surface expression by B/TSMC using flow cytometry. Confluent B/TSMC (P3-6) obtained from Clonetics were cultured and analyzed by flow cytometry for cell surface expression of CCR3. In the left panel, B/TSMC were labelled using a rat anti-CCR3 Ab and as a negative control, cells were labelled with isotype matched IgG_{2A}. A representative experiment out of five is shown. In the right panel, biotinylated human recombinant eotaxin was added to cultured B/TSMC in the absence or presence of anti-human eotaxin blocking Ab. A representative experiment out of two is shown. *D,* Immunofluorescent staining of CCR3 in unstimulated permeabilized B/TSMCs. Nuclei are stained blue with DAPI.



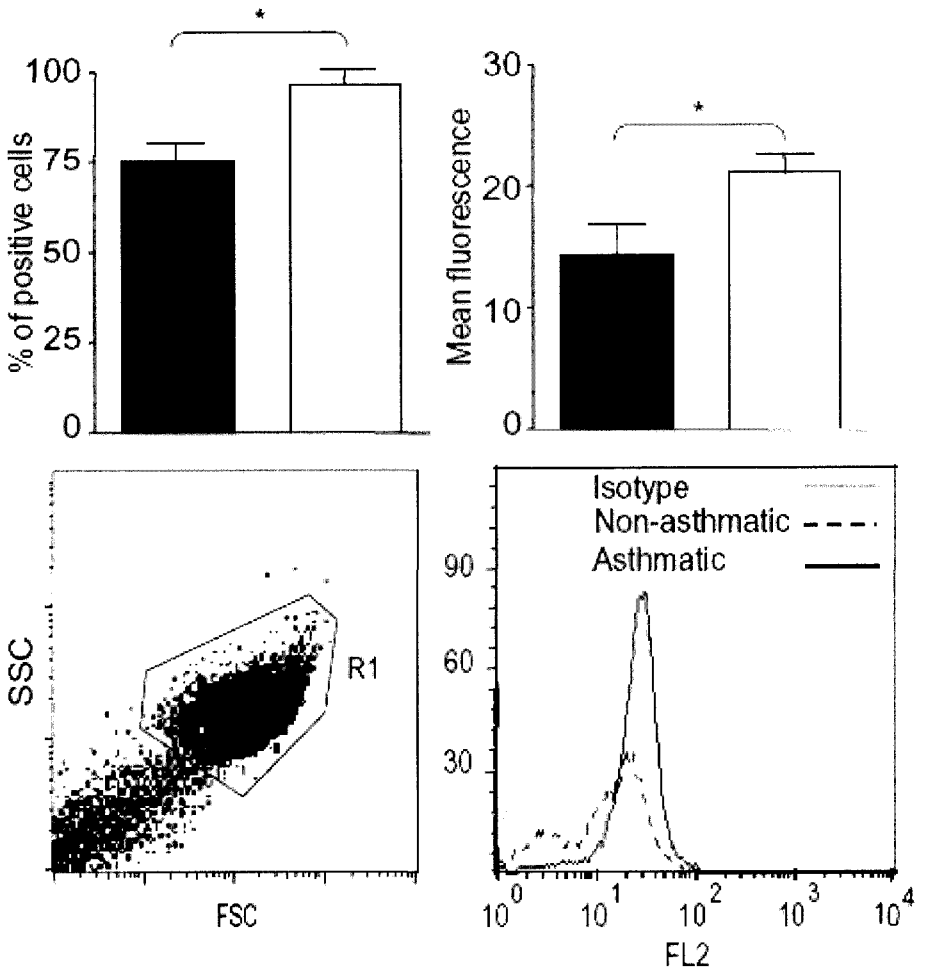
2.6 Figure 2

Expression of CCR3 in ASM *in vivo*. Cross-section of an intermediate airway from asthmatic *(A)* and control *(B)* subjects (representative of n=4 for each group) showing eotaxin immunoreacitivity and isotype control *(C)* in smooth muscle bundle (*large arrows*). Paraffin-embedded sections were prepared from human lung biopsies, and slides were incubated with anti-CCR3 polyclonal Ab, the appropriate secondary Ab, and a tertiary layer of streptavidin-HRP-conjugated. Sections were developed with DAB, with positive cells staining brown.



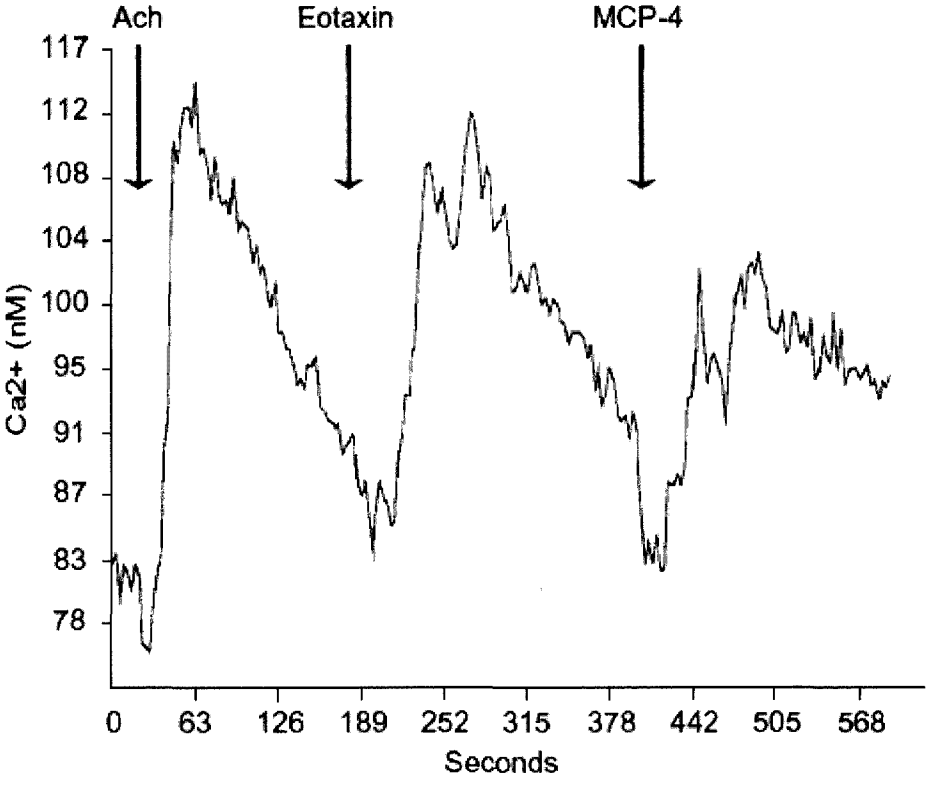
2.7 Figure 3

Regulation of CCR3 expression by B/TSMC using flow cytometry. Confluent B/TSMC (P3-6) cultured in serum-free medium were stimulated with or without TNF-α (10 ng/ml), IL-4 (20 ng/ml), IL-13 (20 ng/ml) or IFN-γ (10 ng/ml) for 24 hrs. Expression of CCR3 was measured by flow cytometry using a rat anti-CCR3 Ab. As a negative control, cells were labelled with isotype matched IgG_{2A} and the percentage of positive cells was calculated by subtracting the isotype control from the specific signal.



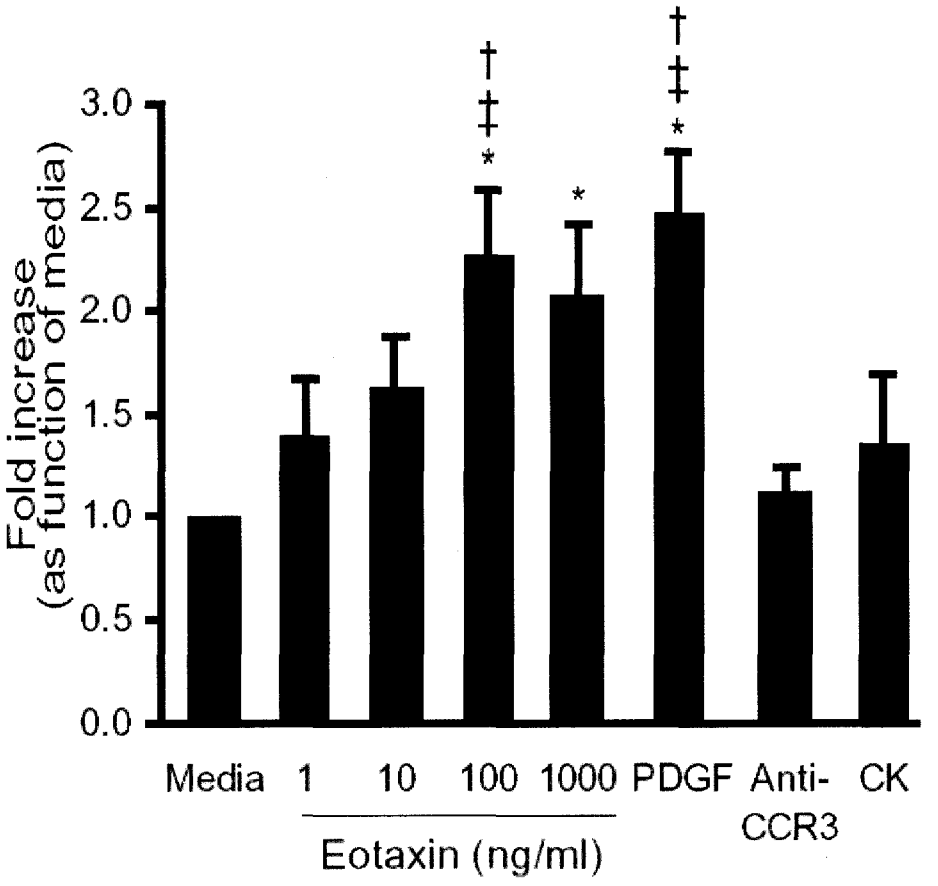
2.8 Figure 4

CCR3 expression by asthmatic and non-asthmatic ASMC. ASMC (P3) were obtained from Dr Michel Laviolette and Dr Jamila Chakir's group as previously described in Materials and Methods. CCR3 surface expression was assessed by flow cytometry using a rat anti-CCR3 Ab. As a negative control, cells were labelled with isotype matched IgG_{2A}. The percentage of positive cells and Mean Fluorescence Intensity (MFI) was calculated by subtracting the isotype control from the specific signal. * $p<0,01$ (n=3 for asthmatic group and n=4 for control group).



2.9 Figure 5

Measurement of Ca^{2+} in cultured B/TSMC in response to eotaxin and MCP-4. For calcium measurements, confluent serum fed B/TSMC were loaded with fura-2 as described in Materials and Methods. Cells were stimulated with either eotaxin or MCP-4 (50 ng/ml) and intracellular Ca^{2+} was measured for at least 350 s thereafter. Ach (10-60M) was used as a positive control. Data shown is representative of 3 experiments.



2.10 Figure 6

Cellular migration of B/TSMC in response to eotaxin. B/TSMC were incubated in a Boyden chamber for 4 h with increasing concentrations of eotaxin (1, 10, 100 and 1000 ng/ml). Eotaxin was loaded in both upper and lower wells to address whether the obtained findings were the result of chemotaxis or chemokinesis (CK). Platelet-derived growth factor (PDGF) was used as a positive control (20 ng/ml). * Significantly different from media, † significantly different from anti-CCR3, ‡ significantly different from CK; $p < 0,05$ (n=6, except for anti-CCR3 n=3).

