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Cytokine Regulation in Allergic Inflammation: Role of Transcription Factors

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1. **Christodoulopoulos P**, Cameron L, Nakamura Y, Shigeo M, Dugas M., Lemière C, Boulet LP, Laviolette M, Olivenstein R, Hamid Q. Th2 cytokine-associated transcription factors in atopic and non-atopic asthma. evidence for differential STAT-6 expression. *J Allergy Clin Immunol* 2001. 107:586-91.

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ABSTRACT

The allergic inflammatory response is orchestrated by Th2 cytokines, particularly IL-4, IL-5 and IL-13. Th2 cytokines have therefore become therapeutic targets in the management of allergic disease. Current therapeutic strategies inhibiting individual cytokines have not been proven effective at inhibiting the allergic response, therefore finding a common factor controlling the expression of all Th2 cytokines would provide a more ideal target. A number of gene-specific factors have been identified which regulate the transcription of cytokine genes. Based on *in vitro* studies and animal models, GATA-3 appears to be necessary for Th2 cytokine gene expression. In this thesis, we provide first time evidence for the expression of GATA-3 in humans, where levels were significantly higher in asthmatics compared to controls. The high expression of GATA-3 mRNA was also observed within allergic nasal mucosa which significantly increased in response to local allergen challenge. To assess the contribution of resident cells expressing GATA-3, we performed *in vitro* allergen challenge of isolated nasal explant. Using *in situ* hybridization, we demonstrated a significant increase in GATA-3 mRNA+ cells in response to local allergen challenge, excluding the possibility that the increase in GATA-3 mRNA was solely due to the infiltrating inflammatory cells. We also investigated the expression of STAT6 and cMaf transcription factors in bronchial biopsies from human asthmatics, which have also been described to be associated with Th2 responses. We demonstrated a significantly higher expression of cMaf and STAT6 immunoreactivity in asthmatic compared to control subjects. We then went further to determine whether the expression of GATA-3, STAT6 and cMaf was dependent on atopic status. To do

this, we investigated their expression in bronchial biopsies from both atopic and non-atopic asthmatics. Our results demonstrate an upregulation of GATA-3 and cMaf in both variants of asthma, whereas STAT6 was significantly higher in atopic compared to non-atopic asthma. The idea that allergies arise from an imbalance between Th1 and Th2 cells, has focused attention on Th1 cytokines and the factors regulating their expression. YY1 is a novel transcription factor recently described to upregulate the expression of IFN-γ *in vitro*. Our objective was to characterize the expression of YY1 in a Th1 milieu, and to determine if Th1 stimulating factors can induce the upregulation of this transcription factor. We used nasal mucosal explants from allergic patients, and cultured them in the presence and absence of LPS (a bacterial product known to induce Th1 differentiation), or ragweed allergen for 24 hours. Using immunocytochemistry, we demonstrated a significant increase in the number of YY1 immunoreactive cells following LPS stimulation, which was not seen in tissue, cultured with medium alone or with ragweed extract. This data suggests that LPS may potentially skew the Th2 response in allergic tissue towards Th1, through the induction of Th1 transcription factors. Identification of the factors responsible for cytokine gene regulation and modulation, may be of value for the development of alternative, and more specific approaches in the treatment of allergy.

SUMMAIRE

La réponse inflammatoire allergique est orchestrée par les cytokines de type Th2, en particulier l'IL-4, -5 et -13. Ces cytokines sont ainsi devenues des cibles dans le traitement des maladies à composante allergique. Les stratégies thérapeutiques actuelles visant à inhiber sélectivement certaines cytokines s'étant révélées incapables de bloquer la réponse allergique, la mise en evidence d'un facteur controlant l'expression de l'ensemble des cytokines de type Th2 devrait permettre de proposer une cible plus efficace. De nombreux facteurs de transcription sont connus pour réguler sélectivement l'expresion des genes codant pour les cytokines. Des modèles animaux et des études *in vitro* ont ainsi montré que le facteur de transcription, GATA-3 est nécessaire à l'expression des cytokines de type Th2. Nous rapportons ici pour la première fois que GATA-3 est exprimé chez l'homme, et que cette expression est augmentée au niveau de l'arbre respiratoire des patients asthmatiques par rapport aux sujets sains. L'ARNm de GATA-3 est également fortement exprimé au niveau de la muqueuse nasale des sujets allergiques, et cette expression est augmentée par une provocation allergénique locale. Afin d'évaluer la contribution des cellules résidentes dans l'expression de GATA-3, nous avons réalisé une provocation allergénique d'explants nasaux isolés et mises en culture. Dans ces conditions, nous avons mis en évidence par hybridation *in situ* une augmentation significative de l'ARNm de GATA-3, ce qui exclue la participation unique des cellules inflammatoires infiltrantes à ce phénomène. Nous avons ensuite évalué l'expression de facteurs de transcription également associés aux réponses de type Th2, STAT-6 et c-Maf, au niveau des biopsies bronchiques des sujets asthmatiques. Nous avons alors montré une

expression significativement augmentée de ces protéines chez les asthmatiques par rapport aux sujets contrôles. Afin de déterminer si le statut atopique influence l'expression de GATA-3, STAT-6 et c-Maf, nous avons évalué l'intensité de leur expression au niveau de biopsies bronchiques de patients asthmatiques atopiques et non-atopiques. Nos résultats montrent que GATA-3 et c-Maf sont augmentés dans les deux groupes de patients, alors que l'expression de STAT-6 est plus élevée chez les sujets atopiques que chez les non-atopiques.

Il est actuellement accepté que les allergies sont la conséquence d'un dérèglement dans la balance de production des cytokines de type Th1 et Th2. Ce concept a conduit à s'intéresser également aux cytokines de type Th1. Il a été récemment montré que le nouveau facteur de transcription YY1 induit l'expression de l'IFN-γ *in vitro*. Notre objectif était d'évaluer l'expression de YY1 dans un milieu Th1 en utilisant des facteurs bactériens capables de stimuler une telle reponse. Pour cela, des explants de muqueuse nasale de patients allergiques ont été cultivées en absence ou en présence de LPS ou de pollen pendant 24 heures. Des expériences d'immunohistochimie nous ont permis de démontrer une augmentation significative du nombre de cellules exprimant YY1 en réponse à l'exposition au LPS, par rapport à la culture en milieu seul ou en présence de pollen. Ce resultat suggère que les produits bactériens peuvent déplacer la réponse Th2 présente au niveau d'un tissu allergique vers un phénotype Th1 à travers l'induction de l'expression de facteurs de transcription de type Th1. L'identification des facteurs capables de moduler l'expression des cytokines devrait permettre de développer dans le futur des stratégies thérapeutiques plus spécifiques pour le traitement des maladies allergiques.

ABBREVIATIONS

ADAM: a disintegrin and metalloprotease

AHR: Airway hyperresponsiveness

APAAP: Alkaline phosphatase anti-alkaline phosphatase

APC: Antigen presenting cell

AP-1: Activator protein 1

AS: Antisense

BCG: Bacille Calmette-Guerin

CpG: Cytosine and guanosine dinucleotide repeat motifs

ECP: Eosinophilic cationic protein

E coli: Escherichia coli

GC: Glucocorticoid

GCR: Glucocorticoid receptor

GM-CSF: Granulocyte macrophage - colony stimulating factor

ICC: Immunocytochemistry

IgE: Immunoglobulin E

IL: Interleukin

IFN: Interferon

ISH: *in situ* hybridization

JAK: Janus kinase

LPS: Lipopolysaccharide

LTC_4: Leukotriene C_4

MBP: Major basic protein

NFAT: Nuclear factor of activated T cells

NF-kB: Nuclear factor kB

PBS: Phosphate- buffered saline

PLL: Poly-L-lysine

RW: Ragweed

STAT: Signal transducer and activator of transcription

TB: Tuberculosis

T-bet: T box expressed in T cells

TBS: Tris buffered saline

Th cell: T helper cell

TNF: Tumor necrosis factor

VCAM: Vascular cell adhesion molecule

YY1: Ying yang 1

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CHAPTER I: INTRODUCTION

1.1 Allergy

The term "allergy" was first introduced by von Pirquet in 1906 who recognized that in both protective immunity and hypersensitivity reactions, antigens had induced changes in reactivity. Although his intent was that the term be applied to the biologic response, which may either lead to protective immunity or allergic disease, the word is now frequently used synonymously with IgE-mediated allergic disease, even though it is the term "atopy" that is often used to describe IgE-mediated diseases. Atopic individuals have a hereditary predisposition to produce IgE antibodies against common environmental allergens. As a result, they develop one or more allergic diseases such as allergic rhinitis or asthma, which are chronic inflammatory conditions of the upper and lower airways, respectively.

1.2 Epidemiology

Reported prevalence rates of allergic diseases vary within and between countries around the world. In the last 20 years, allergic diseases have had a high and increasing prevalence rate, particularly in Western countries (1), (2). According to a 1997 report from the World Health Organization, the total prevalence of asthma is estimated to be 7.2% of the world population, with a prevalence of about 6% in adults and 10% in children. In fact, at least 40 000 deaths per year worldwide may be attributed to asthma (3). In spite of a mortality rate associated with this disease, which had stabilized in the United States in the 1990s, at present, this rate is more than 50% higher than in 1979 (4). According to the Canadian Institute for Health Information,

over two million Canadians are currently affected by asthma (5). Epidemiological studies would suggest that allergic rhinitis is found in approximately 10% of the general population, and in 15-20% of the North American population, making it the most common allergic disorder in western countries (6), (7). Overall, allergic rhinitis affects an estimated 20-40 million people in North America, and this incidence is increasing (8). Studies have shown a significantly lower prevalence of asthma and allergic diseases in Eastern Europe than in western countries. Allergic diseases were less common in former East Germany than West Germany, however, since reunification, the prevalence of these disorders has increased among those who spent their early childhood in East Germany (9), (10).

1.3 Etiology

1.3.1 Genetic Factors

Genetic epidemiology confirms that there is a strong heritable component to allergic diseases, particularly in asthma (11). There are difficulties, however, in conducting genetic studies due in part to the multiple markers for atopy, and the heterogeneity of the disease. To date, genetic studies have linked several loci to atopy, however the clinical relevance of these findings is unclear. Examples are the associations between an allele of the HLA-DR locus and reactivity to the ragweed allergen (12), as well as the linkage of atopy to a polymorphism of the gene for the β chain of the high affinity receptor for IgE (FcεRI-β) (13), and to the interleukin-4 family of cytokine genes on chromosome 5q31 (14), (15), (16). In fact, genetic mapping studies reveal that chromosome 5q31-q33 is of particular interest since it

contains numerous gene candidates that may potentially play a role in airway inflammation, associated with allergic disease. In particular, the IL-9 cytokine was suggested as a likely candidate gene based on unbiased human genetic mapping studies on airway hyperresponsiveness and linkage homology (17). Furthermore, polymorphisms in the gene encoding the high affinity receptor for bacterial lipopolysaccharide, CD14, located on chromosome 5q31.33, have also been linked to total serum IgE levels and associated atopy (18). Most recently, a member of the ADAM (a disintegrin and metalloprotease) family, ADAM33, has been identified and characterized as a putative asthma susceptibility gene by positional cloning. ADAM33 was identified on chromosome 20p13 on which a locus has been linked to asthma and atopy (19).

1.3.2 Environmental factors

Although allergic diseases have a strong genetic component, there are a number of important environmental factors interacting with genes that are of central importance in the clinical manifestation of the disease. In fact, the marked increase in the prevalence of allergic diseases, particularly in Western countries, in which public hygiene measures are well implemented, indicates the importance of environmental influences. Indicative of this would be the increased incidence of allergic disorders after the reunification of Germany. In modern metropolitan communities, the developing immune system is deprived of the microbial antigens that stimulate Th1 cells, given that the environment is relatively clean, and the use of antibiotics for minor illness in early life is widespread (20). On the contrary, allergic diseases have

been reported to be less frequent in populations living in farms or in rural areas which increases the likelihood of exposure to bacteria, which is thought to protect against atopic disease (21). These observations have given rise to the 'hygiene hypothesis', in which reduced exposure to bacterial products predispose to the persistence of allergies in childhood (22). Other factors that may favor atopy in infants include diet, and being born when pollen counts are high (16). Furthermore, allergic diseases are less common in younger children who have older siblings, and most interestingly among children who have had viral infections such as measles or hepatitis A. This indicates that repeated immune stimulation may protect against atopic allergy (23). This idea is supported by a study conducted by Ball et al demonstrating that exposure of young children to older children at home or at day-care centers protected them against the development of asthma and frequent wheezing in childhood (24). These factors alone or in combination, may alter immunoregulatory mechanisms at mucosal surfaces in ways that promote an allergic inflammatory response.

1.3.3 In Utero events

It is believed that environmental exposure begins at conception. During pregnancy, the maternal-fetal interface is an immunologically active site producing many cytokines. Numerous lines of evidence point to a dominance of Th2 type cytokines in the uterine environment as well as fetal cord blood lymphocytes, which are skewed towards the Th2 type. Neonatal T cells have been shown to produce low levels of IFN-γ, yet, an overproduction of Th2 cytokines (25). Maternal lymphocytes produce IL-5 in response to normal allogenic placenta, whereas placentas from

abortion-prone pregnancies cause lymphocytes to secrete IL-2 and IFN-γ (26), (27). At birth, the immune system of the fetus has been strongly shifted toward a Th2 lymphocyte profile. In mothers with atopic disease, this effect may be exaggerated. If this skewed fetal immune response is not re-balanced by stimuli that restore the normal Th1/Th2 balance, the child may become predisposed to asthma and atopy.

1.3.4 Hygiene Hypothesis

One of the major influences on the Th1/Th2 balance is exposure to infectious agents. In fact, the increasing prevalence of allergic diseases is thought to be the result of an unopposed Th2 development due to the lack of natural infections resulting in a defective Th1 response (28). This has led to the "hygiene hypothesis". The basic tenet of this hypothesis, as discussed above, is that the immune system of a newborn infant is skewed toward Th2 cells and needs timely and appropriate environmental stimuli to create a balanced immune response (29), (30). Factors that enhance Th1 mediated responses, and that are associated with a reduced incidence of allergy, include infection with Mycobacterium tuberculosis, measles, and hepatitis A virus, increased exposure to infections through contact with older siblings, and attendance at a day care facility during the first six months of life (22). Restoration of the balance between Th1 cells and Th2 cells may be impeded by frequent administration of oral antibiotics, with concomitant alterations in gastrointestinal flora (30) (Fig.1). Although these observations have generated intense interest, conflicting results have prevented researchers from drawing firm conclusions about the validity of the "hygiene hypothesis" (31).

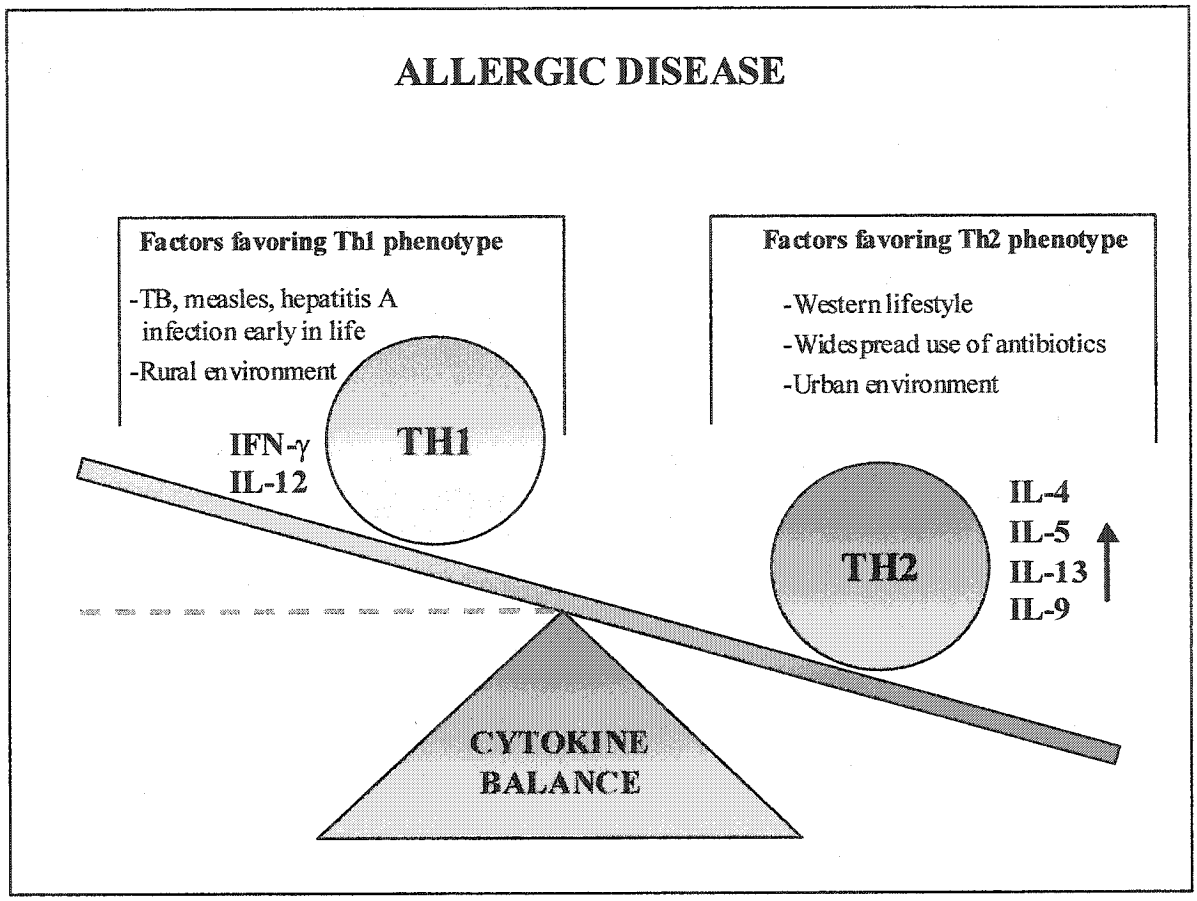


Fig 1. Th1/Th2 imbalance in allergic disease. Numerous factors including alterations in the number or type of infections early in life, the widespread use of antibiotics, adoption of the Western lifestyle, and repeated exposure to allergens, may affect the balance between Th1 type and Th2 type cytokine responses. These factors increase the likelihood that the immune response will be dominated by Th2 cells, and will ultimately lead to the expression of allergic diseases such as asthma.

1.4 Allergic Airway Diseases

1.4.1 Allergic rhinitis

Allergic rhinitis is a chronic inflammatory condition of the nasal mucosa, characterized by episodes of sneezing, itching, rhinorrhea, and nasal obstruction (32), (33). Although frequently trivialized, this condition represents an important cause of morbidity and impaired quality of life. It is frequently divided into two types, seasonal and perennial, based on the patient's location and the time of symptom onset and duration (34). For seasonal disease, the aeroallergen sensitization is commonly due to outdoor allergens such as tree or grass pollens, whereas perennial rhinitis is triggered by indoor allergens such as house dust mites and animal dander.

In this thesis, the majority of experiments were done using nasal specimens from allergic rhinitis patients. This allowed us to study the nature of the disease itself, but at the same time, the nose represents an important 'window' for the events that occur in the lower respiratory tract as it is conveniently accessible, and allows for high quality biopsy material to be obtained. This has provided us with detailed information on the nature of the allergic inflammatory response in rhinitis that is applicable to allergic disease of the lower airways (35).

1.4.2 Asthma

With the advent of fiberoptic bronchoscopy in the 1970s, and the use of novel molecular biology tools, it became clear that asthma is a chronic inflammatory disease of the airways. Clinical manifestations consist of recurrent episodes of wheezing, breathlessness, chest tightness, and cough, due to airway narrowing.

Airway hyperresponsiveness and airflow obstruction are invariably accompanying features of this disease, caused by the inflammation in the airways. Most patients with asthma are atopic although a minority has intrinsic, non-atopic asthma, that often has a later onset and a more protracted course than atopic asthma (36). The most striking difference, however, is the lack of demonstrable specific IgE to an allergen in non-atopic asthma. Nevertheless, recent studies indicate that there are more similarities than differences in the airway abnormalities of atopic and non-atopic asthma (37).

1.4.3 Relationship between allergic rhinitis and asthma

The overall pathogenic view of respiratory allergy has profoundly changed and evolved over the past 10 years. Particular attention has been paid to the relationship between allergic rhinitis and asthma, and between the upper and lower respiratory airways. There is evidence to suggest that there is a link between the two, and dysfunction of either or, frequently coexists. The mucosa of the upper and lower airways is covered by a similar pseudostratified, columnar, ciliated epithelium, with a continuous basement membrane. As such, there is often a close association between allergic inflammation in rhinitis, and in asthma. The only differences are the absence of smooth muscle (except around vessels) in the upper airway, and the lack of a vascular network in the lower airways. Data from epidemiological studies indicate that nasal symptoms are experienced by as many as 78% of patients with asthma, and that asthma is experienced by as many as 38% of patients with allergic rhinitis (38), (39). Although the inflammation initiated by allergens and irritants is probably similar in the upper and lower airways, the consequences of this inflammation differ, based

on the structure and function of the airway involved (e.g. sneezing versus coughing, nasal congestion versus breathlessness, rhinorrhea versus sputum production). Furthermore, there is increasing evidence of an inflammatory process in the lower airways of nonasthmatic subjects with allergic rhinitis, in response to allergen exposure (40). These observations taken together have led to the concept of "united airways disease", where allergy is not a disease confined to a specific target organ, but rather a disorder of the whole respiratory tract with a broad spectrum of clinical manifestations. Since both conditions have similar immunological mechanisms and underlying pathogenesis, and each may potentially affect the other, neither should be treated in isolation (41).

1.4.4 Allergic response

After repeated low-dose exposure to specific allergens, atopic individuals develop specific IgE antibodies to the allergen, leading to an orchestration of cellular immune responses (Fig.2). The potential for this response particularly in people with genetic predispositions, is established with the first exposure to the antigen. The process of initial antigen exposure and priming the inflammatory cells for the response to the antigen is referred to as the sensitization phase. In those destined to develop an allergic response, naïve T cells differentiate to a subtype of T helper cells designated Th2, which secrete cytokines responsible for switching B lymphocytes to produce IgE. Once a baseline level of IgE + cells is attained, at second and subsequent exposures to the same antigen, the primed cells respond through the release of mediators directly responsible for rapid inflammatory changes within

minutes. The kinetics of allergic inflammation involve a migration of inflammatory cells to the mucosa, which becomes apparent, approximately 30 minutes after specific challenge, continues for the following 24 hours, and then slowly subsides (42). The early phase response in allergic rhinitis is evidenced clinically by nasal itch, sneezing, rhinorrhea, and congestion; and in asthma, by wheezing and reduced airflow, accompanied by breathlessness. Depending on the amount of allergen, these immediate hypersensitivity reactions are followed by a clinical late phase response which occurs at 3 to 11 hours, but peaks at 6 to 9 hours, following the immediate phase. The late phase represents the long-term outcome of cellular activities that were set in motion during the early phase. This phase consists principally of congestion and sustained nasal blockage in allergic rhinitis, and a resurgence of bronchoconstriction and further wheezing in asthma (43) (Fig.3).

1.4.5 Histopathology of allergic airway mucosa

At necropsy, asthmatic lungs typically show hyperinflation, mucus plugs, due to a combination of mucus and a cellular infiltrate within the airways, clusters of sloughed epithelial cells, and crystalline precipitates of eosinophil derived proteins (44), (45). The bronchial mucosa appears edematous, with an increase in the number of goblet cells, a thickened basement membrane as well as a hypertrophied smooth muscle cell layer. A wide variety of inflammatory cells are found infiltrated within subepithelial and epithelial layers, and an inflammatory exudate is also present in the bronchus itself (46).

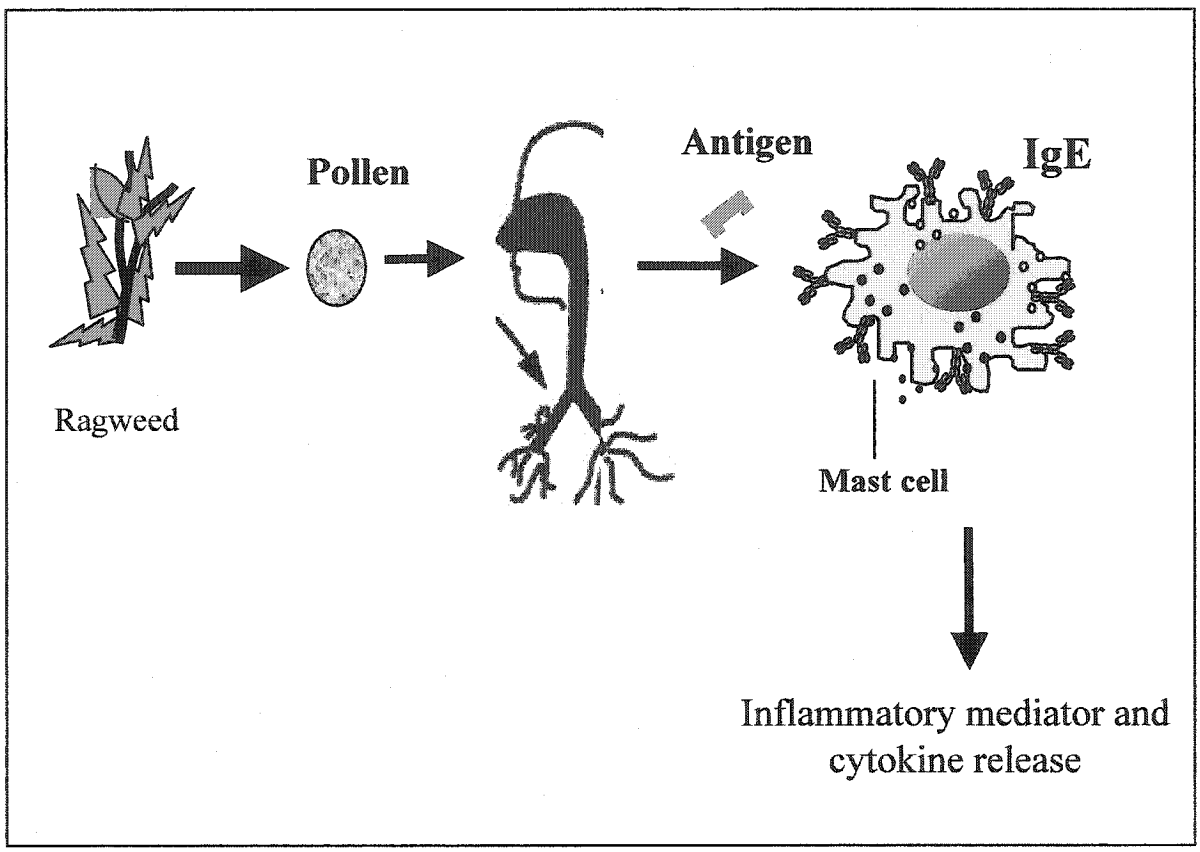


Fig 2. Antigen induced mast cell activation. Response to specific allergen, and subsequent inflammatory mediator and cytokine release

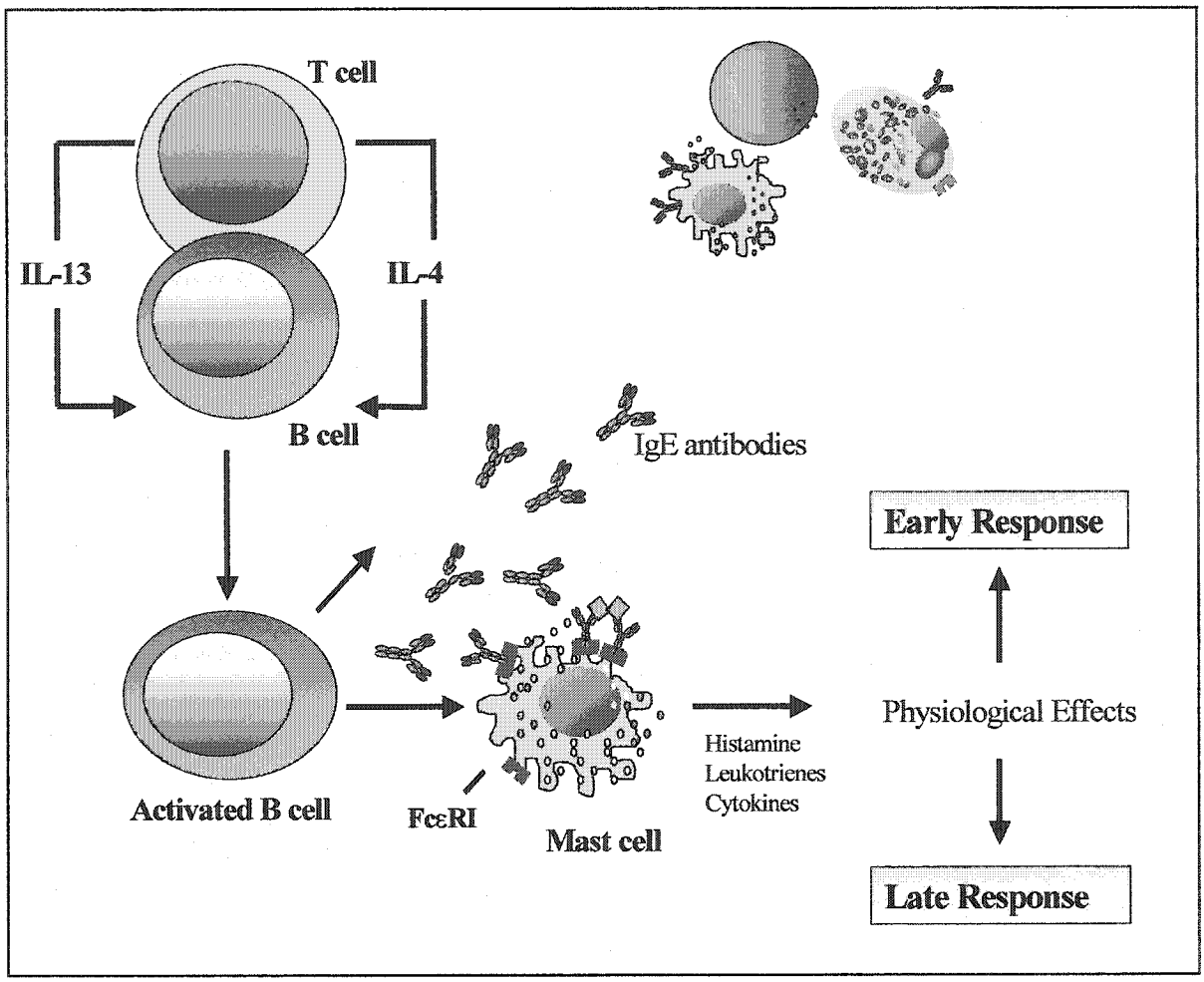


Fig 3. IgE production and the allergic response. IL-4 and IL-13 provide the first signal to B cells to switch to the production of the IgE isotype. Once formed, IgE antibody circulates in the blood, binding to both FcεRI and FcεRII. After subsequent encounters with antigens, binding to the IgE receptors produces the release of preformed and newly generated mediators. Once present in various tissues, mediators may produce various physiological effects, depending on the target organ, manifesting into an early and late response.