2.11 Discussion

In the last few years, studies have shown that ASMC possess properties that would indicate a potential involvement in airway remodelling and inflammation. These properties include the expression of a variety of cytokines and chemokines as well as their receptors. The CC and CXC chemokines are important chemotactic molecules that control leukocycte trafficking and functions. These molecules also play an important role in regulation of leukocyte development, expression of adhesion molecules, cell proliferation and angiogenesis. CCR3 is a CC chemokine receptor that has been traditionally associated with recruitment of inflammatory cells implicated in the pathophysiology of asthma [292, 293]. At sites of allergic inflammation, increased expression of CCR3 and CCR3 ligands, such as eotaxin and RANTES, by inflammatory cells have been well characterized [287]. However, recent work has demonstrated that the expression of chemokine receptors is not restricted to leukocytes. Stellato *et al.* have shown that airway epithelial cells could also express CCR3 [80]. In this study, we examined the expression of CCR3 by ASMC and showed that the receptor is expressed both *in vivo* and *in vitro*. Functional studies demonstrate that CCR3 is a functional receptor, as it transduces intracellular calcium mobilization and induces ASMC migration. We have also demonstrated that CCR3 is up-regulated in bronchial smooth muscle cells of individuals with asthma, as compared with normal control subjects.

Asthma is a disease characterized by marked structural changes of the airway wall. Benayoun *et al.* [89] and Pepe *et al.* [354] have recently demonstrated that there is a decrease in the distance between the airway smooth muscle and

epithelial layers of asthmatic individuals. The possible migration of ASMC, either from the interstitial compartment or from a circulating precursor stem cell population, has been suggested as a possible mechanism to explain the increase in smooth muscle mass observed in the airways of asthmatics [182, 183]. The mediators involved in the migration of ASMC, however, must still be determined. We hypothesized that the increased levels of eotaxin in asthmatic airways could promote the chemotaxis of ASMC through the activation of CCR3. In the present study, we demonstrate that eotaxin is able to induce the migration of ASMC in a dose-dependent manner. Similar observations have recently been made concerning the migration of vascular smooth muscle cells in atherosclerosis [280]. *In vivo*, both epithelial and airway smooth muscle cells are potent producers of eotaxin [234, 294, 295], and thus may be responsible for the generation of an eotaxin gradient, allowing migration of ASMC toward the smooth muscle bundles and the epithelium. To support this hypothesis, Pepe *et al.* [354] have recently shown that there is an increased production of eotaxin by ASMC in severe asthmatics compared to control subjects. Furthermore, this increased production of eotaxin was seen to correlate significantly with the amount of smooth muscle found in the airways of asthmatic patients.

Recent studies strongly suggest that chemokine receptor expression in many cell types can be modulated by both inflammatory and anti-inflammatory signals [80, 228, 293, 296]. Pro-inflammatory, Th1 and Th2-cytokines have been shown to be potent mediators regulating the expression of CCR3 in lymphocytes, eosinophils and neutrophils, while TNF-α was shown to augment the expression

of CCR3 transcripts in epithelial cells. We examined the effects of IL-4, IL-13, IFN-γ and TNF-α on the expression of CCR3 in ASMC. Using flow cytometry analysis, we found that the expression of CCR3 was increased 24 hrs after stimulation with TNF-α. Interestingly, IFN-γ, IL-4 and IL-13 acted to slightly downregulate the cell surface expression of CCR3, suggesting that the Th2-biased immunological state observed in asthmatics is not likely to play a role in the migration of ASMC within airways. Rather, proinflammatory cytokines, such as TNF-α, are more likely to control the migration of ASMC towards the epithelium. Vijh *et al.* reported that the CCR3 promoter contains several transcription factor-binding sites for AP-1 [297]; a transcription factor implicated in the regulation of genes involved in the pathogenesis of asthma [123]. Also, AP-1 is strongly activated by TNF-α [298] and may explain the induction of CCR3 expression by ASMC. It is also possible that ASMC might be able to migrate toward the smooth muscle layer as a result of interactions between eotaxin and CCR3.

We demonstrated in our study that a higher percentage of asthmatic ASMC express CCR3 compared to non-asthmatic patients. This increased expression by asthmatic ASMC might be an inducible phenomenon related to the augmented expression of TNF-α in the airways of asthmatics [72, 299]. The increased expression of CCR3 on the cell surface attributable to an increased expression of TNF-α might render ASMC more responsive to the greater eotaxin levels observed in asthmatic airways [220]. These observations suggest that the cytokine environment in asthma could contribute to increased CCR3 production by ASMC and their increased responsiveness to eotaxin. A different phenotype linked to a

genetic duality between asthmatics and non-asthmatics could also explain this increased expression of CCR3 by ASMC.

We have also demonstrated that the addition of various CCR3 ligands, such as eotaxin and MCP-4, to cultured ASMC induced the release of intracellular calcium, suggesting efficient signalling through CCR3, as shown in epithelial cells [80]. Eotaxin alone was able to trigger an increase in intracellular calcium, supporting the requirement of a GPCR for this effect [183]. However, since RANTES, MCP-3 and MCP-4 are able to activate other G-protein coupled receptors potentially expressed by ASMC, we chose to focus our attention on eotaxin, which solely binds to CCR3. A decrease in the effect of a second ligand stimulus was also observed independent of whether the same chemokine was used in both instances. This phenomenon, known as desensitization, is a well-documented feature of the GPCR response, and is an indication of receptor specificity since only ligands that interact with the same GPCR can desensitize its response [183]. Further studies are under way to establish the implication of other intracellular pathways possibly involved in the activation of ASMC.

The signalling pathways that mediate chemokine-induced trafficking are not well understood. In lymphocytes, activation of G_i and release of $G_{\beta\gamma}$ subunits have been shown to be crucial for induction of chemotaxis in response to a chemokine [300, 301], while in tracheal smooth muscle, $p38^{MAPK}$/HSP27 seems to be involved in initiating migration in response to cytokines and growth factor such as TNF-α, IL-1β and PDGF [180]. In eosinophils, multiple signalling pathways activated by CCR3 participate in the inflammatory response and the initiation of

migration. Eotaxin stimulates intracellular calcium release, production of reactive oxygen species, and changes in actin polymerization through a pertussis sensitive pathway. Rho and ROCK (Rho-associated coiled coil forming protein kinase), a protein kinase activated by Rho, regulate actin stress fiber formation and are required for eosinophil chemotaxis [302]. MAPK pathways are also involved in chemotaxis [75]. However, whether one of these intracellular pathways is involved in the induction of ASMC migration toward a gradient of eotaxin will need to be investigated.

In conclusion, we have demonstrated for the first time the expression of functional CCR3 by ASMC. Our results suggest that the activation of CCR3 by eotaxin could participate in the increased smooth muscle mass observed in the airways of asthmatic subjects; inducing the migration of ASMC towards the smooth muscle layer, and contributing to the airway hyperresponsiveness characterizing an episode of asthma. Further work will be required to demonstrate the migration of ASMC in an *in vivo* model of asthma, as well as to determine the intracellular pathways involved in the activation of CCR3 in ASMC.

2.12 Acknowledgments

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2.13 Footnotes

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[3] Abbreviations used in this paper: ASMC, airway smooth muscle cells; B/TSMC, bronchial/tracheal smooth muscle cells; CK, chemokinesis; PDGF, Platelet-Derived-Growth Factor; RT, room temperature.

CHAPTER III: Expression and Regulation of CCR1 by ASMC in asthma

3.0 Prologue

As discussed in the chapter I, one of the most important features of asthma is the airway remodelling occurring in asthmatic airways. Airway remodelling typically describes increased smooth muscle mass, thickening of the lamina reticularis, hypertrophy and hyperplasia of goblet cells and mucous glands and epithelial shedding. CCR1 is a receptor that has the ability to bind to several chemokines, including CCL3, CCL5, CCL7 and CCL13. These chemokines are increased in the bronchoalveolar lavage of asthmatic individuals. Interest in CCR1 comes from its implication in airway remodelling. In animal models of asthma and lung fibrosis, deletion of its gene resulted in a reduction in airway remodelling features, more particulary in the mucous production and the subepithelial ECM deposition. In the present chapter, we want to assess the expression of CCR1 by ASMC. We also look at the functional relevance of this receptor expressed by ASMC in the context of asthma.

Expression and Regulation of CCR1 by ASMC in asthma[1]

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Running title: Expression of CCR1 by Airway Smooth Muscle Cells.

Keywords: Human, chemokine, airway smooth muscle cells, lung.

3.1 Abstract

C-C chemokines such as CCL11, CCL5 and CCL3 are central mediators in the pathogenesis of asthma. They are mainly associated with the recruitment and the activation of specific inflammatory cells such as eosinophils, lymphocytes and neutrophils. It has recently been shown that they can also activate structural cells such as airway smooth muscle and epithelial cells. The aims of this study were to examine the expression of the CCL3 receptor, CCR1 on human ASMC, and to document the regulation of this receptor by cytokines involved in asthma pathogenesis. We first demonstrated that CCR1 mRNA is increased in the airways of asthmatic versus control subjects. We showed for the first time that ASMC express CCR1 mRNA and protein, both *in vitro* and *in vivo*. Mobilization of calcium by CCR1 ligands confirmed its functionality on ASMC. Stimulation of ASMC with TNF-alpha and, to a lesser extend, IFN-gamma resulted in an up-regulation of CCR1 expression, which was totally suppressed by both dexamethasone or mithramycin. Taken together, our data suggest that CCR1 might be involved in the pathogenesis of asthma, through the activation of ASMC by its ligands.

3.2 Introduction

Asthma is an inflammatory condition of the airways characterized by bronchial hyperresponsiveness, infiltration of inflammatory cells and airway remodelling[281, 303, 304]. Increased airway smooth muscle mass is an important feature of the airway remodelling and has been linked in different ways to asthma pathogenesis[139, 283]. In the last decade, several studies have shown that ASMC may contribute to the airway inflammation through the release of cytokines and chemokines such as TNF-α, IL-1β, CCL11 (eotaxin) and CCL5 (RANTES)[285]. ASMC were also shown to respond to a wide variety of immune mediators and to express receptors for several non-inflammatory cytokines[143]. More recently, expression of CCR3, a receptor for a number of C-C chemokines has been described in ASMC[278, 279]. However, little is known regarding the expression of other relevant C-C chemokine receptors by ASMC.

The C-C chemokine subfamily is composed of 28 members and includes CCL3. This chemokine binds to CCR1 and CCR5 and has been shown to be increased in bronchoalveolar lavage and bronchial biopsies of asthmatic patients[222, 231]. CCL3 is principally involved in the recruitment of eosinophils, basophils and mast cells[267, 305]. Interest in the role of CCR1, which is one of the CCL3 receptors, in allergy comes from its involvement in the development of the airway remodelling[245, 306].