Analysis of allergic nasal biopsies following allergen exposure, reveal an increased number of infiltrating cells within submucosal and epithelial layers, as well as a thickening of the basement membrane. Degranulation of cells leads to mucus hypersecretion, where sinusoidal vessels become engorged, causing nasal obstruction (47). The airway mucosa responds to inflammatory provocations with bulk vascular and mucosal exudation of plasma, a prominent feature of airway inflammation, demonstrated in allergic rhinitis. Submucosal gland area appears to be increased compared to the normal state, with an increase in the number of goblet cells (48). As such, the nasal mucosa of allergic rhinitics is often pale blue and edematous (34).

1.5 Inflammation in Allergic Airways

A hallmark feature of allergic diseases is the increased number of inflammatory cells within the airways, notably CD4 + T lymphocytes, eosinophils, basophils, and mast cells, as well as the activation of structural and resident cells, particularly the airway epithelial cells. Each of these cells has an important contribution, and a specific relation to the development of airway inflammation. The infiltrating inflammatory cells make their way to the target organ through site-specific endothelial adhesion molecules, as well as a wide variety of chemokines, whose expression is induced by cytokines released from these inflammatory cells. Chemokines are relevant in inflammation, not only because of their role in regulating leukocyte recruitment to allergic sites, but also because of their ability to activate inflammatory cells to release pro-inflammatory mediators at the site of inflammation where they work together to promote the Th2 inflammatory response (49). This

interaction between the released mediators, cytokines and chemokines, with the resident and infiltrating inflammatory cells leads to the development of the inflammatory response in allergic diseases.

1.5.1 Epithelial cells

The epithelium has long been considered to act mainly as a barrier participating in mucociliary clearance and removal of noxious agents. However, in addition to being a protective barrier, the epithelium has recently been found to participate in inflammation by producing a wide array of mediators including cytokines, chemokines, eicosanoids, peptidases, and matrix proteins. Inducible nitric oxide synthase has also been found in epithelial cells, which has vasodilating and bronchodilating effects, cytotoxicity, and mucus secretion (50), (51). Epithelial cells can also perform an immune function by their capacity to express human leukocyte-associated antigen-DR (HLA-DR) and present antigen (52). In asthma, epithelial cells are also likely to be important in repair processes as they release extracellular matrix proteins such as fibronectin, which appear to be of importance in cell regeneration (53), (54).

1.5.2 Antigen presenting cells

Antigen presenting cells (APCs) form a morphologically heterogeneous group of cells with the common task of presenting antigen to T cells in the context of the MHC molecules. APCs are therefore mandatory for initiating and controlling the immune response to antigens present at the interface with the environment (55), (56).

The cells most commonly considered APCs are dendritic cells, macrophages, and B cells, all of which express MHC class II molecules as well as adequate levels of T cell costimulatory molecules such as B7.1 and B7.2 (57). Resting APCs in the mucosa normally sample the antigens in their environment by pinocytosis and express relatively low levels of costimulatory molecules. On activation, APCs shut down their antigen uptake, and migrate to the draining lymph nodes, during which time they upregulate MHC and costimulatory molecule expression, thereby enhancing their antigen-presenting capabilities. Hence, T cell mediated immune inflammatory responses can only be generated after APCs have been activated (58).

1.5.3 Mast cells

Mast cells play an essential role in mediating the immediate response to allergen in allergic disease. The cross-linking of mast cell bound IgE by allergen, induces the activation of membrane and cytosolic pathways that cause the release of preformed mediators such as histamine, tryptase, and cytokines such as TNF-α, and IL-4 (59), (60), (61). In patients with allergies, inhaled allergen precipitates initiates the release of mast cell mediators such as histamine, leukotrienes, platelet activating factor, and prostaglandins leading to vascular permeability, bronchoconstriction, and induction of an inflammatory response all leading to obstruction of the airways. There are two subpopulations of mast cells, which reside in airway tissue, based on the enzymes they contain and their tissue location. Mucosal mast cells contain tryptase and are located within the epithelial layer, whereas connective tissue mast cells contain both tryptase and chymase and are located lower within the submucosa of the

airways. Although the total number of mast cells does not change during the allergy season in allergic patients, a higher proportion of these cells is observed just beneath or within the epithelial cell layer (62), (63). Furthermore, there are elevated numbers of mast cells expressing FcεRI, indicating their increased ability to bind IgE (64).

1.5.4 Basophils

Basophils represent less than 1% of the normal blood differential count, being the least numerous of the circulating leukocytes (65). Unlike mast cells, basophils are not normally a component of the inflammatory cell network of the nasal mucosa. Basophils are the only circulating leukocytes containing histamine, but like mast cells, they bind allergen by IgE/FcεRI and release histamine on activation (66). Basophils have also been shown to release LTC_4, IL-4 and IL-13 (67) (68). A marker of systemic effects of allergic disease is a stable basophilia, which increases approximately 2 fold during the allergy season (69). Thus, allergic individuals have a larger pool of basophils that are capable of being recruited to sites of allergic inflammation. In asthmatics, the numbers of blood basophils correlate with bronchial hyperresponsiveness and changes in methacholine reactivity over time (70). Basophil numbers are also increased within the nasal lavage fluid obtained 24 hours after nasal allergen challenge attributing to the increased histamine levels during the late-phase response (71), (66), (72).

1.5.5 Eosinophils

Eosinophils are bone marrow derived granulocytes that are normally not prominent in either peripheral blood or tissue. In allergic diseases however, eosinophilia develops, and are considered critical effector cells (73), (74). Mature eosinophils develop from CD34 pluripotent progenitor cells in the bone marrow and in tissue, under the influence of hematopoietic growth factors. As a consequence of allergen challenge, eosinophils migrate to the airways (75), (71), (76), (77). The accumulation of eosinophils is regulated by the generation of survival and activation factors, IL-3, IL-5, and GM-CSF, which eosinophils have receptors for (78). Eosinophils contribute to allergic inflammation through the release of preformed and newly generated mediators such as histamine, leukotriene C_4, major basic protein (MBP), and eosinophil cationic protein (ECP) (79), (80). MBP, in particular, has been reported to damage respiratory epithelium and to induce degranulation of other inflammatory cells promoting airway hyperresponsiveness (81), (82). Cysteinyl leukotriene C_4 is known to contract airway smooth muscle, to increase vascular permeability, and has chemotactic properties recruiting more eosinophils to the airways (83). Eosinophils also release a collection of pro-allergic/inflammatory cytokines, including IL-4, IL-5, IL-10, GM-CSF and TNF-α (84), (85), (86), (87), which all play an important role in the allergic response. Eosinophils have also been shown to produce and release SCF (stem cell factor), which is known to act on mast cells during an allergic response (88). Most recently, eosinophils were reported to be a cellular source of IL-11 and IL-17 production in human asthmatic airways (89), (90)

1.5.6 T lymphocytes

T lymphocytes are known to coordinate and mediate the adaptive immune response that the body elicits in response to foreign antigen. In fact, the initiation and regulation of allergic inflammatory responses of the nose and lung in allergic individuals, is dependent upon T lymphocyte activity. These cells develop from committed precursors in the bone marrow, and are selected and processed through the thymus during development. T lymphocytes are generally subdivided into two distinct subsets according to their cell surface markers, and distinct effector functions. T cells expressing the CD4 antigen are involved in humoral immunity and are termed helper T cells (Th), whereas those expressing the CD8 antigen are referred to as T cytotoxic / suppressor cells. CD8+ cells are involved in cell mediated responses and interact with intracellular pathogens such as viruses, presented in conjunction with MHC class I. In contrast, CD4+ T cells are capable of recognizing foreign antigen processed in association with MHC class II on the surface of professional antigen-presenting cells. As such, CD4+ T cells have attracted considerable attention in the pathogenesis of allergic disease because of their ability to drive antigen-specific inflammatory responses and regulate Ig production.

Allergic subjects have increased numbers of CD4+ T lymphocytes, which have been associated with both the onset and severity of the disease, and are a frequent finding within the bronchial mucosa of patients with all forms of asthma, from newly diagnosed to severe. Similarly, nasal biopsies have demonstrated that the CD4+ T lymphocyte is the most prominent cell type within allergic nasal mucosa and that following allergen challenge outside the pollen season, there is a marked increase

in the number of CD4+ T cells (91). These lymphocytes, both in bronchial and nasal mucosa, appear to be "activated", as determined by their expression of the IL-2 receptor (CD25), suggesting that they are a cardinal feature of the disorder (92), (93), (94), (95), (48), (96), (97). Furthermore, the local environment in which CD4+ T cells are activated, influences their development further, either towards the T helper 1 (Th1) or Th2 subtypes. Although it is overly simplistic to say that various disease states are either "Th1" or "Th2 like", evidence suggests that Th2-like lymphocytes and their products are important in allergic diseases.

1.5.6.1 Th1 / Th2 lymphocytes

In 1986, it was Mosmann et al who classified CD4+ T cells into "Th1" and "Th2" subtypes, based on their expression of different cytokine patterns as observed in mice (98). Th2 cells are reported to produce IL-4, IL-5, IL-9 and IL-13, and are involved in humoral immune responses and allergy. By contrast, the polarized form of the T helper cell response which is defined by Th1, is characterized by the production of IL-2 and IFN-γ, and involved in cellular immunity (98), (99). Th1 and Th2 cells are derived from a common precursor, Th0 naïve T cells, and acquire their set pattern of cytokine production during differentiation. The specific cytokine profile that develops in CD4+ T cells is influenced by the antigen type and dose, the antigen-presenting cell type, and the cytokine microenvironment (100). It has been shown that the presence of IFN-γ and IL-4 promote the differentiation of naïve T cells into effector Th1 or Th2 cells respectively (101), (102), (103), (104) (Fig.4). Although the Th1/Th2 polarization is clear-cut in murine models, this distinction is less clear in

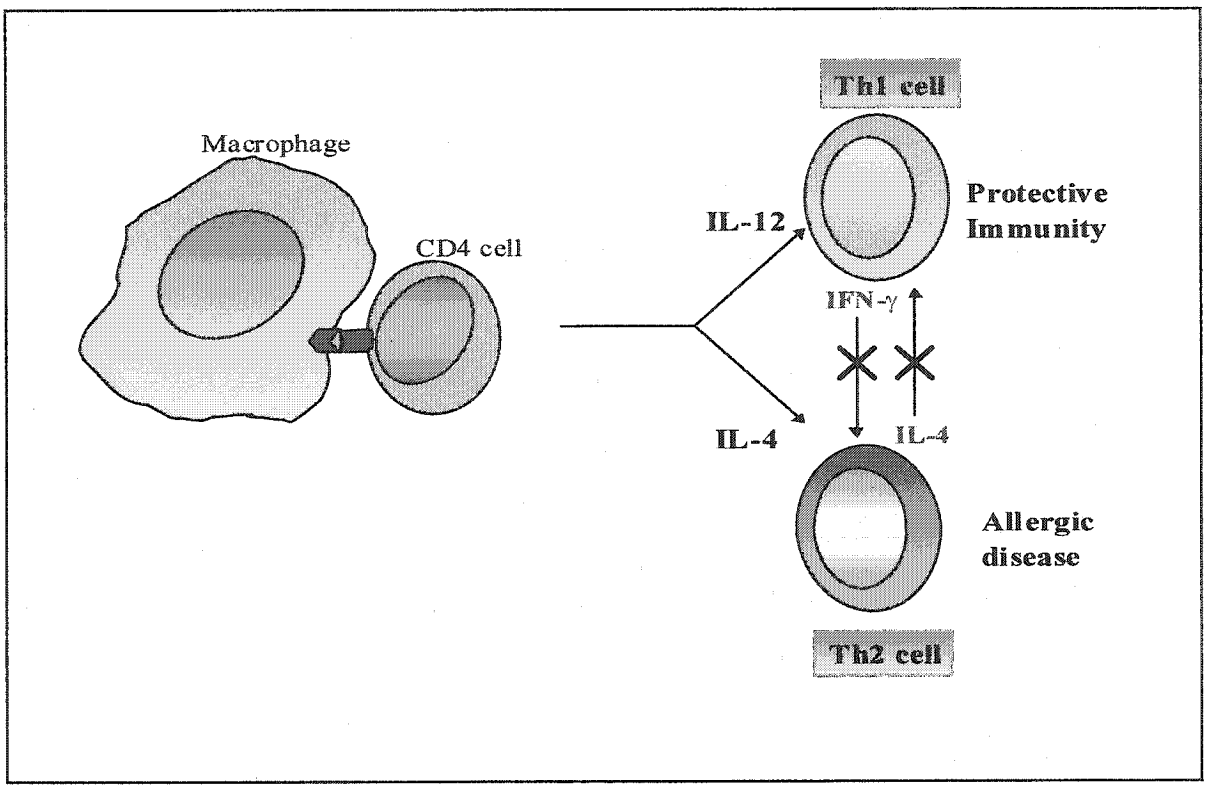


Fig 4. Th1 and Th2 cells. The differentiation of Th1 and Th2 cells depends on IL-12 and IL-4 cytokines, produced by antigen-stimulated precursor CD4+ T cells. In a regulatory loop, IFN-γ from Th1 cells inhibits Th2 cells, and IL-4 from Th2 cells inhibits Th1 cells. An imbalance that favors Th2 cells may be important in asthma.

humans. T cells that produce only Th1 or Th2 cytokines do not exist in humans. Depending on the stimulus, human T cells can make both IL-4 and IFN-γ (105). As such, most researchers use the ratio of IFN-γ to IL-4 to define the phenotype of the human T cell response.

Asthma and allergic rhinitis have been defined as Th2 driven diseases since there are elevated levels of Th2 cytokines in target tissues (106), (107), (108), involved particularly in T cell activation, IgE production, and eosinophilic inflammation. Studies have shown that following allergen challenge, bronchial biopsies and bronchoalveolar lavage fluid from asthmatic patients, as well as nasal biopsies from allergic rhinitic patients, are characterized by CD4+ T cells displaying an increase in IL-4 and IL-5, and low levels of IFN-γ (109), (110). This 'Th2 hypothesis' describes that allergic diseases are caused by a relative increase in Th2 cellular response in combination with a decrease in Th1 response. The consequent alteration in cytokine milieu with excess Th2 cytokines in concert with decreased Th1 cytokines is predicted to drive the allergic phenotype (111).

1.6 Cytokines

Cytokines are low molecular weight glycoproteins involved in cell-to-cell signaling, playing an integral role in specific immune responses. Cytokines provide communication between cells facilitating diverse functions such as growth, chemoattraction, immunoglobulin isotype switching, as well as cellular proliferation and differentiation (112). Cytokines usually have an effect on closely adjacent cells, and therefore function in a predominantly paracrine manner, although they may also

act in an endocrine and autocrine manner. Cytokines act on high-affinity cell surface receptors and it is through the regulation of receptor number, or through the production of membrane-bound versus soluble forms of the receptor that the actions of cytokines are modulated. A number of cytokines recognized to date exhibit pleiotropy and there is often redundancy in their actions, making their role in the pathogenesis of various disease states difficult to unravel. Cytokines may have synergistic or antagonistic effects with other cytokines, with each as part of a complex interacting network. They may act as proinflammatory mediators, but also have the capacity to downregulate inflammation. Cytokines produced by leukocytes which have effects mainly on other white blood cells are termed 'interleukins'. Cytokines that have chemoattractant activity are called 'chemokines'. Those that cause differentiation and proliferation of stem cells are called colony-stimulating factors, while cytokines that interfere with viral replication are called 'interferons'.

1.6.1 Th2 Cytokines in Allergic Inflammation

The study of cytokines in human disease has become very important since the discovery of Mosmann et al. that fully differentiated mouse CD4+ T cells tend to secrete one of the two different sets of cytokines. The initial description of these cytokine identified IFN-γ as a Th1 cytokine, and IL-4 as a Th2 cytokine (98). Subsequent studies have identified additional members of each cytokine set, but established IFN-γ and IL-4 as the prototypic Th1 and Th2 cytokines respectively. In the last decade, one of the most striking advances in the study of allergic diseases has been the recognition that cytokines play a critical role in orchestrating, perpetuating,

and amplifying the inflammatory response in asthma. Th2 cytokines, particularly IL-4, IL-5, IL-13, IL-9 and GM-CSF, play a critical role in allergic diseases. Increasing knowledge in this area has provided the basis for a number of novel therapies for allergic disease (113), (114) (Table 1).

1.6.1.1 Interleukin-4 (IL-4)

The IL-4 cytokine plays a critical role in allergic diseases as it mediates important pro-inflammatory functions. An essential biological activity of IL-4 in the development of allergic inflammation, is its ability to drive the differentiation of naïve T helper lymphocytes into a Th2 phenotype. These Th2 cells are then able to secrete IL-4, IL-5, IL-9 and IL-13 (102), (115), but lose the ability to produce IFN-γ (116). The major cellular sources of IL-4 within the airways of allergic subjects are CD4+ T cells, and to a lesser extent eosinophils, and mast cells (109), (117). IL-4 is critical in the switching of B cells to IgE production. In addition, it has a potential role in regulating the inflammatory response to recall antigens, and in regulating leukocyte trafficking by activating adhesion systems in the vascular endothelium (118), (119), (120). IL-4 has the ability to induce the expression of vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells, producing enhanced adhesiveness on the endothelium for T cells, eosinophils, basophils, and monocytes, for subsequent recruitment, characteristic of allergic reactions (121). IL-4 also has the ability to induce mucin gene expression from bronchial epithelial cells, as well as goblet cell metaplasia (122). IL-4 has been shown to increase the expression of

Table 1. Involvement of Th2 cytokines in allergic inflammation

Stimulation of IgE production	IL-4, IL-9, IL-13
Eosinophil, neutrophil, and mast cell infiltrates	IL-4, IL-5, IL-9, IL-13
Mastocytosis	IL-3, IL-4, IL-9
Goblet cell hyperplasia	IL-4, IL-5, IL-9, IL-13
Th2 differentiation	IL-4
Increased adhesion molecule expression	IL-4, IL-13
Increased vascular permeability	IL-4, IL-5, IL-13
Airway hyperresponsiveness	IL-4, IL-5, IL-9, IL-13
Smooth muscle hyperplasia	IL-4, IL-9, IL-13
Subepithelial fibrosis	IL-4, IL-5, IL-9, IL-13

chemokines such as eotaxin, RANTES and MCP-1 from epithelial cells (123), as well as other inflammatory cytokines such as IL-6, IL-16, TGF-β and TNF-α, all playing a role in allergic inflammation (124), (125), (126), (127), (128), (121) (Fig.5). Studies have shown that inhalation of IL-4 causes the development of sputum eosinophilia and increased airways hyper-responsiveness (129), while inhibition of its actions during allergic airway inflammation attenuates eosinophilia, and IgE production (130), (131), (132). Studies, however, show that mice deficient in IL-4, maintain residual Th2 responses which may be explained by the expression of other Th2 cytokines, particularly IL-5, leading to eosinophilia and consequent airway hyperreactivity (120), (133), (134). Because of these properties, IL-4 has been considered a potential target in allergic diseases.

1.6.1.2 Interleukin-5 (IL-5)

IL-5 is a pleiotropic cytokine that exhibits biological activity on cells of diverse hemopoietic lineages. IL-5 is the cytokine felt to be the most critical in the proliferation, differentiation, survival, and activation of eosinophils (135), (136) (Fig. 6). IL-5 enhances histamine release and leukotriene C_4 generation from eosinophils as well as basophils, and promotes IL-2-induced proliferation and differentiation of human and murine cytotoxic T lymphocytes (137). IL-5 has been shown to stimulate the production of IgA from plasma cells, which are extremely efficient at degranulating eosinophils when bound to antigen. In addition to its hematopoietic properties, IL-5 is a weak eosinophil chemoattractant priming eosinophils for their recruitment by other chemotactic agents, such as eotaxin (138). It is generally thought

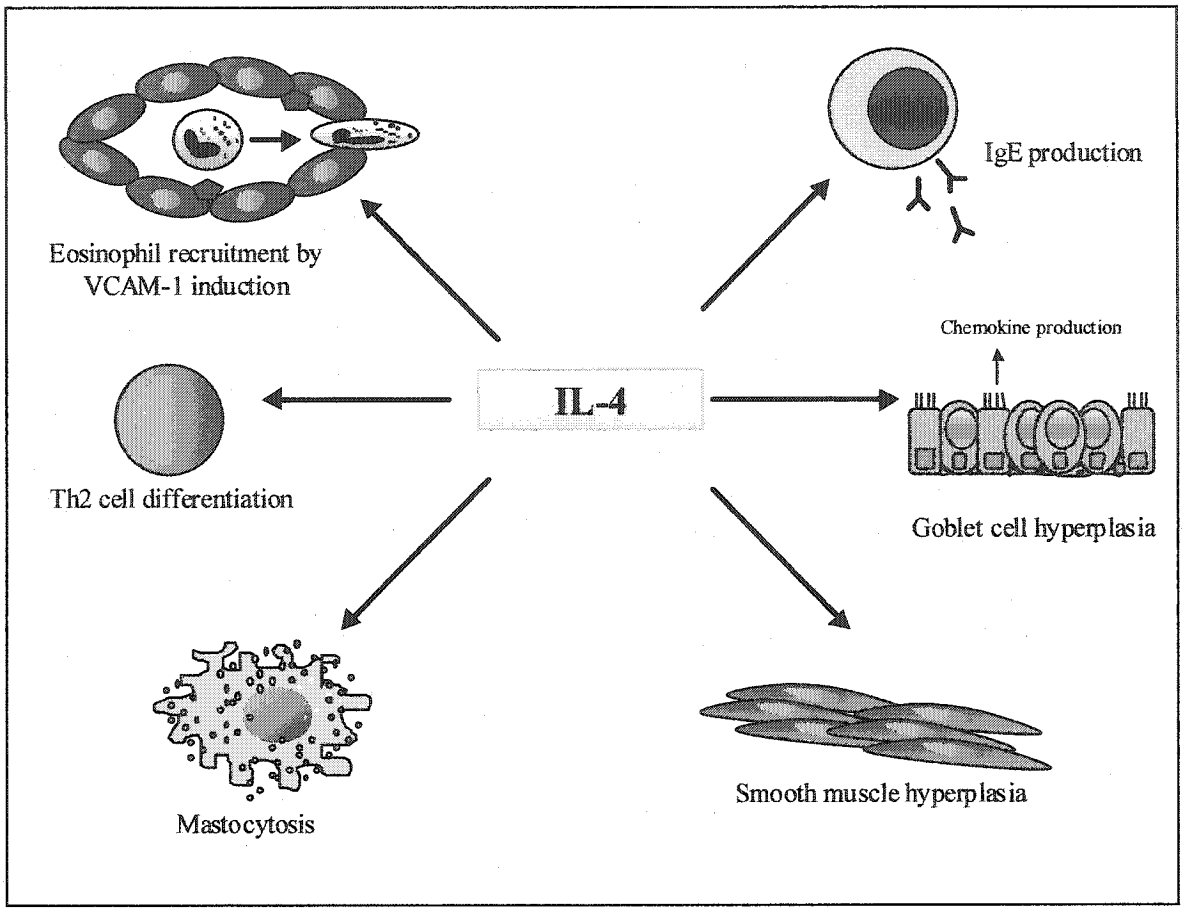


Fig 5. Major actions of IL-4. IL-4 acts on B cells for the production IgE, it drives Th2 differentiation of T cells, promotes eosinophil recruitment through the induction of adhesion molecules (VCAM-1) on endothelial cells, and it acts on epithelial cells for the release of cytokines and chemokines. IL-4 is also responsible for smooth muscle hyperplasia in asthma, mastocytosis, and goblet cell hyperplasia.

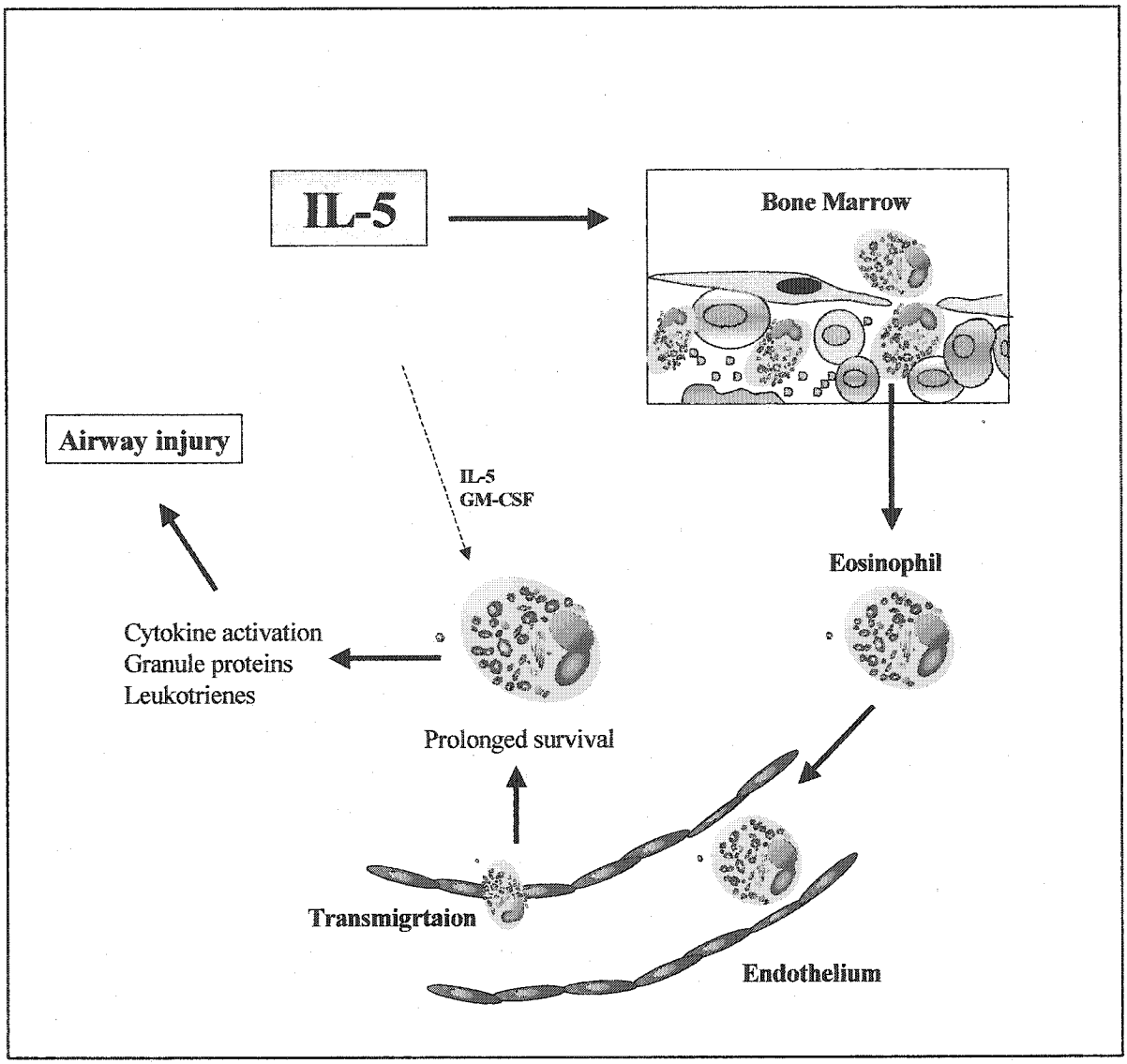


Fig 6. IL-5 effects on eosinophils. IL-5 travels to the bone marrow and causes terminal differentiation of eosinophils. Circulating eosinophils enter the area of allergic inflammation and begin migrating to the inflammatory site by "rolling", which is mediated through the interactions with selectins, and by adhering to the endothelium through the binding of integrins. As eosinophils enter the matrix of the airway through the influence of various chemokines, as well as IL-5, their survival is prolonged by IL-5 as well as GM-CSF. On activation, the eosinophil releases inflammatory mediators such as leukotrienes and granule proteins leading to airway injury.

that T cells, mast cells, basophils, and eosinophils are the principal cellular sources IL-5 (117).

The importance of IL-5 in murine models of airway inflammation is highlighted by a number of studies. IL-5 deficient mice indicate that IL-5 is critical for regulating eosinophilia during allergic inflammation (139), (140). Moreover, IL-5 was found to be essential for the development of airway epithelial cell damage and bronchial hypereactivity in response to inhaled allergen (139). Airway hyperresponsiveness, however, can sometimes be dissociated from IL-5 regulated eosinophilia, suggesting that alternative pathways also act to modulate enhanced bronchial reactivity. This dissociation was also demonstrated by the ineffectiveness of IL-5 antibody in blocking allergen-induced late asthmatic responses and hyperresponsiveness in human asthmatics (141).