The aims of this study were to examine the expression and the regulation of CCR1 on ASMC and to assess its expression in asthma. Our findings show that ASMC express CCR1 and that the receptor expression is increased by TNF-α and

IFN-γ while IL-4 and IL-13 have no effect. Binding of either CCL3 or CCL23 to CCR1 induces the release of intracellular calcium, demonstrating the functionality of the receptor. We have also shown that asthmatic patients express higher levels of CCR1, compared to controls, suggesting a potential function of this receptor in the pathogenesis of asthma.

3.3 Materials and Methods

3.3.1 Cell culture

Primary human airway smooth muscle cells (ASMC) were obtained from main bronchial airway segments (0.5–1.0 cm diameter) in pathologically uninvolved segments of resected lung specimens using isolation methods described previously[307, 308]. Cells were then seeded at a density of 10^5 cells/cm^2 and grown at 37°C in a humidified incubator with 5% CO_2 in Smooth Muscle Growth Medium (SmGm-2; Clonetics, San Diego, CA, USA). At confluence, primary human ASMC exhibited spindle morphology and a hill-and-valley pattern characteristic of smooth muscle in culture. In cultures up to passage 5, over 90% of the cells at confluence retained smooth muscle-specific α-actin, SM22, and calponin protein expression, and were able to mobilize intracellular Ca^{2+} in response to acetylcholine. Growth rate (determined by cell number) of ASMC from all lung resection donors was similar to that reported previously for ASMC cultures from healthy human transplant donors. Morphologically, the ASMC from lung resection donors and from healthy human transplant donors were indistinguishable. Cell viability was always above 95% as assessed by Trypan Blue dye exclusion.

3.3.2 Cell stimulation

ASMC were growth-arrested by FBS-deprivation for 24 hours prior to stimulation with cytokines. After starving, cells were stimulated with fresh, serum-free media, containing IL-4, IL-13, TNF-α or IFN-γ (R & D Systems, Minneapolis, MN, USA) in a concentration and time-dependent manner. In inhibition

experiments, mithramycin and dexamethasone (Sigma-Aldrich, Oakville, ON, Canada) were added in a dose-dependent manner 1 hour prior to the stimulation of the cells with cytokines.

3.3.3 RNA extraction

Total cellular RNA was isolated from human ASMC, epithelial cells (A549), fibroblasts (ATCC) purified peripheral blood eosinophils, and endothelial cells (HUVEC; ATCC). RNA was extracted using the RNeasy mini kit extraction columns (Qiagen, Mississauga, ON, Canada) as directed by manufacturer. RNA was eluted in 35 µl nuclease-free water, and cDNA was generated in a 30 µl reaction, using 0.5 µg of total RNA, oligo(dT)12-18 primers (Amersham Pharmacia Biotech, Baie d'Urfé, QC, Canada) and Superscript II (Invitrogen, Burlington, ON, Canada), in the presence of RNAguard (Amersham Pharmacia Biotech, Baie d'Urfé, QC, Canada).

3.3.4 RNA extraction from human airway biopsies

The biopsies were obtained from the Tissue Bank (MCI/Meakins-Christie Tissue Bank, McGill University). A clinical diagnosis of asthma was made based on the evaluation of the patient's medical file and by a respiratory physician. Individuals in moderate and severe asthma groups were all taking inhaled corticosteroids, while people in the mild group were using B_2-agonist. Age and predicted FEV_1 for each group were as follows: control, 42.3 y.o., 105%±23%; mild, 40.8 y.o.,95.5%±20%; moderate, 44.8 y.o., 86%±9%; severe, 50 y.o., 56%±20%.

Tissue RNA was extracted using the RNeasy micro kit extraction columns (Qiagen, Mississauga, ON, Canada) as directed by manufacturer. RNA was eluted in 12 ul nuclease-free water, and cDNA was generated as described above, using 9 ul of extracted RNA.

3.3.5 RNase Protection Assay

Total RNA was extracted as previously described. 20 μg of total RNA from cell cultures was used. Riboprobes were synthesized using T7 RNA polymerase and [^{32}P]CTP (Amersham Biosciences, Piscataway, NJ), from human multiprobe set (Riboquant; BD Biosciences, Mississauga, ON, Canada) containing template for CCR1 receptor. [^{32}P]-labeled riboprobes were hybridized with RNA samples overnight at 56°C and processed using the manufacturer's protocol. Protected RNA fragments were separated using a 5% acrylamide gel and analyzed by autoradiography (Kodak, Rochester, NY).

3.3.6 PCR and Preparation of standards

Quantification of the housekeeping gene ribosomal protein S9 and CCR1 was achieved by constructing a standard curve from serial dilutions of a known amount of gel-purified cDNA. This latter consisted of the quantified amplicon extracted from a gel. To do so, studied genes were first amplified using conventional PCR. The PCR mixture consisted of 1.5 mM $MgCl_2$, 1X PCR buffer, 0.25 mM dNTP mixture , 2.5 units *Taq* Platinum polymerase (Invitrogen, Burlington, ON, Canada), 0.4 μM each of the sense and antisense primers, as well

as 1 µl of cDNA. Primers for S9 and CCR1 were generated by Invitrogen (Invitrogen, Burlington, ON, Canada) using the following sequences: S9 sense 5'-TGCTGACGCTTGATGAGAAG - 3'; antisense 5'-CGCAGAGAGAAG-TCGATGTG-3'; CCR1 sense 5'- GACAAAGTCCCTTGGAACCA - 3'; antisense 5'- ACCAGGATGTTTCCAACCAG – 3'. Sequences of the primers were designed in two different exons with a big intronic sequence between the exons, to avoid any possible amplification of genomic contamination. The samples were amplified in a thermal cycler (PTC-100, Programmable Thermal Controller, MJ Research Inc. Watertown, MA, USA) for 40 cycles consisting of denaturation at 95°C, annealing at 57°, and extension at 72°C. The PCR products were visualized on a 1% agarose gel containing 0.2 ug/mL ethidium bromide. The correct band size was determined by comparison with a 100 bp DNA ladder (Invitrogen, Burlington, ON, Canada). Amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Mississauga, ON, Canada) and 10-fold series were prepared in Tris-HCl pH 8.0. Sequential dilutions ranged from 10^{-1} to 10^{-10} ng/ul.

3.3.7 Quantitative real-time PCR

Quantification of CCR1 and S9 mRNA expression by ASMC was done by quantitative PCR (QPCR) using the LightCycler (Roche Diagnostics, Laval, QC, Canada) following reverse transcription, as previously described. The same primers as the ones described for preparation of standards were used. PCR reactions were performed in a volume of 20 µl containing 1 µl of cDNA, 0.3 µM

of each primer, 10 ul of QuantiTect SYBR Green PCR Master Mix (Qiagen, Mississauga, ON, Canada) containing HotStarTaq DNA Polymerase, QuantiTect SYBR Green PCR Buffer, dNTPs and SYBR Green I. The PCR protocol consisted of three programs: denaturation, amplification and melting curve analysis for product identification. The denaturation and amplification conditions for both S9 and CCR1 were 95°C for 15 minutes followed by 45 cycles of PCR. Each cycle included denaturation at 95°C for 10 sec, annealing of 30 seconds at 60°C and extension of 20 secondes at 72°C. The temperature transition rate was 20°C/s, except when heating at 72°C, when it was at 5°C/s. Fluorescence was measured at the end of every cycle to allow quantification of cDNA. To eliminate the formation of primer dimers, a melting curve was obtained, after a normal cycle, by slowly increasing temperature of the samples to 95°C with fluorescence detection every 0.2°C following normal cycle.

3.3.8 Immunohistochemistry

To determine whether ASMC have the capacity to produce CCR1 protein *in vivo*, immunohistochemistry was performed on sections of major airways from asthmatic subjects. The biopsies were obtained from the Tissue Bank (MCI/Meakins-Christie Tissue Bank, McGill University). A clinical diagnosis of asthma was made based of the evaluation of the patient's medical file by a respiratory physician. Diagnostic criteria included prior diagnosis and treatment for asthma, documented evidence of variable airflow obstruction greater than 15%, and bronchial hyperresponsiveness. Following resection of the biopsies, lung specimens were prepared for immunohistochemistry. Briefly, formalin-fixed

tissues were paraffin-embedded and 5-μm-thick sections were prepared, sections were deparaffinized in xylene, and hydrated through a graded alcohol series. Endogenous peroxidase was quenched by incubating the slides in 0.5% hydrogen peroxide in PBS for 30 min. After rinsing in PBS, sections were blocked with blocking solution (Dako Corporation, Carpinteria, CA) for 20 min at room temperature (RT). Primary Ab against CCR1 diluted in diluting buffer (Dako Corporation, Carpinteria, CA) was applied (25 μg/ml, MAB145, clone 53504.111; R & D Systems, Minneapolis, MN) and sections were incubated overnight at 4°C. Control sections were incubated with isotype control (25 μg/ml, MAB004 clone 20116; R & D Systems, Minneapolis, MN). After rinsing in PBS, a biotinylated rabbit anti-mouse Ab (1:100 dilution; Dako Corporation, Carpinteria, CA) was applied, and sections were incubated for 60 min at RT. Sections were thoroughly washed in PBS and incubated with the streptavidin-HRP conjugated for 60 min at RT. After PBS washes, the reaction was revealed using 0.5 mg/ml diaminobenzidine tetrahydrochloride (DAB) in Tris buffer (pH 7.6) as the chromogen and 0.05% hydrogen peroxide as the substrate for 5 min. Sections were counterstained with hematoxylin and mounted.

3.3.9 Flow cytometric analysis

FACS analysis was performed as follows: ASMC were detached from the flask by addition of PBS containing EDTA (0.5 M) for 20 minutes at 37°C. Cells were resuspended at a concentration of $1x10^6$ cells/ml and washed once with PBS. ASMC were labeled with a Fluorokine Kit for Human CCR1 (Fluorokine; R&D

Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, 10 µl of biotinylated recombinant CCL3 reagent was added to 25 µl aliquots of washed cells (10^5) and incubated for 60 min on ice. Following the incubation period, 10 µl of streptavidin-FITC reagent was added, and cells were incubated for an additional 30 min at 4°C in the dark. Cells were then washed twice, resuspended in 200 µl of PBS, and analyzed by flow cytometry. As a negative control, an identical sample of washed cells was incubated with 10 µl of biotinylated negative control reagent (supplied with the kit). The specificity of the reaction was assessed by mixing 20 µl of anti-human CCL3 blocking Ab with 10 µl of biotinylated CCL3 and incubated for 15 min at RT. CCR1 expression was analyzed using fluorochrome-labeled CCL3 (Fluorokine; R&D Systems, Minneapolis, MN, USA), and analyzed via flow cytometry (BD FACSCalibur System. BD Biosciences, Mississauga, ON, Canada). At least 10000 cells were counted per analysis. As a positive control, the same protocol as above was applied to CCR1-transfected Ghost-cells (National Institute of Health, AIDS reagent program, #3682).

3.3.10 Lasercapture microdissection of ASMC

In order to assess the capacity of ASMC *in vivo* to express CCR1 mRNA, lasercapture microdissection was performed on asthmatic biopsies. The biopsies were obtained from the Tissue Bank (MCI/Meakins-Christie Tissue Bank, McGill University). They were cut into 5 µm sections on a cryostat and placed on charged slides prior to fixation in 70% ethanol. The slides were stained with hematoxylin and eosin, rinsed in an ethanol gradient, and dehydrated in a mixture of xylenes.