1.6.1.3 Interleukin-13 (IL-13)

IL-13 has an overlapping biological profile with IL-4, as it shares the IL-4 receptor α subunit for its high affinity receptor formation (142). IL-13 is a pleiotropic cytokine and induces B cells to undergo Ig isotype switching to IgE, promotes differentiation of Th2 lymphocytes, and enhances eosinophil activation (143), (144), (145). IL-13 has also been shown to downregulate the transcription of IFN-γ and IL-12 and as such, may modulate the cytokine environment at the time of antigen presentation (142). The main source of IL-13 production is circulating basophils, however CD4 + T cells also produce IL-13 (146). IL-13 is thought to have a close association with the pathophysiology of asthma and allergic rhinitis as the increased

expression of IL-13 mRNA has been demonstrated in both conditions (147), (148). In mice, it has been shown that administration of IL-13 was able to induce AHR, and administration of soluble IL-13Rα_2 completely reversed IL-13 mediated AHR (149), (150). IL-13 is also critical factor for mucin synthesis in airway epithelial cells, which is a characteristic feature of asthma. Studies using murine models of allergen-induced airway inflammation have shown that blocking IL-13 results in the complete abrogation of mucin synthesis in airway epithelial cells (151), (152). Conversely, the exogenous administration of IL-13 stimulates mucin production via an IL-4Rα-dependent pathway, upregulates IgE production, and induces eosinophil recruitment (152), (151).

1.6.2 Th1 Cytokines

The idea that allergic inflammation in diseases such as asthma and rhinitis arises from an imbalance between Th1 and Th2 cells has focused attention on the Th1 cytokines, particularly IFN-γ. A Th1 cytokine profile is thought to be associated with inhibition of the production of inflammatory cells and their cytokine synthesis. Since IFN-γ inhibits the synthesis of IgE and the differentiation of precursor cells to Th2 cells, a lack of IFN-γ would induce the Th2 type cytokine pathway promoting allergic inflammation. In humans, Th1 cytokines consist mainly of IL-2 and IFN-γ, and although not produced by T cells, IL-12 can also be considered a Th1-like cytokine. IL-12, which is produced by antigen presenting cells such as macrophages and dendritic cells, is the key cytokine primarily responsible for regulating the balance between Th1 and Th2 cells (153). In the presence of IL-12, the development of Th1

cells is favored since Th2 cells are usually not responsive to IL-12 due to a selective downregulation of the IL-12 receptor β2 subunit (IL-12R β2) that occurs during the developmental commitment of these cells (154). In general Th1 responses play an integral role in cellular inflammation as seen in diseases such as tuberculosis and sarcoidosis (155), (156).

1.6.3 Imbalance between Th1 and Th2 cytokines

T cells of the fetus are primed by common environmental allergens that cross the placenta. As a result of Th2 trophic factors from the placenta, immune response of virtually all newborn infants is dominated by Th2 cells (157). It has been proposed that during subsequent development the normal infant's immune system shifts in favor of a Th1-mediated response to inhaled allergens, a process known as immune deviation (158) In the potentially atopic infant on the other hand, there is further increase in Th2 cells that were primed in utero. Microbes may be the chief stimuli of protective Th1 mediated immunity. Macrophages that engulf microbes secrete IL-12, which induces Th1 cells and natural killer cells to produce IFN-γ thereby shifting the immune system into an "allergy protective" Th1 mediated response. Other factors may also influence whether Th1 or Th2 cells dominate the response, including the amount of allergen, the duration of exposure to the allergen, and the avidity of allergen-specific interactions between T cells and antigen-presenting cells (159), (160). The extent of the imbalance between Th1 cells and Th2 cells during the neonatal phase may be useful in predicting the subsequent development of allergic disease. To reduce the risk of allergies in childhood, some have suggested that infants

at high risk for these conditions should be exposed to stimuli that up-regulate Th1 mediated responses, so as to restore the balance during a critical time in the development of the immune system and the lung (161).

1.7 Transcription Factors

It is now well established that the expression of many inflammatory genes involved in the pathogenesis of allergic diseases is primarily regulated at the level of transcription. Cellular responses are coordinated by a genetic regulatory network in which a given transcription factor controls the expression of a diverse set of target genes depending on the cell type and/or the nature of cellular stimuli. As such, the expression and regulation of the factors that control transcription may play important roles in allergic inflammation. Thus blocking of transcription factors, which are specifically involved in the expression and/or activity in atopic diseases, might provide the basis for the development of novel therapies in allergic diseases.

1.7.1 Mechanism of action

Extracellular signals such as cytokines, leukotrienes and other inflammatory stimuli, generally act on cells via cell surface receptors. Ligand receptor interaction initiates various intracellular signaling cascades that result in the activation and/or induction of transcription factors, which are specific DNA-binding proteins (162). Functional diversity of such transcription factors is dependent on their modification via phosphorylation or dephosphorylation and/or interaction with other transcription factors. Although transcription factors have diverse primary structures, they form a

tertiary structure that is compatible with the DNA sequence with which they must interact. The greater affinity of transcription factors for specific sites determines their binding specificity. After an initial contact is made between the protein and DNA, these interactions become enhanced as a result of the formation of numerous hydrogen bonds, hydrophobic and ionic interactions. This is followed by the formation of a highly specific three-dimensional nucleoprotein complex involving extensive protein-DNA and protein-protein interactions. Formation of the specific three-dimensional nucleoprotein complex is often accompanied by conformational changes in the protein and the DNA, and many transcription factors induce DNA bending upon specific binding (163), (164).

1.7.2 Transcription factors in allergic disease

A number of transcription factors such as NF-κB, C/EBPβ, AP-1 and NFAT have been reported to play a role in Th2 gene expression in allergic diseases. NF-κB (nuclear factor-kappa B) is known to regulate the expression of multiple inflammatory genes such as IL-1β, TNF-α, GM-CSF, and IL-8, and is involved in a number of chronic inflammatory diseases. Several NF-κB proteins exist, however, the classic NF-kB complex is a heterodimer composed of two polypeptide subunits p50 and p65. It is a ubiquitous transcription factor that appears to be of particular importance in inflammatory and immune responses (165). In fact studies have shown that targeted deletion of the NF-kB gene in mice results in immune deficiency which may be lethal (166). There is also evidence for activation of NF-κB in biopsies of patients with asthma and in inflammatory cells in the sputum (167). The transcription

factors C/EBPβ and AP-1, which are widely expressed in many cell types, have also been shown to be important for the expression of Th2 cytokine genes (168), (169). AP-1 is a collection of related transcription factors belonging to the Fos and Jun families which dimerize in various combinations as Fos/Jun heterodimers binding with high affinity, or Jun/Jun homodimers which bind with low affinity. There is evidence for increased expression of c-Fos in epithelial cells in asthmatic airways, and many of the stimuli relevant to asthma that activate NF-κB will also activate AP-1. AP-1. Like NF-κB, regulates many of the inflammatory and immune genes that are over-expressed in asthma. Indeed many of the genes require the simultaneous activation of both transcription factors that work together cooperatively (170). AP-1 also interacts with cell-specific transcription factors, such as nuclear factor of activated T-cells and is thus involved in the regulation of IL-4 and IL-5 (171).

CCAAT/enhancer-binding proteins (C/EBP) belong to the bZIP class of transcription factors and bind as hetero- and homo-dimers to C/EBP sites (172). C/EBP is important in IL-1, IL-6 and LPS-dependent signal transduction, and plays a major role in the induction of many immune and inflammatory response genes (173).

Nuclear factor of activated T cells (NFAT) has been shown to activate the IL-4 promoter. NFAT proteins contain a cytoplasmic subunit and an inducible nuclear component. Four related genes encoding the cytoplasmic subunit are currently known, including NFAT1 to NFAT4. NFAT proteins are present both in Th1 and Th2 cells, and have been implicated in the expression of both Th1 and Th2 genes in *in vitro* studies (171). Although NFAT, NF-κB, C/EBPβ and AP-1 are all involved in cytokine gene expression associated with allergic diseases, they are not specific for

Th2 cytokine gene expression, but rather more general factors associated with different inflammatory gene expression. Recently however, other transcription factors such as GATA-3, STAT-6 and c-Maf have been characterized, and described to be specific for Th2 cytokine gene expression.

1.7.2.1 GATA-3

GATA-3 is a pleiotropic transcription factor of the C4 zinc finger family expressed mainly in T cells (174), (175), (176). Six members (GATA-1 to-6) of the GATA family of proteins have been identified to date, and based on their expression profile and structure, the GATA proteins may be classified as hemopoietic (GATA-1 to -3) or non- hemopoietic (GATA-4 to –6). GATA-3 is selectively expressed in Th2 cells, and is known to play an essential role in T cell development, Th2 differentiation, and Th2 cytokine gene regulation. (177), (178), (179). As such, GATA-3 has attracted considerable attention in the study of allergic disease.

1.7.2.1a GATA-3: a critical factor in early T cell development

GATA-3 has been shown to be essential for the development of the earliest T cell progenitor cells (180). In fact, targeted disruption of the GATA-3 gene in mice results in embryonic death with a failure of fetal liver hematopoiesis and defects in the central nervous system (174). The lethal effects of GATA-3 deficiency were bypassed by generating GATA-3 knockout chimeras and by using antisense oligonucleotides for GATA-3 in fetal thymus organ cultures (175), (181), establishing an essential role for GATA-3 in the earliest steps of T cell development.

1.7.2.1b The Role GATA-3 in Th2 differentiation factor

In addition to its essential role in T cell development, GATA-3 has also been identified as a Th2 differentiation factor. Naïve CD4 + T cells express low levels of GATA-3 mRNA. GATA-3 expression, however, is markedly upregulated in cells differentiating along the Th2 lineage, and downregulated in cells differentiating along the Th1 pathway (182), (178). Subsequently by using established clones as well as Th1 or Th2 cells generated from naïve CD4+ T cells (177), Th2 specific expression of the GATA-3 gene was demonstrated. Ouyang et al have shown that overexpression of GATA-3 in developing Th1 cells resulted in abolition of IL-12Rβ2 expression with abrogation of IFN-γ production (178). Interestingly, ectopic expression of GATA-3 at sufficiently high levels in already committed Th1 cells was also shown to induce Th2 cytokine production and to inhibit IFN-γ production (183). Thus, by inhibiting IL-12Rβ2 expression, GATA-3 represses the responsiveness of Th1 cells to IL-12 (178). The ability of GATA-3 to promote Th2 lineage commitment with concomitant inhibition of Th1 development suggests a key role for this transcription factor in determining a Th1 or Th2 fate. Studies in retrovirally infected T cells have shown that the activation of GATA-3 occurs upon stimulation of the IL-4/STAT6 signaling pathway suggesting that the exposure of naïve T cells to IL-4 may be an early event that induces GATA-3 activation and Th2 cell differentiation (184). However, GATA-3 can fully reconstitute Th2 development in STAT6 deficient T cells suggesting that it is a master switch both in STAT6-dependent and –independent Th2 development (178).

1.7.2.1c The Role of GATA-3 in Th2 cytokine gene expression

A role for GATA-3 in the expression of a Th2 cytokine was first established by Siegel et al, who demonstrated the critical importance of GATA-3 binding site in the IL-5 promoter (168), (179), (185). Although GATA-3 has been shown to directly activate the IL-5 promoter, studies also suggest the involvement of GATA-3 in active transcription of the IL-4/IL-13 genes (186) as several regions around the IL-4/IL-13 locus have been shown to bind GATA-3 (187). GATA-3 has been reported to play an essential role in the control of production of all key Th2 cytokine genes (177), (186), (188), and has thus been implicated to play a critical role in allergic inflammation.

To date, the relevance of GATA-3 in allergic diseases is evident mainly from *in vitro* and animal studies. Because deficiency of the GATA-3 genes causes embryonic lethality, it was unclear whether GATA-3 inhibition would block the production of all the key Th2 cytokines that have been implicated in asthma. As such, transgenic mice expressing a dominant-negative mutant of GATA-3 were developed to address this question. This study demonstrated that inhibition of GATA-3 activity causes a severe blunting of Th2 effects such as eosinophil influx and mucus production in the lung, and systemic IgE production (188), indicating that GATA-3 plays a crucial role in the development of Th2-mediated lung inflammation. Furthermore, inhibition of GATA-3 expression in a murine model of asthma by antisense oligonucleotides to GATA-3 resulted in significant inhibition of airway hyperresponsiveness (189). Thus, the importance of GATA-3 in asthma is underlined in the observations of these studies.

1.7.2.2 Signal transducer and activator of transcription 6 (STAT6)

The signal transducers and activators of transcription (STATs) proteins play a fundamental role in relaying intracellular signals elicited by cytokines. The STAT factors exist as latent monomeric precursors in the cytoplasm. After stimulation with cytokines, STAT proteins undergo phosphorylation by the Janus family of kinases (Jak) (190). This causes dimerization and nuclear translocation of the proteins, wherein they induce transcription of target genes (191). To date, six human STAT factors have been isolated and cloned. STAT-1 is activated by IFN-α and γ, while STAT-2 is only activated by IFN-α (192). STAT-3 is tyrosine phosphorylated in response to IL-6 and other cytokines that act through related receptor subunits (193). STAT4 is involved in the differentiation of Th0 to Th1 cells, and STAT5 is important for the IL-2 mediated cell cycle progression of T cells, and for NK proliferation and cytolytic activity (194), (195). Finally, STAT-6 is required for Th2 gene induced expression, and as such has been implicated in the pathogenesis of allergic disease (196), (197).

1.7.2.2a Mechanism of Activation

The principle pathway leading to activation of STAT6 is through the IL-4Rα chain, which dimerizes either with the common gamma chain (γc) or with the IL-13α chain, which are activated when engaged by IL-4 or IL-13 respectively. Subsequently, the Jak/STAT pathway is activated where Jak 1 and Jak3 phosphorylate STAT6 forming biological active homodimers (191). STAT6 dimers then move from the cytoplasm to the nucleus where they bind to specific promoter

sequences regulating target gene transcription of IL-4 regulated genes such as IL-4R, IgE, FcR, and MHC class II molecules (198), (199), (200) (Fig.7).

1.7.2.2b The role of STAT6 in TH2 responses

In vitro studies have shown that ectopic expression of activated STAT6 in developing Th1 cells induces the expression of Th2 specific cytokines, the downregulation of IFN-γ and the IL-12Rβ2 chain, as well as the induction of GATA-3 and cMaf mRNA. (184), (201), (202), (203). Furthermore, blocking STAT6 in Th2 cells with the use of an antisense oligonucleotide was shown to inhibit IL-4 induced STAT6 promoter transactivation, to effectively ablate mRNA expression and production of IL-4, and to inhibit IL-4-mediated cell proliferation in Th2 cells. The importance, however, of the STAT6 pathway in allergic responses was mainly demonstrated in studies using STAT6-/- knockout mice which failed to develop airway hyperresponsiveness, to produce excess mucus in response to allergen challenge, and to mount allergen specific IgE responses (204), (205). Furthermore, STAT6 appears to be the principle signaling pathway involved in the commitment of CD4+ T cells to the Th2 phenotype, and IgE isotype switching in B cells (201), (144). These observations underscore the importance of STAT6 in the regulation of Th2 responses.

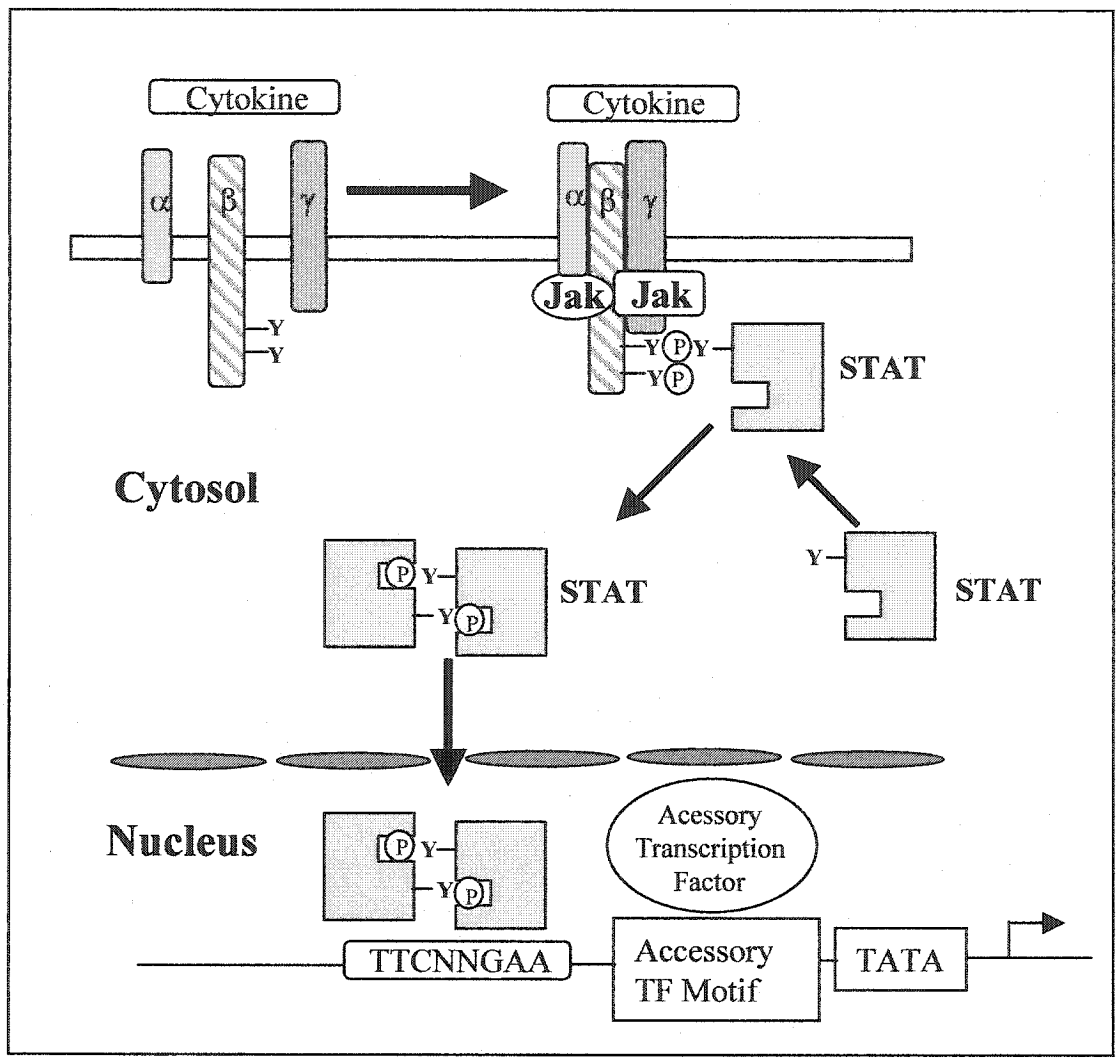


Fig 7. Cytokine induced STAT regulation. Cytokines such as IL-4 and IL-13 activate Jaks, which in turn phosphorylate tyrosine-based docking sites on the receptor. STATs then bind via their SH2 domains. As a consequence, the STATs are then phosphorylated, form homo- or heterodimers, and then translocate to the nucleus where they bind target sequences, allowing for the transcriptional activation of genes.

1.7.2.3 c-Maf

The c-Maf protooncogene is a basic region/leucine zipper transcription factor that belongs to the subfamily of AP-1 proteins. cMaf is expressed in Th2 but not in Th1 clones, and is induced during the differentiation of normal Th0 cells along a Th2 but not a Th1 lineage (206). cMaf was shown to bind to a consensus site MARE, a maf response element, within the proximal IL-4 promoter (207). Mutation of the MARE binding site has been shown to significantly reduce IL-4 promoter-mediated transcriptional activation (208). c-Maf has been implicated as a critical factor in regulating the pattern of cytokine expression by T cells through its direct action on IL-4 transcription (209). Ectopic expression of c-Maf in virtually any cell, including Th1 cells and B cells, transactivates the IL-4 promoter (210). It has been shown to be selective for IL-4, but not for all Th2 cytokine genes (207).

1.7.2.3a The role of cMaf in Th2 responses

To date, most of the findings implicating cMaf as an important regulator in Th2 responses come from animal models. In particular, studies of cMaf-/- (knockout) mice have demonstrated a crucial role for cMaf in IL-4 gene expression (211). Although cMaf -/- mice displayed impaired IL-4 production by CD4+ T cells, the production of IL-5 and IL-13 was not affected indicating that they are cMaf independent. Also, upon immunization, c-Maf-/- mice expressed normal IgE levels which was probably due to unimpaired IL-13 production. Therefore, unlike GATA-3, c-Maf directly activates the IL-4 promoter, but does not appear to regulate the expression of all Th2 cytokine genes (211). Furthermore, overexpression of c-maf in

animal models results in a significant increase in the IL-4 cytokine with preferential Th2 responses, yet an inhibition of Th1 cytokines such as IFN-γ (210). In addition, cMaf, in the absence of IL-12 attenuates the differentiation of Th1 cells by a mechanism that is independent of Th2 cytokines (210). Taken together, these observations suggest that cMaf is a critical factor in regulating the pattern of cytokine expression by T cells through its direct action on IL-4 transcription, a critical cytokine in allergic inflammation.

1.7.3 Th1 associated transcription factors

Although a number of studies have been done investigating Th2 associated transcription factors, little is known about the factors that regulate Th1 cytokines. The idea that allergies arise from an imbalance of Th1 and Th2 cytokines has recently focused attention on Th1 cytokines. To date, several factors have been identified which are associated with Th1 responses such as BCL-6, STAT4, and most recently, T-bet. BCL-6 is a proto-oncogene as well as a transcriptional regulator which is found at the highest levels in B cells and T cells of the germinal center immune reaction. BCL-6 is a potent transcriptional repressor that appears to repress Th2 responses *in vivo*. It has been shown to bind the STAT6 DNA-binding sequence in the CD23 promoter, and to repress IL-4-induced activation of CD23 expression (212). Interestingly, targeted deletion of the BCL-6 gene in mice has been shown to result in a massive inflammatory response in the lungs characterized by eosinophilic infiltration (212), (213).

STAT4, a member of the signal transducers and activators of transcription, is predominantly activated in response to IL-12, which is critical for controlling T helper cell differentiation along the Th1 pathway. Studies using STAT4 knockout mice showed impaired IL-12-mediated functions. IL-12-mediated increases in IFN-γ production, cellular proliferation, and NK cell cytotoxic activity of lymphocytes were impaired in these STAT4 deficient mice. Furthermore, IL-12 induced development of Th1 cells was not observed in these mice (214), (215).

T-bet (T box expressed in T cells) is a recently identified transcription factor that is Th1 specific and has been shown to transactivate the IFN-γ gene in Th1 cells. T-bet also has the unique ability to redirect fully polarized Th2 cells into Th1 cells, as demonstrated by simultaneous induction of IFN-γ and repression of IL-4 and IL-5 (216), (217), (218). T-bet expression, is in fact downregulated in human asthmatic airways of Th2 phenotype compared to nonasthmatic airways (219). T-bet, thus appears to function by initiating a Th1 differentiation program while repressing Th2 differentiation.

1.7.3.1 Ying Yang 1 (YY1)

YY1 is a DNA-binding zinc finger transcription factor that has been shown to function as an activator, a repressor, and an initiator of transcription, and appears to be expressed in most cell types (220), (221). The observation that YY1 can simultaneously activate and repress different genes in the same cell illustrates the role of promoter-specific factors in regulating activities of YY1 (222), (223). While the identification of separate activation and repression domains in YY1 has shed some

light on its multifunctionality, the mechanisms by which YY1 carries out and regulates its transcriptional activities remain unclear. YY1 has recently been shown to regulate the expression of several T cell cytokines. The interferon- γ promoter contains at least two YY1-binding sites and in a recent report, Sweetser *et al.* concluded that YY1 acts in a complex fashion to regulate the IFN-γ promoter. They found that YY1 cooperates with NFAT to activate the IFN-γ promoter in primary mouse splenocytes (224). YY1 was also shown to down-regulate the expression of the IL-5 promoter in a human T cell line. Repressor activity appears to involve YY1 together with Oct-1 and octamer-like proteins, which bind to the negative regulatory elements of the hIL-5 promoter (221). These results underscore the complex nature of transcriptional regulation by YY1 and suggest that the effect of YY1 on cytokine gene expression will be highly dependent on the promoter context. Based on these findings YY1 may be associated Th1 immune responses, but its precise role has yet to be determined.

1.8 Treatments of Allergic Diseases

1.8.1 Antihistamines

Antihistamines have long been used in the treatment of atopic diseases (225), and are the most commonly prescribed class of medication particularly for allergic rhinitis. Although these drugs act primarily by blocking the H_1-histamine receptor, many of these agents have also been shown to have mild anti-inflammatory properties. As a general rule antihistamines reduce symptoms of sneezing, itching, and rhinorrhea in allergic rhinitis, but have no clear benefit in asthma (226). A newer

class of antihistamines however, has been claimed to have additional anti-asthma effects that are not mediated through the H_1-receptor blockade, including an inhibitory effect on eosinophil chemotaxis into the airways following allergen exposure (227). Because antihistamines have a relatively rapid onset of action, these agents are frequently and effectively used on an intermittent, as needed basis. Although antihistamines are effective, they readily cross the blood-brain barrier, which helps account for the adverse effects associated with the use of these agents.

1.8.2 Corticosteroids

Corticosteroids are currently the most effective treatment for allergic diseases as they control symptoms by reducing airway inflammation. They act at a number of levels, inhibiting the production of inflammatory mediators including cytokines, chemokines, and adhesion molecules

1.8.2.1 Mechanisms of action

Corticosteroids act by binding to a cytosolic glucocorticoid receptor (GC), dimerize and translocate to the nucleus where they bind to a specific DNA consensus sequence resulting in the activation of inflammatory genes. However, the major anti-inflammatory effect of corticosteroids is by suppression of multiple inflammatory genes, including cytokines, inflammatory enzymes, adhesion molecules, and inflammatory mediator receptors; this is why corticosteroids are so effective in complex inflammatory conditions, such as atopic diseases. Most of the anti-

inflammatory actions of corticosteroids can be accounted for by inhibiting transcription factors, such as activator protein 1 (AP-1), nuclear factor-*k*B (NF-*k*B).

1.8.2.2 Side Effects

Although effective, oral corticosteroids have systemic side effects limiting the dose that can be given over long periods. Concerns have been raised about adrenal suppression and inhibition of growth and bone formation (228), (229), (230). As such, in the early 1970s, this led to the development of inhaled steroids, which have local anti-inflammatory effects, however the suppression of airway inflammation is only temporary in this case. In fact inhaled steroids are the most effective treatment for allergic rhinitis (231). Newer inhaled corticosteroids for asthma, including budesonide, fluticasone propionate, and mometasone fuorate, have a high level of anti-inflammatory action with minimal side effects, because the swallowed fraction of the drug is largely removed by the hepatic metabolism (232). However, these drugs are absorbed from the lung or nasal mucosa and therefore may have some systemic effects at high doses.

1.8.2.3 Corticosteroid insensitivity

There exists a subset of allergic patients who do not respond to steroid therapy and have been termed 'steroid insensitive' The molecular basis of steroid insensitivity remains poorly understood, however, a possible mechanism has been postulated based on studies demonstrating that alternative splicing of the GR pre-mRNA generates a second GR, GRβ, which does not bind GC, but instead antagonizes the

activity of GRα, possibly accounting for steroid insensitivity. Studies have shown that a significant reduction in GRα DNA binding capacity can be reproduced by transfection of cell lines with the GRβ gene (233). More recently our group has demonstrated that patients with GC insensitivity express higher numbers of GRβ cells in peripheral blood and BAL, compared to GC sensitive asthmatics or normal controls. Furthermore we demonstrated a prominent airway expression of GRβ positive cells in fatal asthma (234). These findings suggesting that GRβ may is a contributing factor to steroid insensitivity leading to asthma mortality (235).

Although highly effective, corticosteroids are not curative, and inflammation recurs when they are discontinued. Furthermore their use is associated with many well described side effects and may even lead to general immunosuppression. Thus selective approaches that block Th2 cell activation would be more suitable.

1.8.3 Antileukotrienes

Although steroids are extensively used, antileukotrienes, the first novel class of therapy introduced specifically for asthma in the last 30 years, have attracted considerable attention. Antileukotrienes act against cysteinyl leukotrienes, which are associated with airway edema, smooth muscle contraction, and altered cellular activity in the inflammatory process (236). Cysteinyl leukotriene mediators are not necessarily or consistently controlled by corticosteroids, therefore, the combination of a leukotriene modifiers with inhaled glucocorticoids may be of added benefit, minimizing the doses of inhaled corticosteroids that are needed (237). The bulk of patients treated with antileukotrienes have mild to moderate asthma, and when added

to low- and high-dose inhaled corticosteroids, antileukotrienes have shown to be effective (238). They appear to improve asthma symptoms and lung function, and reduce the need for rescue bronchodilator treatment (236). Leukotriene modifiers were designed either to inhibit leukotriene synthesis or to prevent the leukotrienes from binding to their specific receptors on airways and inflammatory cells (239). Although antileukotrienes may also be effective in treating allergic rhinitis, when compared to other anti-inflammatory agents, they have relatively little benefit (240).

1.8.4 Other anti-inflammatory agents

There has been an intensive search for anti-inflammatory treatments that are effective as corticosteroids, but with fewer side effects. Cyclosporin A, which is a fungal metabolite proved to be an extremely useful immunosuppressant agent for organ transplants, may be of potential benefit in the treatment of allergic disease. The principle action of cyclosporin is to inhibit T cell activation, and to suppress the production of cytokines. It has inhibitory effects on a number of cells including mast cells, monocytes, neutrophils, and basophils. Cyclosporin was shown to block the late asthmatic reaction and to inhibit eosinophil-associated cytokines after allergen challenge (241), (242). Cyclosporin is associated with a number of unwanted sequelae, requiring very careful monitoring (243). At present there appears to be insufficient evidence of benefit, recommending its use.