The smooth muscle cells bundles were carefully captured using the Pixcell laser capture microscope (Arcturus Bioscience, Inc., Mountain View, CA). During this process, cellular material was transferred to CapSure HS LCM Caps (Arcturus Bioscience, Inc., Mountain View, CA) and digested in RLT lysis buffer (Qiagen, Mississauga, ON, Canada). mRNA was extracted using RNeasy® Micro Kit (Qiagen, Mississauga, ON, Canada) following manufacturer's instruction. mRNA was eluted in 12 ul of water. Reverse transcription was performed as described above. Because of the low amount of RNA present in the samples, two series of amplification were performed on the sample. The first one consisted of 30 cycles of the PCR program described above. 1 µl of the PCR product of each sample was then re-amplified in a similar PCR reaction for 45 cycles. The PCR products were visualized on a 1% agarose gel containing 0.2 ug/mL ethidium bromide.

3.3.11 Measurement of intracellular free Ca^{++}

For the measurement of calcium cells were loaded with the Ca^{2+}-sensitive dye, fura-2 AM (Molecular Probes, Eugene, OR) according to the previously described methods [309] and imaged using an intensified charge-coupled device camera (IC200) and PTI software at a single emission wavelength (510 nm) with a double excitatory wavelength (340 and 380 nm). Fluorescence ratio (340/380) was measured in cells stimulated with CCL3 or CCL23 (1, 10 and 100 ng/ml) or appropriate vehicle. Intracellular calcium concentration ($[Ca^{2+}]_i$) was calculated according to the formula of Grynkiewicz et al. [310]. Each experimental group consisted of 102–115 cells. Studies were performed using three cell lines, each acquired from a different donor.

3.3.12 Statistical analysis

Statistical significance was determined using a Student's *t* test. P values < 0.05 were considered statistically significant.

3.4 Results

3.4.1 Biopsies obtained from airways of mild, moderate and severe asthmatics express higher levels of CCR1 compared to controls

One of CCR1's ligand, CCL3, has been shown to be upregulated in asthma[311, 312], though, expression of this receptor in asthmatic airways has never been documented. We used quantitative real-time PCR to compare the levels of CCR1 mRNA in biopsies obtained from the airways of control, mild, moderate and severe asthmatics. Figure 1 shows relative expression of CCR1 mRNA (expressed as function of S9) in the four groups. Mild and severe asthma groups showed a significantly increased expression ($3.4x10^3 \pm 1.7x10^3$ and $5.7x10^2 \pm 2.8x10^2$ for mild and severe asthma groups respectively), while no CCR1 mRNA was detected in the control. Although no significant difference was seen between moderate and control groups, a general trend towards an increased expression of CCR1 mRNA was observed ($p=0.10$). No significant differences were observed between the asthmatic groups. The housekeeping gene S9 was detected in all the groups, confirming the presence of mRNA in all the samples processed for PCR analysis.

3.4.2 ASMC constitutively express CCR1 at mRNA and protein levels

In order to demonstrate the expression of CCR1 by ASMC *in vitro* at both mRNA and protein levels, RT-PCR and flow cytometry analyses were performed. PCR analyses were carried out on cultured ASMC and different populations of structural cells to assess the expression of CCR1 mRNA. Peripheral blood eosinophils were used as a positive control. Gel electrophoresis (figure 2A)

revealed bands corresponding to the expected size of the CCR1 cDNA product (197 bp). Surface expression of CCR1 by ASMC was confirmed using flow cytometry, and revealed a high percentage of unstarved serum cells expressing the receptor (figure 2B, upper and middle panels). This signal was completely suppressed when anti-CCL3 was simultaneously added (figure 2B, middle panel), confirming the specificity of the signal. As positive control, CCR1-transfected cells (CCR1+ cells) were analyzed (figure 2B, lower panel).

3.4.3 CCR1 is expressed by ASMC in vivo

Using immunocytochemistry, CCR1 immunoreactivity was detected in ASMC in bronchial biopsies obtained from subjects with asthma. CCR1 protein was mainly localized in the smooth muscle bundles (Figure 3A), airway epithelium and some inflammatory cells (data not shown). Expression of CCR1 by ASMC *in vivo* was confirmed using lasercapture microdissection. Indeed, RT-PCR analysis of mRNA obtained from ASMC microdissected from four severe asthmatic biopsies revealed the presence of the receptor (figure 3C), confirming the results obtained *in vitro.*

3.4.4 TNF-α and IFN-γ upregulate the expression of CCR1 by ASMC

Expression of chemokine receptors has been shown to be regulated by different inflammatory mediators [291]. In asthma, the cytokine environment is characterized by increased TNF-α, IL-4 and IL-13 levels [60]. However, there are conflicting results regarding the amount of IFN-γ found in asthmatic airways[54, 57, 58]. We

therefore investigated the effects of IL-4, IL-13, IFN-γ and TNF-α on ASMC mRNA and surface expression of the CCR1 receptor. Addition of IL-4 and IL-13 did not modulate the expression of CCR1 (1.4±0.6 and 2.2±0.7 fold, respectively), while TNF-α and IFN-γ significantly upregulated the expression of CCR1 at mRNA level (11.0±4.8 and 4.8±1.4 fold; Figure 4B) and also increased surface expression levels (Figure 4D) after 12 (mRNA) and 24 (surface expression) hours of stimulation. The combination of TNF-α with either IL-4, IL-13 or IFN-γ did not potentiate the surface expression of CCR1, as seen with TNF-α or IFN-γ alone (data not shown). Addition of TNF-α or IFN-γ both resulted in a dose and time-dependent augmentation of CCR1 mRNA expression with a plateau dose at 1 ng/ml for TNF-α and 10 to 50 ng/ml for IFN-γ and a maximal effect after 48 hours of stimulation (Figure 4C).

3.4.5 CCR1 mRNA upregulation by TNF-α and IFN-α is sensitive to dexamethasone and mithramycin

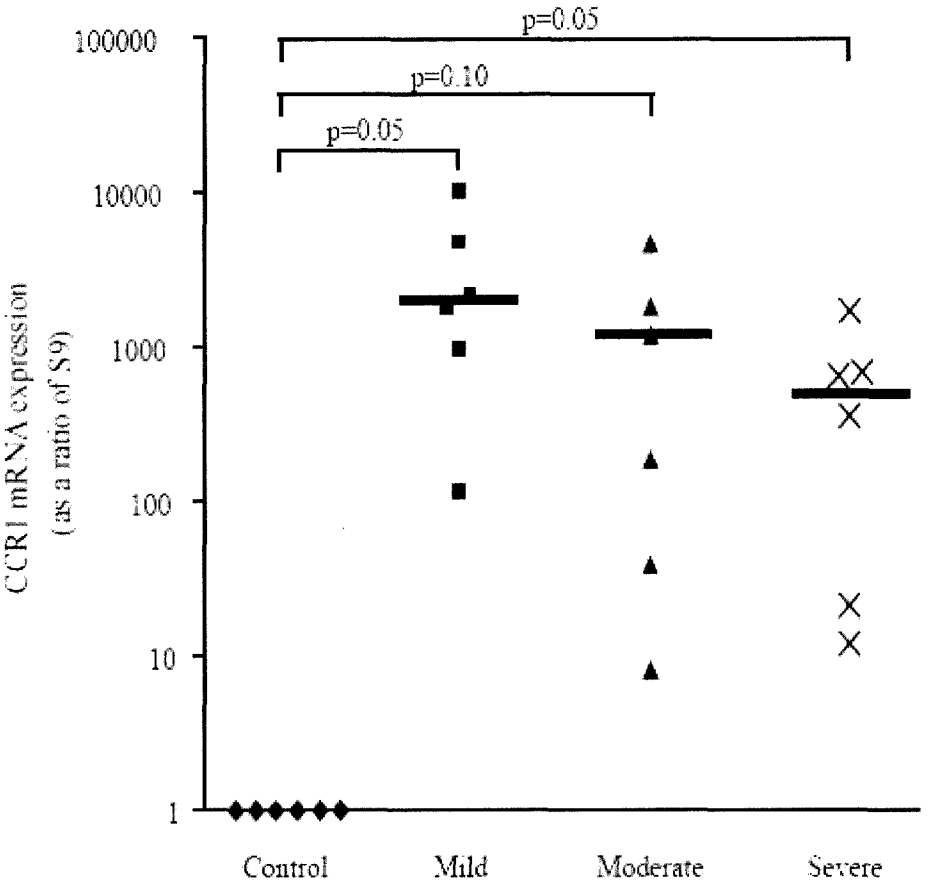
Corticosteroids are widely used for the treatment of asthma, but their exact mechanism of action remain unclear. However, they have been shown to inhibit signal transduction of pro-inflammatory cytokines through the inhibition of the NF-κB pathway. We therefore looked at the capacity of dexamethasone to inhibit TNF-α and IFN-γ-induced CCR1 mRNA. We observed a significant inhibition of CCR1 mRNA expression at low doses of dexamethasone when cells were stimulated with TNF-α or IFN-γ (Figure 5). The inhibition was dose-dependent

with a maximal effect using 0.1 μM and 1 μM of dexamethasone for TNF-α and IFN-γ respectively (Figure 4).

Because the CCR1 promoter contains several binding sites for stimulatory-protein-1 (Sp1) [313] we looked at the potential involvement of this transcription factor in the induction of CCR1 mRNA expression with TNF-α or IFN-γ. We used mithramycin, an inhibitor of Sp1 binding, and found that it completely inhibits TNF-α and IFN-γ-induced CCR1 mRNA upregulation at concentrations of 100 nM and 250 nM (Figure 5B).

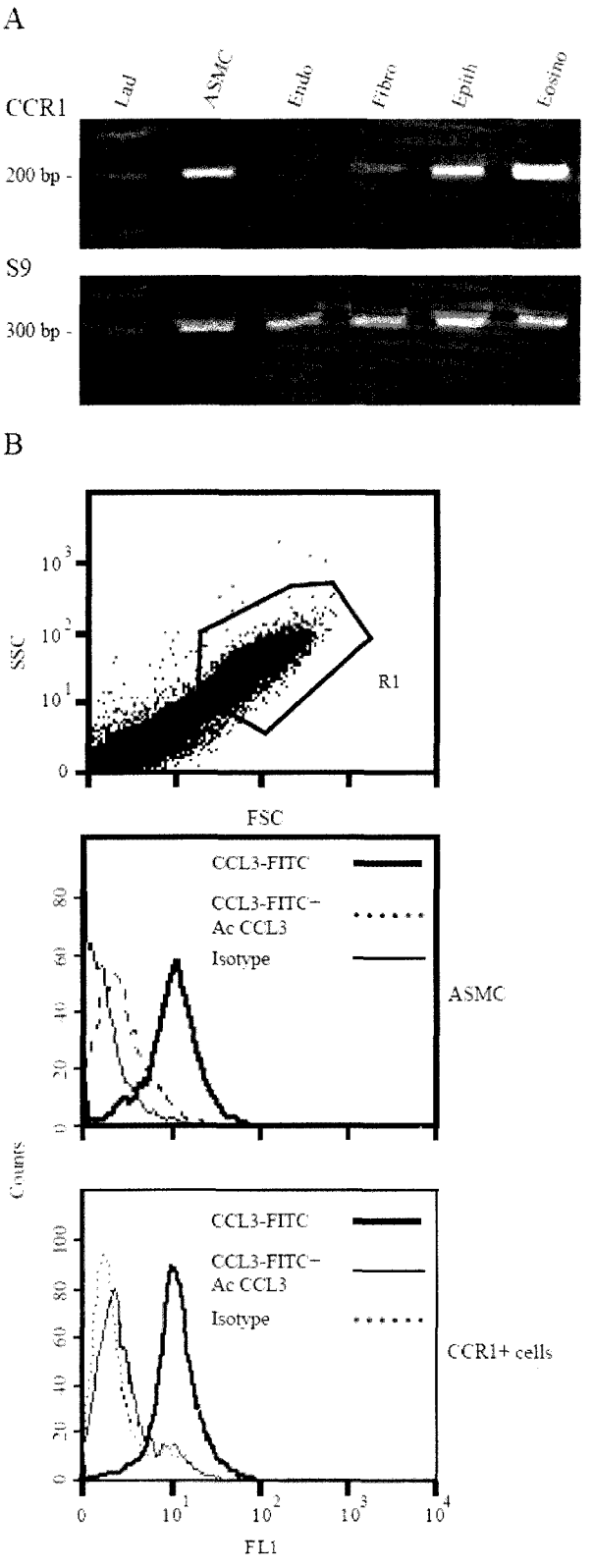
3.4.6 Addition of CCR1 ligands induces an increase in intracellular [Ca^{2+}]

In order to assess the functionality of CCR1, we stimulated ASMC with increasing doses of CCL3 and CCL23. CCL3 and CCL23 triggered calcium responses indicating that the receptors are functional. The number of cells responding and the magnitude of the response were concentration-dependent, with 100 ng/ml of CCL23 causing the most pronounced response (Figure 6). However, even in this group, not all cells were responsive, as suggested by the results obtained with flow cytometry where just a percentage of cells express the receptor.



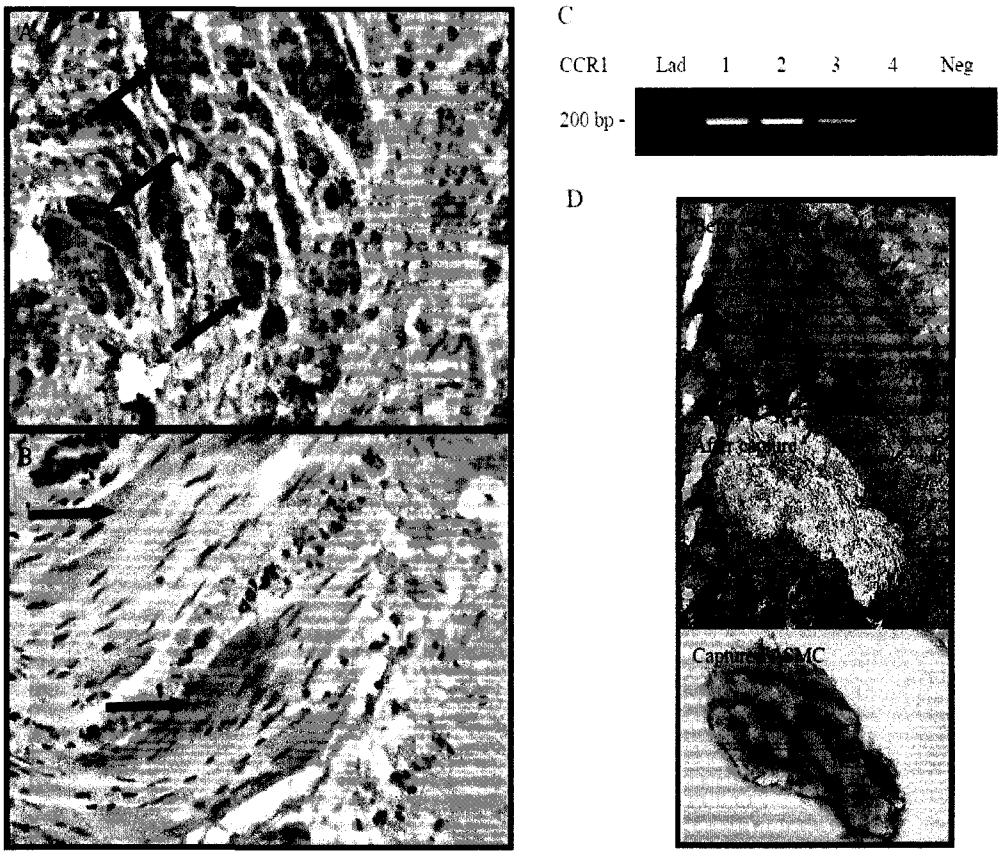
3.5 Figure 1

CCR1 mRNA expression in asthma. Quantitative RT-PCR analysis of mRNA extracted from airway biopsies obtained from mild (♦), moderate(■), severe(▲) asthmatics and control patients (X).



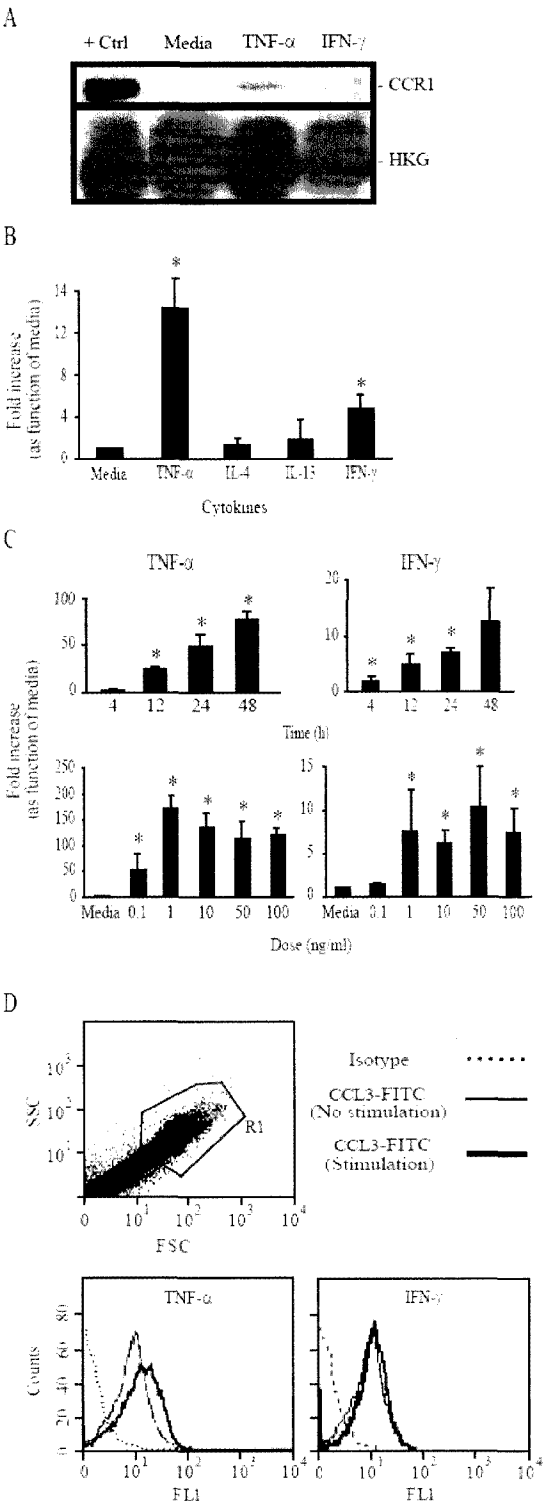
3.6 Figure 2

Detection of CCR1 expression in ASMC. *A*, RT-PCR analysis of constitutive mRNA CCR1 expression by structural cells. Endothelial cells (Endo), epithelial cells (Epith), fibroblasts (Fibro) and ASMC were examined while eosinophils were used as positive control (representative of n = 3). *B*, Determination of CCR1 surface expression by ASMC using flow cytometry. ASMC were cultured and analyzed by flow cytometry for cell surface expression of CCR1. Biotinylated human recombinant CCL3 was added to confluent cultured ASMC (P3-7) in the absence or presence of anti-human CCL3 blocking Ab (representative of n = 3).



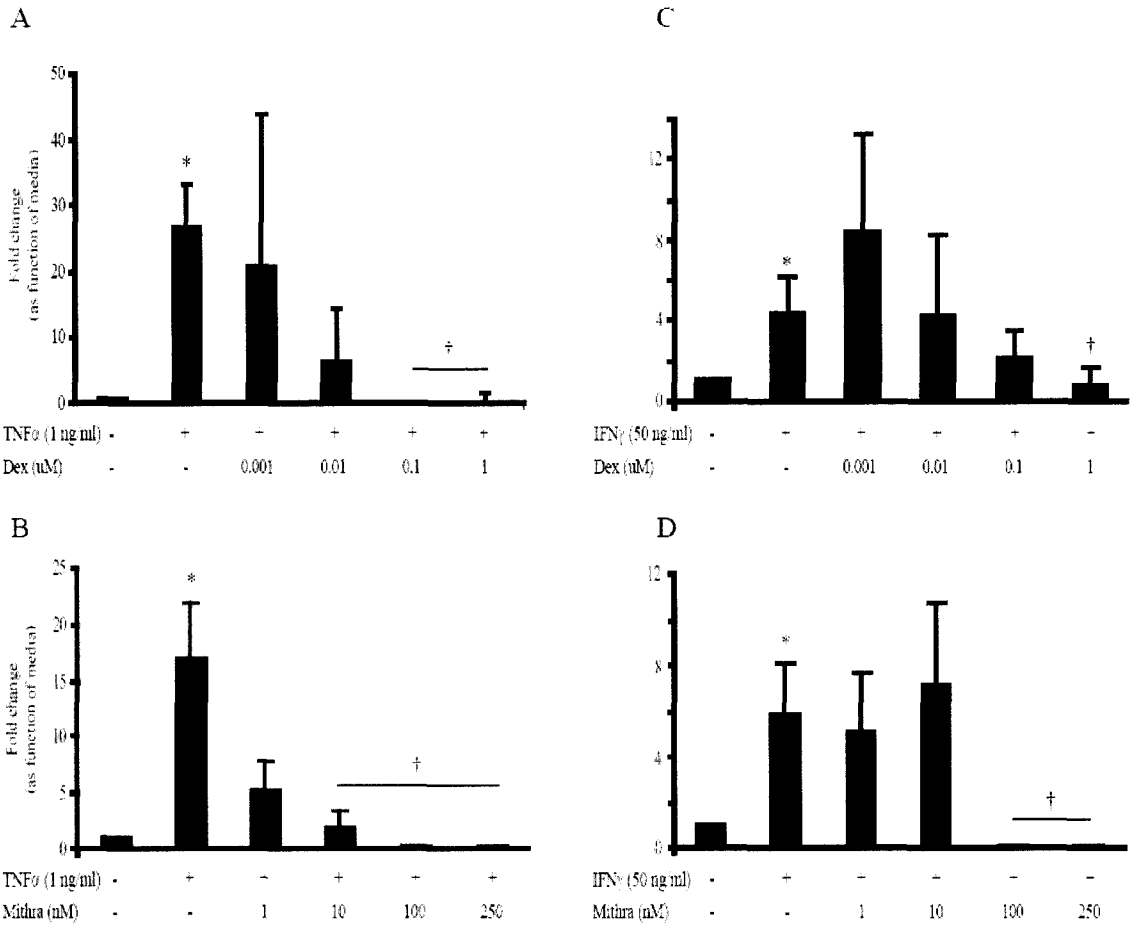
3.7 Figure 3

Expression of CCR1 in ASMC *in vivo*. Cross-section of an intermediate airway from asthmatic *(A)* subjects showing CCR1 immunoreacitivity and isotype control *(B)* in smooth muscle bundle (*large arrows*) (representative of n=3). Paraffin-embedded sections were prepared from human lung biopsies, and slides were incubated with anti-CCR1 monoclonal Ab, the appropriate secondary Ab, and a tertiary layer of streptavidin-HRP-conjugated. Sections were developed with DAB, with positive cells staining brown. *C* and *D*, CCR1 mRNA detection of microdissected ASMC from human airway biopsies, using RT-PCR. ASMC were captured using lasercapture microdissection from airway biopsies (D) obtained from four patients (1,2,3 and 4 in the figure) with severe asthma.