Phosphodiesterase (PDE) inhibitors are potentially useful as anti-inflammatory treatments in allergic disease. PDEs are known to break down cyclic nucleotides that inhibit cell activation. PDE4 is the predominant family of PDEs in

inflammatory cells, including mast cells, eosinophils, T lymphocytes, macrophages, and structural cells such as epithelial cells (244), which has been shown to be overexpressed in atopic patients (245). In animal models of asthma, PDE4 inhibitors reduce eosinophil infiltration and AHR responses to allergen, however, in human clinical trials, PDE4 inhibitors have been associated with unacceptable side effects (244), (246).

1.8.5 Cytokine Antagonists

Recent research into the therapeutics of allergic disease has focused on strategies for inhibiting proinflammatory cytokines. IL-4, IL-5, and IL-13 cytokines are considered to be potential candidates for critical intervention in Th2 driven inflammatory processes, and thus represent valuable therapeutic targets in allergic disease. There are several approaches which can be taken to inhibit specific cytokines including monoclonal antibodies against the particular cytokine or cytokine receptors. Inhibition of IL-5 in allergic diseases is one possibility of paralyzing Th2 inflammation. Clinical trials with humanized monoclonal antibodies to IL-5 have been developed demonstrating that a single injection can reduce blood and sputum eosinophils for several weeks and prevent eosinophil recruitment into the airways after allergen challenge. No effect, however, was seen on either the early or the late response to inhaled allergen. Moreover, IL-5 antibody treatment failed to reduce airway hyperresponsiveness in these patients (141). There are well known series of antagonists that will inhibit the effect of cytokines at the receptor level and inhibit cytokine-mediated signal transduction and may therefore be candidates for

immunopharmacologic intervention in allergic disease. IL-4 receptor antagonists have been developed as IL-4 presents a promising target for allergy therapy. As demonstrated by Borish et al, IL-4 receptor antagonists moderately improved asthma in a placebo-controlled trial, but showed no significant clinical effect (247). These findings suggest that targeting a single cytokine is not sufficient in blocking the entire allergic response to airway allergen challenge.

1.8.5.1 Antisense Oligonucleotides

Another approach to target Th2 cytokines would be antisense (AS) technology the translation of specific mRNA is blocked through the use of antisense oligonucleotides. AS oligonucleotides are short chains of nucleic acids based upon nucleotide sequences of targeted genes that selectively bind to the targeted region of the mRNA, blocking its access to ribosomes, thus preventing synthesis of the corresponding protein. As a result, the production of the targeted protein is inhibited without affecting the translation of other proteins. Although antisense applications have been used for HIV, cancer, and organ transplantation, to date, AS technology has not been investigated *in vivo* in human allergic disease. Nevertheless, AS oligos for the treatment of allergies represent enormous potential as they can target virtually any mediator or receptor, cytokine, chemokine, adhesion molecule, enzyme, or transcription factor. AS oligos targeting individual cytokines have in fact been investigated in animal models of asthma. Although effective at blocking certain features of the airway response to allergen, targeting individual cytokines such as IL-

4 and IL-5, did not appear effective at abolishing the late airway response in a rat model of asthma (248).

1.9 Immunomodulators

1.9.1 Immunotherapy

Immunotherapy has been recognized as an effective treatment for allergy and has been used for the treatment of allergic disease for nearly 100 years. It involves subcutaneous injections of small amounts yet increasing doses of purified allergen over a prolonged period of time, in effect allowing the development of a protective response.

The exact mechanisms underlying the efficacy of this treatment are not well understood, however, it appears that immunotherapy is associated with alterations in serum antibody levels with a blunting of the seasonal increase in IgE and an increase in "blocking" IgG antibodies which compete with IgE for allergen (249). As such immunotherapy results in a shift from the production of Th2 to Th1 type cytokines (249), (250). Immunotherapy has been shown to be effective particularly for seasonal rhinitis, and may induce prolonged remission especially when treatment is continued for several years. (251). Unfortunately, immunotherapy is less effective in asthma, and its use is associated with a number of disadvantages. The associated risk of anaphylaxis, the inconvenience and discomfort of frequent dosing, and the duration of several years of therapy are all factors that limit its efficacy (252), (253).

1.9.2 Endotoxins and other microbes

The potential of Th1 inducers such as endotoxins and other microbial components to mitigate allergy is consistent with clinical association studies of the hygiene hypothesis and is also supported by studies using rodent models of asthma. Experimental results in murine models of asthma have invariably supported this hypothesis because treatment with microbes such as Bacille Calmette-Guerin (BCG), or microbial components such as lipopolysaccharide LPS, and bacterial CpG DNA, have been shown to mitigate allergen sensitization and prevent the eosinophilic inflammation and airways hyperresponsiveness that characterize the asthmatic phenotype in these mice (254), (255), (256). As such, microbe-derived products are being developed as potential therapies for allergy and asthma.

1.9.2.1 Bacille Calmette-Guerin (BCG)

A relative lack of infections may be a factor predisposing to the development of atopy in genetically predisposed individuals, leading to the concept of vaccination to induce protective Th1 responses preventing sensitization and thus the development of allergic diseases. Since infections with intracellular pathogens stimulate Th1 driven inflammatory responses, it has been suggested that atopic disorders represent the default inflammatory pathway in the absence of these infections (28). Studies have shown that Bacille Calmette-Guerin (BCG) vaccination may have protective effects as it associated with a reduction in atopic disease (257). BCG inoculation in mice has been shown to reduce the formation of specific IgE in response to allergen as well as eosinophilia and AHR, but rather with an increase in the production of

IFN-γ (255). Similar results have been obtained in mice with a single injection of heat-killed *Mycobacterium vaccae*, another potent inducer of Th1 responses (258).

1.9.2.2 CpG DNA

Attempts at improving immunotherapy by reducing allergenicity have been paralleled by reduced immunogenicity, and have not resulted in effective immunotherapy. The latest potential method concerns the administration of allergens together with adjuvant DNA oligonucleotides, which may preferentially induce counterbalancing Th1 responses, while also having reduced allergenicity (259). These adjuvant immunostimulatory sequences favor Th1 responses by containing unmethylated cytosine and guanosine dinucleotide repeat motifs (CpGs) in DNA with adjuvant properties. In mice, administration of CpG oligonucleotides was shown to increase the ratio of Th1 to Th2 cells, decreases formation of specific IgE, and reduces the eosinophilic response to allergen (260), (261). In our laboratory, we have demonstrated that allergen-linked immunostimulatory DNA is effective in stimulating an allergen specific Th1 response, and decreasing a Th2 response *ex vivo*, in nasal biopsies obtained from allergic rhinitic patients (submitted).

Thus CpG sequence allergen immunotherapy may represent a more advanced molecular adjuvant, however the benefit and safety in humans has not yet been determined.

1.9.2.4 Lipopolysaccharide

Lipopolysaccharadide (LPS) is a major component of the outer most layer of the cell membrane of gram-negative bacteria. Its potent immune stimulatory capacity is largely attributed to the Lipid A moiety of endotoxin, which is highly conserved across different bacterial species (262). LPS exerts its biological actions through its interaction with CD14, the primary receptor for LPS, and through Toll-like receptor-4, a co-receptor essential for LPS signaling (263), (264). The genetic locus of CD14 is close to the genomic region controlling levels of IgE. LPS has been reported to be a potent inducer of IL-12 and IFN-γ, which are key regulators of Th1 type immune development (265), (266). In fact, LPS has been shown to strongly influence innate antigen-presenting immune cells, particularly dendritic cells, to produce IL-12, and to costimulate T cells to become effector cells that primarily secrete IFN-γ (267), (268). IL-12 production by dendritic cells appear to be an obligatory signal for the differentiation of naïve T cells into Th1-like cells As such, reduced exposure to LPS may be responsible for the delayed development of the mature, Th1-dominated immune response through the deficient maturation of APCs and subsequent insufficient IL-12 mediated stimulation of uncommitted naïve Th cells (18), thereby promoting Th2 cytokine production and the consequent atopic immune development and associated disease.

1.10 Rationale

The redundant expression of Th2 cytokines appears to play a critical role in orchestrating, and perpetuating the inflammatory response, which is a critical

component of the allergic airways diathesis. Although corticosteroids are effective at reducing airway inflammation, they do not act specifically on Th2 cytokines as they are general immunosuppressants, and their use is associated with a number of side effects. As such, alternative therapeutic approaches aimed at selectively targeting Th2 cytokines are being developed. Clinical trials using blocking monoclonal antibodies abrogating specific cytokines, demonstrate that targeting a single cytokine is not sufficient in blocking the allergic airway response. A common factor that regulates the expression of all Th2 cytokines would therefore provide an ideal target to inhibit the spectrum of features that characterize allergic airway diseases. Studies have recently shown that central to the control of cytokine gene expression are transcription factors, which may be of therapeutic value. It is believed that switching off the Th2-driven allergic inflammation can also be accomplished by stimulation of Th1 cells promoting the production of IFN-γ and IL-12. The idea that allergy arises from an imbalance between Th1 and Th2 cytokines has led to novel therapeutic approaches aimed at increasing Th1 cytokine levels in the immune system. Although allergen immunotherapy, which is currently used as a form of treatment, is thought to act through this mechanism, it is not always effective, and it is associated with the risk of anaphylaxis. According to the 'hygiene hypothesis', atopy appears to be due to immune deviation from Th1 to Th2, which may arise because of a failure to inhibit the normal Th2 preponderance at birth, due to an absence of the bacterial infections and endotoxins, that stimulate the Th1 response. As such it has recently been argued that a shift of the balance towards Th1 cytokines can be achieved through the use of infectious agents

1.11 Objectives

1.11.1 General Aim I

In the first part of this thesis our aim was to determine whether the Th2 specific transcription factors GATA-3, STAT6 and cMaf are involved in human allergic diseases.

1.11.1.1 Specific Aims

a. To determine if GATA-3 transcription factor is increased in human asthma, and if this increase correlates with the levels of Th2 cytokines.
b. To determine whether GATA-3 increases in response to *in vivo* allergen challenge in patients with seasonal allergic rhinitis.
c. To determine whether *ex vivo* allergen challenge using explanted nasal mucosa tissue, from asymptomatic seasonal allergic rhinitis patients would also exhibit an increase in the expression of GATA-3 mRNA.
d. To determine whether cMaf and STAT6 transcription factors are expressed in human allergic disease.
e. To determine whether the level of expression of GATA-3, STAT6, and cMAF is dependent upon atopic status of the patient.

1.11.2 General Aim II

In the second part of this thesis, our aim was to examine the expression of the Th1 associated transcription factor, YY1, in Th1 immune responses.

1.11.2.1 Specific Aims:

a. To compare the *in vivo* expression of YY1 in tuberculosis, a Th1 mediated disease, and in asthma, a Th2 mediated disease.

b. To determine whether LPS can induce a change in cytokine production in human allergic nasal tissue, along with an increase in the local expression of YY1.

CHAPTER II: METHODS AND MATERIALS

In order to complete the objectives listed above, we used several different experimental approaches in this thesis. To address the question of whether or not Th2 transcription factors are expressed in human airways, in the first part of the study, we used bronchial biopsies, as well as bronchoalveloar lavage fluid from atopic and nonatopic asthmatics, and control individuals. In the second part of the study, using the allergen-induced rhinitis model, we assessed the response of the nasal mucosa from allergic rhinitics to specific allergen. The efficacy of this model has been proven in previous studies investigating the allergic response in the nose of rhinitics (48), (110). Subsequently, to confirm that the response to allergen was not only a consequence of cellular infiltration rather than a local tissue response, we used the explant model in which nasal mucosa from allergic rhinitics was cultured with specific allergen. The nasal explant model was established and previously used in our laboratory demonstrating local eosinophil differentiation, as well as the local synthesis of ε germline transcripts of IgE, within allergic tissue (269), (77). Using this model, the possibility of systemic infiltration was excluded, and the local response of resident cells was assessed. In the third part of this thesis, which focuses on Th1 responses and associated factors, we used bronchial biopsies from subjects with tuberculosis as a model of a Th1 mediated response in order to examine the expression of Th1 transcription factors. Subsequently, the explant technique was also used as our tool, this time to assess the effects of LPS on human allergic tissue. Following these experimental procedures, human specimens underwent the

techniques of immunocytochemistry and/or *in situ* hybridization in order to detect the signal of interest.

2.1 Patients

2.1.1 Asthmatic patients

In order to examine the expression of GATA-3 in human airways, a total of 20 subjects recruited for this study, 10 of which were atopic asthmatics, and the other 10, normal controls. This study was approved by the ethics committee of the Montreal Chest Institute of the Royal Victoria Hospital in Montreal, Canada. Informed written consent was obtained from each subject prior to commencement of the study. Subjects with atopic asthma and healthy nonallergic normal subjects were carefully defined according to the criteria of the American Thoracic Society (270). In brief, asthma was defined on the basis of (1) a clear clinical history with current clinical symptoms (episodic wheezing, chest tightness, and dyspnea) and (2) 15% or greater reversibility of FEV_1 spontaneously or after 200 μg salbutamol and/or a methacoline provocation test result with a PC_{20} of less than 8 mg/ml in the previous 2 weeks. Atopy was defined by (1) a positive skin prick test response with extracts of 1 or more common aeroallergens and (2) a positive RAST response of greater than 0.70 IU/ml to 1 or more of these allergens. None of the patients were taking inhaled steroids. Normal subjects demonstrated negative skin prick test responses to a wide range of local aeroallergens in the presence of a positive histamine control. The nonatopic control subjects also had negative RAST responses to 25 common aeroallergens. All subjects were nonsmokers or ex-smokers that had stopped smoking

for greater than 12 months. Asthmatic subjects were treated with β_2-agonists only when required; inhaled corticosteroids (<1000μg/day) or nonsteroidal anti-inflammatory medications were discontinued at least 30 days before the study. Treatment with greater than 1000mg/day of inhaled steroids, long-acting β_2-agonists, leukotriene antagonists, or antihistamines was discontinued at least 3 months before the study. Exclusion criteria included age less than 18 years or greater than 65 years. FEV_1 less than 60% of the predicted value on the proposed bronchoscopy day, and evidence of acute and chronic infection, pregnancy, breast feeding, or any chronic medical illness other than asthma.

In order to determine whether the level of expression of GATA-3, STAT6, and cMAF is dependent upon atopic status of an individual, a second group of patients was recruited consisting of a total of 23 patients 7 of which were nonatopic asthmatics, 8 atopic asthmatics, and 8 controls. The patients were recruited from the Quebec Respiratory Health Network, including the Montreal Chest Institute, Sacré Côeur Hospital, and Laval University, in Quebec. Atopy was defined on the basis of clinical history, positive skin prick test results to one or more common aeroalleregens, and total serum IgE concentration greater than 100 IU/ml. Patients with nonatopic asthma had a clinical history of perennial symptoms of asthma with no clear allergic trigger, negative skin prick test result with positive histamine reaction, and total serum IgE concentrations less than 100 IU/ml. Control subjects were asymptomatic and nonatopic with normal spirometry. All subjects were nonsmokers or ex-smokers with smoking history of less than 6 pack-years and had ceased smoking for at least 3 years. All patients with asthma were treated with short-acting β2-agonists as needed

and inhaled corticosteroids at a beclomethasone dipropionate daily dose equivalent ranging from 200 to 4000 mg for a minimum of 1 month before the study. None of the patients had received oral leukotriene antagonists, methylxanthines, antihistamines, or oral corticosteroids for at least 3 months before the study. Exclusion criteria included age less than 18 or greater than 55 years, FEV_1 less than 60% predicted, evidence of upper respiratory tract infection within the previous 6 weeks, pregnancy, breast-feeding, or any chronic medical illness other than asthma.