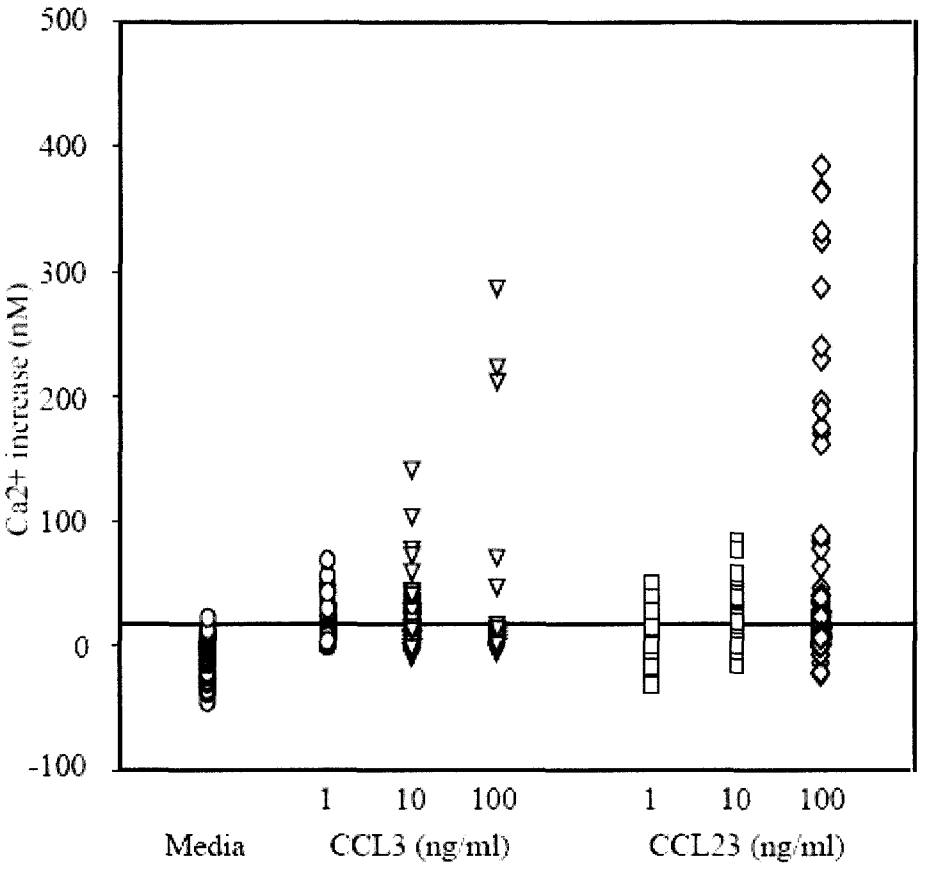
3.8 Figure 4

Effect of cytokines on CCR1 mRNA and protein expression by ASMC. Regulation of CCR1 mRNA using *A*, RNase Protection Assay (RPA) and *B*, QPCR using TNF-α (10 ng/ml), IFN-γ (50 ng/ml), IL-4 (50 ng/ml) and IL-13 (50 ng/ml) for 12 hours. * p<0,05 (n=4).*C*, CCR1 mRNA expression using increasing doses of TNF-α and IFN-γ at different time points, using QPCR. Results are expressed as a ratio of housekeeping gene S9. * p<0,05 (n=3).*D*, Expression of CCR1 was qualitatively evaluated by flow cytometry using a biotinylated human recombinant CCL3. As a negative control, cells were incubated with a biotinylated soybean trypsin inhibitor (supplied by the manufacturer). Cells were stimulated with TNF-α or IFN-γ for 24 hours. Viable cells were gated (R1) and analyzed (representative of n=3).



3.9 Figure 5

Effects of mithramycin and dexamethasone on TNF-α (panels A and B) and IFN-γ-induced (panels C and D) CCR1 mRNA upregulation. Confluent ASMC (passages 3-7) cultured in serum-free medium were stimulated with TNF-α (10 ng/ml) or IFN-γ (50 ng/ml) for 24 hours. Increasing doses of mithramycin (panels B and D) or dexamethasone (panels A and C) were added 1 hour prior to stimulation with cytokines. mRNA expression was evaluated using QPCR. Results are expressed as a ratio of housekeeping gene S9 expression. * Different from media, $p<0,05$ (n=3); † Different from TNF-α, $p<0,05$ (n=3).



3.10 Figure 6

Measurement of Ca^{2+} in cultured ASMC in response to three different concentrations of CCL3 or CCL23. For calcium measurements, confluent serum fed ASMC were loaded with fura-2 as described in Materials and Methods. Cells were stimulated with either CCL3 or CCL23 and intracellular Ca^{2+} was measured for at least 300 s thereafter. Figure shows difference between peak response and baseline level calcium. Histamine ($10E^{-6}M$) was used as a positive control. Data shown are representative of 3 experiments.

3.11 Discussion

In the last few years, the role of ASMC in the pathogenesis of asthma has considerably evolved. A large body of literature has clearly shown that functions of ASMC extend beyond their contractile properties. They can contribute to the airway inflammation through the release of inflammatory mediators, including cytokines and chemokines. They also express a wide variety of receptors which make them potential targets for the inflammatory mediators involved in the pathogenesis of asthma. Several C-C chemokines have been shown to be upregulated in the airways of asthmatic patients. Their functions are mainly associated with the recruitment of inflammatory cells towards the site of inflammation, although studies have also shown that chemokines can promote angiogenesis and proliferation.

Expression of CCR1 has been described mainly in leukocytes such as macrophages, eosinophils, basophils and dentritic cells[267] , However few studies have demonstrated the expression of CCR1 by cells other than leukocytes such as osteoclasts and platelets[231, 314]. In the present study, we showed for the first time that ASMC express CCR1 both *in vitro* and *in vivo*. Using quantitative PCR technique, we also demonstrated that asthmatic airways contain higher levels of CCR1 mRNA, compared to normal airways. Functional studies revealed that the CCR1 ligands, CCL3 and CCL23, induce the mobilization of intra-cellular calcium in ASMC. Taken together, these data suggest a potential role for CCR1 on ASMC, in the context of asthma.

Asthma is a disease typically characterized by an increase in Th2 versus Th1 cytokine ratio. Th2 cytokines include IL-4, IL-5 and IL-13 while IFN-γ is the prototypical Th1 cytokine. Both Th1 and Th2 cytokines seem to have the potential to modulate the expression of CCR1, depending on the type of cells involved [267, 315, 316]. Effects of IFN-γ on CCR1 expression has been reported in monocytes and neutrophils, however, little is known concerning the effects of TNF-α on cell populations expressing CCR1[267]. In the present study, we found that both TNF-α and IFN-γ increase CCR1 mRNA and protein while IL-4 and IL-13 had no effect. It is not surprising that TNF-α induces a strong upregulation of the receptor. Such an effect has been reported with several chemokine receptors, including CCR3 and CCR5[278, 317]. CCR1 is known to be involved in host defense where high levels of TNF-α are usually found. TNF-α has also been shown to be upregulated in various inflammatory conditions, including asthma [59, 72, 299]. There is no consensus as to whether or not IFN-γ is diminished in asthmatic airways. Recent studies have shown that IFN-γ positive T cells are increased in asthmatic blood and airways [54, 57, 58]. Therefore, the effect of IFN-γ on CCR1 expression is not necessarily in contradiction to the concept of asthma pathogenesis.

In the present work, we showed that asthmatics express higher level of CCR1 mRNA, which could be related to the higher levels of both TNF-α and IFN-γ asthmatic airways. However, we have also shown that the corticosteroid dexamethasone strongly downregulates the expression of CCR1 mRNA by ASMC when stimulated with TNF-α. Since severe and moderate asthmatic patients that were used in our study were all treated with corticosteroids, it is

possible that the level of mRNA in these patients is not reflecting the real amount of CCR1 in asthma. It is interesting to notice that mild asthmatics without corticosteroid treatment showed the highest level of CCR1 mRNA, suggesting that corticosteroids could downregulate the expression of CCR1 in asthma. Surprisingly, CCR1 mRNA was undetected in the control group. Because we have been able to detect CCR1 mRNA expression in normal cultured ASMC, it is likely that the amount of CCR1 mRNA present in the RNA extracted from the control airway biopsies is below our detection level.

Using an online promoter analysis program (Consite; mordor.cgb.ki.se/cgi-bin/CONSITE/consite/) and the published sequence of the CCR1 promoter[313], we identified several Sp1 binding sites. The Sp1 transcription factor binds to GC-rich sequences and is necessary for the activation of many genes, including cytokines[318-320]. In our study, we used mithramycin, a DNA-binding antibiotic which binds GC-rich regions, to evaluate the contribution of Sp1 to TNF-α and IFN-γ-induced CCR1 upregulation. We showed that a moderate dose of mythramycin (10 nM for TNF-α and 100 nM for IFN-γ) totally abrogates CCR1 mRNA production, suggesting a preponderant role for Sp1 in activation of the CCR1 gene in ASMC, when stimulated with either TNF-α or IFN-γ. Interestingly, dexamethasone, which is known to inhibit NF-kB[321], also totally inhibited both TNF-α and IFN-γ-induced CCR1 mRNA expression, suggesting an involvement of this pathway in CCR1 expression. It is noteworthy to mention that a collaboration between Sp1 and NF-kB pathways is required for the induction of CXCL2 in a macrophage cell line[319], raising the possibility that a similar

phenomenon occurs in the induction of CCR1 mRNA expression in ASMC. This might explain the complete inhibition obtained with either mithramycin or dexamethasone. It is also possible that the inhibition of CCR1 mRNA expression by dexamethasone occurs through the blockade of Sp1 since this effect of the drug has been described in the expression of CD14 by macrophages, when stimulated with LPS[322].

Chemokine receptors with defective signalling function have been reported [323]. In particular, exposure of dendritic cells to IL-10 and LPS has been shown to suppress the intracellular signal mediated by CCR1[324]. In the present study, we have shown that the addition of CCR1 ligands induces intracellular mobilization of calcium, supporting the requirement of a GPCR (G-protein coupled receptor) for this effect [325]. As expected, not all cells responded to CCR1 ligands, presumably because they were not expressing the receptor, as suggested by our flow cytometry results (Figure 2), or possibly because the receptors expressed at the surface of these cells were not functional or too low in number to induce the intracellular mobilization of calcium.

Functions of chemokines in human diseases have been mainly associated with recruitment and activation of inflammatory cells. Chemokines also regulate angiogenesis, Th1/Th2 development and the release of cytokines [167, 326]. Two CCR1 ligands, CCL3 and CCL23, have been associated with recruitment of monocytes and T cells[305], specific inhibition of myeloid progenitor cells and activaton of monocytes and eosinophils[327]. Interestingly, in a CCR1 -/- model of chronic allergic asthma, the airway remodelling features observed in wild-type

animals were greatly reduced (goblet cells hyperplasia and deposition of collagen), suggesting an association between CCR1 and the development of airway remodelling [245]. These results were also strengthened by a similar study in which neutralization of CCR1 using antibody totally abrogated the fibrosis in an animal model of pulmonary fibrosis [328]. In these two studies, CCL3 was suggested as one of the potential mediators involved in the activation of CCR1. Since ASMC have been shown to produce ECMP that are involved in airway remodelling[3, 329], such as versican, lumican and collagen, we initially hypothesized that activation of CCR1 by CCL3 or CCL23 could induce the release of ECMP by ASMC. In the present work, we could not detect any effect of CCR1 ligands on the production of collagen-I, decorin, lumican or versican at mRNA level (data not shown). However, it is possible that activation of CCR1 in ASMC leads to the modulation of ECMP by other types of cells such as mast cells, fibroblasts and myofibroblasts[87, 306, 330].

In conclusion, we have demonstrated for the first time that ASMC express CCR1. We showed that TNF-α and, to a lesser extend, IFN-γ, upregulate CCR1 expression at both mRNA and protein levels in a Sp1 and NF-kB dependent pathways. We also documented an increased expression of CCR1 in airways of asthmatic patients, more particularly in patients who are not taking any corticosteroids. The expression of a functional CCR1 by ASMC indicates that CCL3, a chemokine increased in asthmatic airways, might play a role in the pathogenesis of the disease through the activation of ASMC. We are presently

investigating whether activation of CCR1 mediates synthetic, proliferating or migrating responses in ASMC.

3.12.Acknowledgments

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3.13 Footnotes

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[3] Abbreviations used in this paper: ASMC, airway smooth muscle cells; RT, room temperature; QRT-PCR, quantitative reverse transcriptase polymerase chain reaction.

CHAPTER IV: DISCUSSION

The role of ASMC in asthma pathogenesis has long been regarded as a purely contractile element within the airways. Its main involvement was associated with bronchoconstriction during an asthma episode. However, in the last two decades, data has emerged highlighting the possible contribution of structural cells to inflammation in different diseases including asthma. Cells such as epithelial cells, fibroblasts and ASMC have been shown to produce a wide variety of inflammatory mediators and receptors that extends their role beyond their structural function. In particular, the synthetic capabilities of ASMC in the context of asthma have been extensively studied in the last ten years[1]. As discussed in the first chapter, it is well recognized that they can produce an impressive array of cytokines, chemokines, ECMP and lipid mediators. They can express receptors for pro-inflammatory, as well as Th1 and Th2 cytokines, making them sensitive to the specific cytokine milieu associated with asthma.

One of the most important features characterizing the inflammatory response observed in asthma is the release of certain chemokines. The function of these proteins is mainly associated with the recruitment of inflammatory cells within the airways. C-C chemokines such as CCL11, CCL3 and CCL5 have been implicated in the recruitment and the activation of eosinophils and Th2 cells in asthma. A study published by Stellato *et al.* showed for the first time the expression of a chemokine receptor, CCR3, by a structural cell in the context of asthma[80]. Although the exact function of this receptor at the surface of these cells was unknown, it raised the possibility that chemokines may modulate functions or behaviour of structural cells. The main objective of this thesis was to study the

expression and the function of chemokine receptors by ASMC as it related to asthma. We focused on two C-C receptors: CCR3 and CCR1.

4.1 CCR3 expression and functions in asthmatic ASMC

Among all the chemokine receptors expressed in asthma, CCR3 is probably the the most extensively studied. This particular interest towards CCR3 comes from its ability to bind CCL5, CCL7, CCL8, CCL11 and CCL13, which have all been shown to be increased in asthmatic airways and involved in asthma pathogenesis. Interestingly, deletion of CCR3 in animal model of asthma has also been shown to be associated with a marked reduction of airway eosinophilia [246, 331, 332]. We demonstrated for the first time that cultured ASMC express a functional CCR3. It is also expressed by smooth muscle bundles in human airways. Our results demonstrated that the CCR3-ligand, CCL11, was able to induce ASMC migration. This was indeed the first report showing the ability of a chemokine to induce the migration of ASMC. A similar phenomenon was simultaneously described in vascular smooth muscle in the context of atherosclerosis[280]. Our findings provide a novel explanation for the increased smooth muscle mass characterizing asthmatic airways. Since the publication of this work, another study showed the migration of ASMC in response to a CCR7 ligand, CCL19 [279]. Similarly, the authors showed that ASMC could migrate towards a gradient of CCL19, and suggested that this migration, in combination with CCL11-induced migration, could participate to increased airway smooth muscle mass. Another phenomenon related to migration is the recent description of fibrocytes, a progenitor cell sharing features of both leukocytes and

mesenchymal cells[333, 334], in that they express both CD34 -a marker of haematopoietic cells- and collagen I. These cells were shown to migrate within the lung from the peripheral circulation in response to allergen challenge, and to mature further into myofibroblasts, assessed by their ability to express α-smooth muscle actin[85]. Although the exact nature and role of myofibroblasts remains unclear, they are also thought to originate from ASMC and represents an intermediate form between fibroblasts and ASMC[99, 117]. Collectively, these findings suggest that the increase in ASM mass may be a consequence of fibrocytes influx into the airway and are consistent with mechanisms proposed in skin wound healing[335], and is analogous to the current concepts of cardiac myocyte progenitors contributing to cardiac repair after myocardial infarction[319]. Another possibility is that ASMC might originate from precursor cells present in the basal layers of the airways and following maturation with different mediators or physical stimuli, migrate towards the smooth muscle layer[117, 126]. However, this hypothesis needs further investigation.

Effects of cytokine milieu on chemokine production and chemokine receptor expression have been well described in the literature[336]. We showed in our study that TNF-α increases the surface expression of CCR3, as assessed by flow cytometry. This observation may suggest that asthmatic ASMC could be more sensitive to the presence of eotaxin in the airways. In addition, eotaxin levels are much higher in asthmatic versus non-asthmatic airways [220, 337]. We showed that ASMC migrated in a concentration-dependent manner to CCL11 with a maximal effect at 100 ng/ml. Although the levels of eotaxin in asthmatic

airways were shown to be less than the one used in our study, the local concentration of chemokines within the tissues is likely to be higher than the one measured in bronchoalveolar lavage. This might be due to their ability to bind ECMP or other surface proteins such as syndecan-1 [338].

An important finding is the higher level of cell-surface CCR3 in ASMC is obtained from asthmatic airways compared to those from control. In order to preserve the initial phenotype of the cells, we analysed the cells at a very low passage and cells from both groups were always grown in identical conditions. This might suggest that the asthmatic ASMC were either exposed to different conditions *in vivo or* have an intrinsic difference in their expression of this chemokine receptor. As mentioned, it is possible that the effect of cytokines on ASMC in the asthmatic airways explains this difference observed in CCR3 expression, once *in vitro*. To our knowledge, there is no information regarding the duration of chemokine receptor expression following the withdrawal of a cytokine stimulus. It seems unlikely that ASMC would retain their TNF-α-induced CCR3 upregulation throughout the culturing, unless chromatin-modifying factors *in vivo* have permitted the CCR3 locus to be more open and accessible to transcription than in their non-asthmatic counterparts.

Results obtained from CCR3 knockout mice provide valuable information regarding the role of this receptor in asthma pathogenesis. Three different studies reported that disruption of CCR3 gene in animal models of asthma significantly decreased migration of eosinophils within the airways[246, 332, 339]. Unfortunately, animal models of asthma poorly reproduce some of the airway remodelling

features typically found in human asthma, including increased smooth muscle mass. This is probably because none of the above studies reported any change in the airway smooth muscle layer in CCR3-knockout mice. However, an interesting observation consistently reported is an unexpected increase in AHR in the CCR3-deficient animals. Since the functions of eosinophils have been linked to AHR in several publications[340-342], this finding was in contradiction with the previous concept of eosinophils requirement for AHR. However, in accordance with the murine data, human asthmatic treated with an antibody directed against IL-5 decreased eosinophilia without significantly reducing AHR[69]. These observations highlight the complexity of cytokines-chemokines network and their cognate receptors in the regulation of the immune response characterizing asthma. Development of animal models reproducing typical features of asthma, as well as the real course of the disease, will be required in order to dissect the involvement of each cellular actor involved in asthma and to fully understand the pathogenesis of the disease. The results of our first work provide a new direction to investigate the implication of ASMC in airway remodelling and propose a new mechanism to explain the increase in smooth muscle mass characterizing the airways of asthmatic patients.

Figure 5 summarizes a possible mechanism of action for CCR3 and eotaxin and their role in smooth muscle mass increase in asthmatic airways.

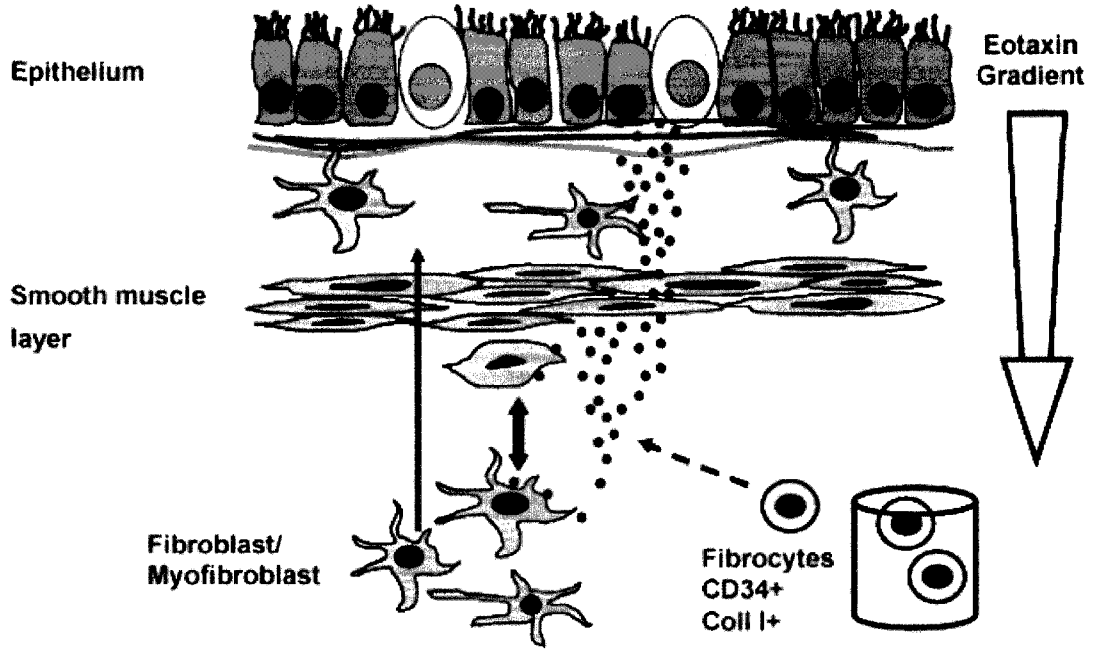

Figure 5. Schematic representation of smooth muscle increase in asthma through a possible mechanism driven by CCR3 and eotaxin. Epithelial cells and ASMC generate a gradient of eotaxin that attracts ASMC and/or myofibroblasts towards an existing smooth muscle bundle, thus increasing its size. A potential source of ASMC could also include fibrocytes, a precursor cell type expressing CD34+ and collagen I. This cell was shown to differentiate into myofibroblasts following allergen challenge.

4.2 Expression and regulation of CCR1 by ASMC in asthma

CCR1 is a C-C chemokine receptor that was initially described in three different laboratories as the high-affinity receptor for CCL3 and CCL5[343-345]. Additional studies subsequently showed that several other chemokines could also bind to CCR1 with a different range of affinity. These chemokines include CCL6, CCL7, CCL8, CCL9, CCL13, CCL15, CCL16 and CCL23. However, most of the work looking at the cellular response, following CCR1 activation, has been done using CCL3, CCL5, CCL7 and CCL13. CCR1 shows the highest tissue expression in lungs, although several other organs express the receptor[346]. Expression of CCR1 has been initially described on lymphocytes (Th2

lymphocytes in particular), monocytes/macrophages, mast cells, basophils and eosinophils[345]. Since then, several other cell types have been shown to express a functional CCR1, including platelets, osteoclasts and fibroblasts[185, 188]. Interest in CCR1 in the context of asthma comes from the ability of some of its ligands, in particular CCL3, CCL5 and CCL13, to recruit eosinophils[305]. Furthermore, these three chemokines have been shown to be increased in asthmatic airways[347-349]. For example, Lamkhioued et al. showed that a combination of antibodies against CCL5, CCL11 and CCL13 decreases by about 50% the eosinophil chemotactic activity of the bronchoalveolar lavage from asthmatic patients[220]. Relevance of CCR1 in asthma was also highlighted in animal models of allergic asthma and pulmonary fibrosis. Deletion of CCR1 resulted in a significant decrease in the number of goblet cells and subepithelial fibrosis, two important aspects of airway remodelling. Furthermore, these animals showed a marked reduction in Th2-cytokine production, as well as a decrease in CCL11 and CCL22[245]. Interestingly, in this particular model, only a slight decrease in the number of eosinophils in the bronchoalveolar lavage was observed and no difference in the airway hyperresponsiveness was observed. These results suggest a partial contribution of CCR1 ligands to eosinphils recruitment to the airways, but suggests a more pronounced role for them in Th2-lymphocyte recruitment and in remodeling [245]. These observations are coroborated by another study carried out in an animal model of lung fibrosis in which CCR1 was blocked using a monoclonal antibody. Deposition of collagen I was substantially reduced and survival increased as a result of the CCR1 blockade [328]. A possible mechanism behind these observations was subsequently provided by Ma *et al*[303], where the authors linked IL-13-

induced airway remodelling to CCL6/CCR1 signalling. Indeed, using CCR1-deficient mice they showed a marked reduction in several features of airway remodelling through the inhibition of proteases and antiproteases production.

The expression level of CCR1 in asthma has never been determined. To assess this, we examined CCR1 mRNA in airway biopsies obtained from mild, moderate and severe asthmatics, compared to non-asthmatics. All three asthmatic groups showed a significantly increased level of receptor expression. Interestingly, the group with mild asthma showed the highest level of CCR1 mRNA. We followed by showing baseline expression of CCR1 by cultured ASMC, which was readily upregulated by TNF-α and, to a lesser extent, IFN-γ. TNF-α is a very potent proinflammatory cytokine produced by inflammatory cells such as macrophages, neutrophils and mast cells, but also by structural cells such as ASMC[1]. In asthma, levels of TNF-α have been shown to be elevated in both BAL and biopsies[59, 72]. As discussed in section 1.3.1, IFN-γ may also be elevated in asthmatic airways. We observed that CCR1 message induced by TNF-α and IFN-γ- could be totally abrogated by the synthetic corticosteroid dexamethasone. These *in vitro* results showing steroid sensitivity of CCR1 might provide a possible explanation as to the reason why mild asthmatics demonstrated higher CCR1 mRNA expression than the other groups. In fact, patients with mild disease were steroid-free, unlike their moderate and severe counterparts who were taking inhaled steroids as part of their treatment regimen.

Using promoter analysis software, we detected several Sp-1 binding sites in the regulatory region of the CCR1 gene. Sp-1 is usually translocated by the

action of several pro-inflammatory mediators[350]. The antibiotic mithramycin A is a potent inhibitor of Sp-1 binding and we thus investigated its effect on CCR1 message. Treatment with mithramycin A completely suppressed the effect of both TNF-α and IFN-γ on CCR1 mRNA production, suggesting the requirement of Sp1 for the action of these cytokines. Collectively, these data imply that anti-inflammatory agents inhibit CCR1 mRNA levels, possibly through a transcriptional mechanism that would implicate repression of its promoter activity.

Since CCR1 seems to be associated with airway remodelling, we looked for the effect of CCR1 ligands on production of components of the ECM. Release of ECMP by ASMC has been well documented in the past few years[82, 153]. ASMC was stimulated with high doses of CCL3 or CCL23 and mRNA expression for collagen-αI, decorin, lumican and versican was evaluated using quantitative PCR at 4, 12, 24 and 48 hours. Booth CCL3 and CCL23 failed to significantly regulate the message of any of these ECM components (figure 6).

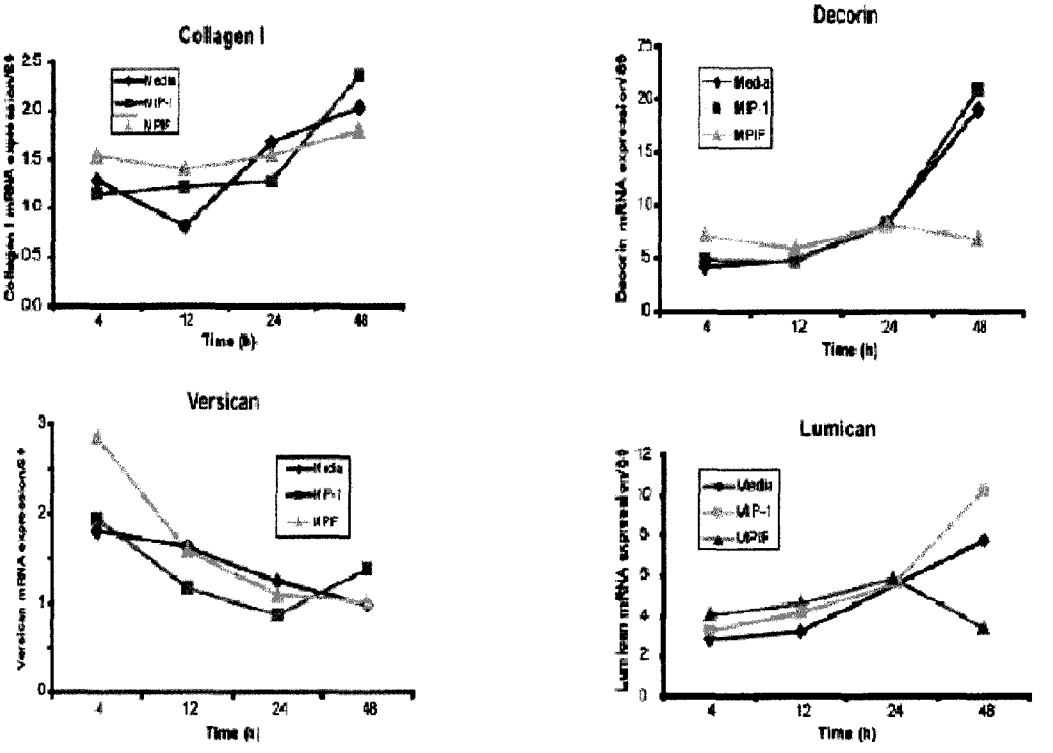

Figure 6. Production of extracellular matrix components by ASMC following stimulation with CCL3 or CCL23. mRNA for collagen I, decorin, versican and lumican was evaluated 4, 12, 24 and 48 hours following stimulation with 100 ng/ml of CCL3 or CCL23. Expression was analyzed by quantitative real-time PCR.

However, the contribution of ASMC to airway remodelling has also been associated with the release of matrix metalloproteinases (MMP)[351, 352]. Supported by recent results published by Ma *et al.*[303], it is possible that CCR1 ligands induce the release of these MMPs. We also investigated the effects of CCL3 and CCL23 on cytokines and chemokine production. Using a cytokine array (RayBio® Human cytokine array III, Norcross, GA) , we examined the production of over 40 different cytokines, chemokines and growth factors after the stimulation with either CCL3 or CCL23 for 4 and 24 hours. We were unable to detect any effect for both chemokines on cytokine release by ASMC.

Finally, CCL3 or CCL23 were tested for their ability to chemoattract ASMC. Using a similar protocol as the one used with CCR3, we incubated ASMC in a Boyden's chamber (see materials and methods, chapter II) with increasing concentrations of CCL3 or CCL23. However neither CCL3 nor CCL23 had any effect on ASMC recruitment. Based on these results, we have yet to identify the precise function of CCR1 on ASMC. Because CCR1 has been linked to airway remodelling in animal models[245, 328], it is plausible that this chemokine receptor is linked directly or indirectly to the modulation of airway remodelling features in human asthma.

4.3 Perspectives and futures directions

Work done with human tissues and cells serve as critical additions and often validate data derived from animal models. All the experiments performed in this thesis have been carried out using primary human ASMC. The data collected herein on chemokine receptors were necessary to determine their presence and possible function in humans. Indeed, working in an *in vitro* setting does not recreate the "real" physiological conditions found in asthmatic airways. Stimulation of cells, for instance, with a single or a combination of cytokines is not representative of the mixture of mediators to which cells are exposed *in vivo*. On the other hand, it is possible to thoroughly dissect single pathways and helps us clearly understand the effect of a single mediator on a given cell behaviour. The inherent advantage to the use of animal models is that it allows us to recreate physiological and pathological conditions to which cells might be exposed in the human disease it models. It is therefore possible to appreciate the consequences of

the addition or the deletion of a single gene/protein on a more complete scale. Several argue that murine models of allergic asthma do not always exactly recreate the disease and key pathological features associated with it, such as airway remodelling [353]. Our work would nonetheless benefit a mice model that could reproduce some aspects of airway remodelling. It would be interesting to selectively knock-out or knock-in CCR3 or CCR1 on smooth muscle, to evaluate our hypothesis on the importance of these receptors on ASMC behaviour in asthma.

The path towards a fuller comprehension of asthma pathogenesis seems distant with so many questions remaining. It is our belief that the work presented here provided new and exciting elements that could potentially be used for developing novel therapies.

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