2.1.2 Allergic Rhinitic Patients

A total of 36 patients, were recruited from the Nasal Clinic at the Sir. Mortimer B. Davis-Jewish General Hospital, and Nôtre-Dame Hôspital in Montreal, Canada, throughout the course of this thesis work. All protocols were assessed and granted approval by the ethics committee of the respective institutions and patients were informed of the procedures in great detail before giving their written consent. Prior to consideration for the study, patients underwent skin testing using a panel of common seasonal and perennial aeroallergens. A positive histamine control is a standard part of the study. Inclusion criteria for the allergic subjects included: (i) history of seasonal rhinitis, (ii) a positive skin-prick test (>5mm) to ragweed extract. Patients were excluded if they had perennial allergies (eg. dust, pets), had previously received immunotherapy or were taking oral anti-inflammatory medication.

2.1.3 Tuberculosis Patients

To address the question of whether YY1 is a Th1 transcription factor expressed in Th1 mediated disease, we set out to examine its expression in human tuberculosis, previously described to be characterized by a Th1 immune response (155). To do this, bronchial biopsies from a total of 12 subjects, 6 with pulmonary tuberculosis, and 6 normal controls, were recruited from the Montreal Chest Institute. Ethical approval for this study was obtained from the Montreal Chest Institute and the Calmette Hospital Ethics Committees. Written informed consent was obtained from all patients and control subjects prior to bronchoscopy. Pulmonary tuberculosis patients were recruited after they had been referred to the clinic for bronchoscopy on the basis of an abnormal chest radiograph and the possibility of a diagnosis of pulmonary tuberculosis. All chest radiographs were reviewed by a respiratory physician who deemed that the probability of pulmonary tuberculosis was likely enough to warrant further investigation. All had strongly positive tuberculin testing (skin-test-positive for PPD > 10 mm) and no history of signs of any other medical condition requiring current treatment, and all were HIV seronegative. Patients who were already receiving anti-tuberculosis chemotherapy were excluded from the study, as were those with known HIV infection, and women who were pregnant. All subjects were nonatopic. Patients had minimal disease with a relatively small area of lung affected. Pulmonary tuberculosis was defined as microbiologically confirmed tuberculosis on the basis of positive cultures for M. tuberculosis (sputum or BAL fluid). Patients were followed for 6 months, and all microbiologic and clinical data were used to establish the final diagnosis of active tuberculosis. Control subjects were

healthy volunteers or patients undergoing bronchoscopy for diagnostic purposes where no disease was detected. Control subjects were selected to include smokers and older subjects, reflecting the expected population risk group for pulmonary tuberculosis. Fiberoptic bronchoscopy was performed on all patients as described below (*Fiberoptic Bronchoscopy*) in more detail. Bronchial biopsies were processed and stored for further experimentation.

2.2 Study design

2.2.1 Fiberoptic bronchoscopy

Prior to the bronchoscopy procedure, each subject, both asthmatic and control, was given nebulized salbutamol (2.5 mg). Sedation was achieved with intravenously administered midazolam, and supplemental oxygen was given throughout the procedure. After 1% lidocaine spray was applied to the nose and throat of each subject, the bronchoscope was introduced. Local anesthesia of the larynx was produced with topical 4% lidocaine, and 2% lidocaine was used below the vocal cords. Bronchial biopsies were obtained from the subsegmental airways and processed and stored appropriately for future experiments.

For bronchoalveolar lavage, the bronchoscope was wedged in a segmental division of the right middle lobe or lingula where four 60 ml aliquots of warmed, pH balanced sterile saline was introduced, and immediately collected into a siliconized glass bottle by gentle aspiration. Total volume of BAL fluid recovered from asthmatics and control subjects did not differ significantly (mean values: 120 ml and 130ml, respectively). Cells from BAL fluid were filtered through a double layer of

sterile gauze to remove any mucus. Cells were pelleted by centrifugation at 400g for 7 minutes at 4°C, followed by a washing step with RPMI medium with HEPES buffer, and then resuspended in 1.5 ml of RPMI medium with 0.5% bovine serum albumin, and 0.1% sodium azide. After being counted on a hemocytometer, cells were resuspended and adjusted to a concentration of 5 X 10^5/ml for cytospin preparations. For cell differentiation, slides were stained with May-Grünwald-Giemsa. For *in situ* hybridization, slides were coated with poly-L-lysine. Samples were air dried for 10 minutes, fixed in 4% paraformaldehyde, and washed in 15% sucrose/PBS. Cytospin samples were then incubated at 37°C overnight and stored at –80°C until use.

2.2.2 Allergen-induced rhinitis model

Antigen challenge of the nasal mucosa has been a successful tool for monitoring allergic inflammatory responses during both the early and late phase reactions, and provides evidence for a step-like progression of allergic inflammation. Importantly, the clinical response to antigen provocation and the ensuing inflammation are comparable to what is observed in patients after natural allergen exposure. Similarly, the inflammatory changes in the nasal mucosa accompanied by the wide release of mediators, as well as the infiltration of inflammatory cells mimic that which is seen during natural allergen exposure. This model was used to address the question put forward in this thesis as to whether the *in situ* expression of GATA-3 increases in response to specific allergen.

2.2.2.1 Study Protocol

A total of 6 allergic rhinitic patients and 6 normal controls included in the study underwent local nasal provocation with ragweed extract. This was done by applying a 4mm filter paper disc presoaked with 1000 biological units of ragweed extract to the undersurface of the inferior nasal turbinate 2 cm distal to its anterior insertion, for a period of 10 minutes (Fig. 8). The filter paper disks were previously attached to a cotton thread and inserted under direct vision. Pre- and post-challenge biopsies were obtained from all subjects. Baseline inferior nasal turbinate biopsies were taken out of season at a time when the subjects were asymptomatic. After the 6 weeks of treatment all subjects underwent a nasal allergen challenge using ragweed extract (1000 PNU aerosol per nostril) followed by a second nasal turbinate biopsy, which was obtained 24 hours after challenge. In order to confirm clinically the adequacy of the challenge, patient symptoms after challenge were noted, specifically the number of sneezes and the degree of nasal obstruction (rated on the scale of 4). Nasal biopsies (5.0-10.00 mm) were taken from the inferior turbinate just beyond the anterior tip. They were transported back to the laboratory in 4% paraformaldehyde, where they were processed appropriately for further experimentation.

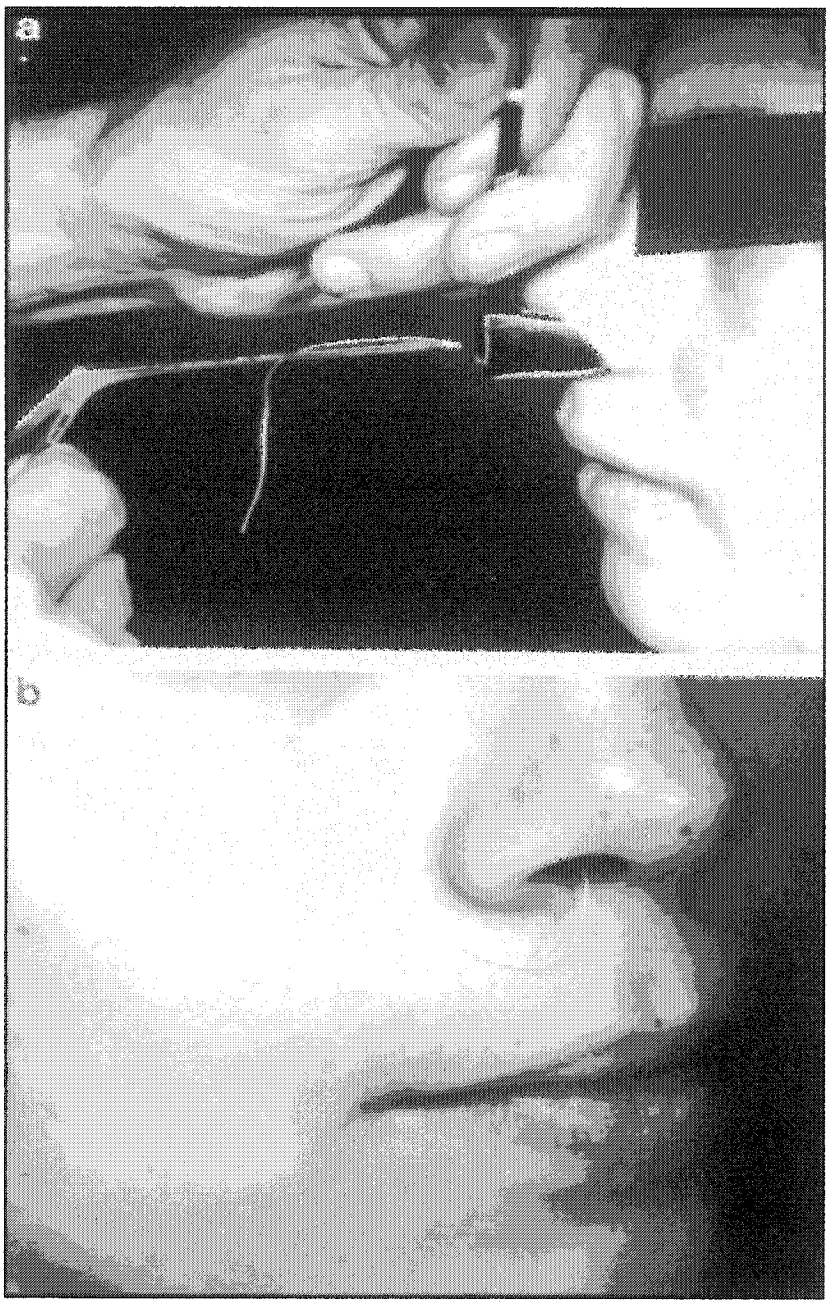


Fig 8. Intra-nasal allergen challenge. In vivo allergen provocation is performed by applying a filter paper disc soaked in allergen extract to the undersurface of the nasal inferior turbinate (a). A string attached to the disc is taped to the subjects' cheek for easy and quick removal (b).

2.2.3 Nasal Explant Model

Until recently, allergic tissue has been mainly examined following *in vivo* exposure to antigen. Although informative, these studies do not distinguish direct local-induced local effects from changes that may be secondary to antigen-induced cellular infiltration. In our laboratory at the Meakins-Christie we recently developed *ex vivo* techniques for culturing human mucosal biopsies to study local events following *in vitro* antigen challenge. In isolation from systemic variables, biopsy explants provide a useful system for delineating local inflammatory events while maintaining normal structural and cellular inter-relationships. We used the explant model to determine whether the increased GATA-3 expression in response to allergen was due either to the *de novo* infiltration of inflammatory cells, or to the local activation of resident cells. The explant model was also used in the second part of this thesis to determine whether ex vivo stimulation with LPS can induce a Th1 response in allergic nasal tissue.

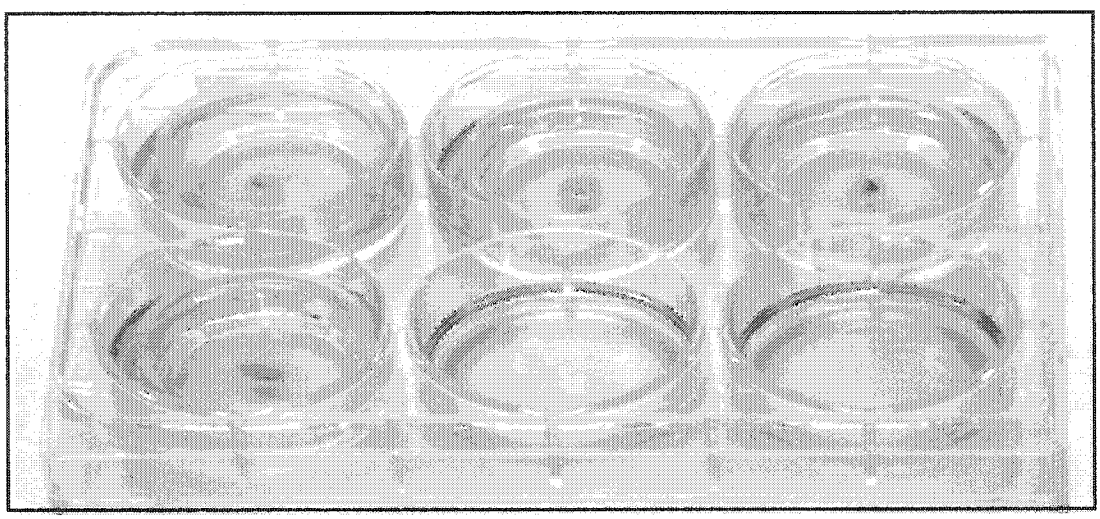
2.2.3.1 Study protocol

Human nasal mucosa biopsies were obtained from the inferior turbinate of 6 allergic rhinitis patients and 6 normal control subjects outside the ragweed season. Tissue was resected from patients undergoing sinus or nasal septal surgery, who had given informed consent before the procedure. Once resected, tissue was immediately placed in bicarbonate medium (BCM) in the operating room for transport to the laboratory. The time frame between tissue resection and culturing procedure was approximately 1 hour, in which the tissue remained immersed in BCM. BCM was

made by dissolving mineral essential medium (MEM) powder with Earl's salts, L-glutamine and sodium bicarbonate in distilled water. This solution was supplemented with 20ml/litre of MEM amino acid solution 50X, 10ml/litre of sodium pyruvate, 10 ml/litre of vitamin solution, 0.1μg/ml of hydrocortisone and 50μg/ml of gentamycin, adjusted to a pH of 7.25 and filter sterilized, leaving it at a final pH of 7.35. Under sterile conditions, the tissue was serially sectioned (2.0-3.0 mm) carefully making sure that each section contained both epithelial and submucosal layers. In a six well culturing dish, each section was placed on 30mm well inserts consisting of a 0.4μm pore diameter filter allowing the tissue to come in contact with, but not to be immersed in medium (Fig. 9). In order to retain an epithelial/air interface and to mimic the *in vivo* situation of the nasal airway, tissue sections were placed with the epithelium exposed to the air. For the first part of the study tissue was cultured with either 250μl of ragweed allergen (1000 PNU/ml), or in medium alone. The optimum concentration of ragweed allergen used for this study was previously determined (77). Although previous work has demonstrated no significant difference in symptom scores when doses of 10-1000 PNU of ragweed allergen were used in nasal provocation studies (271), the conditions for *ex vivo* challenge of nasal mucosal tissue with ragweed allergen had to be determined. Dose response studies demonstrated that 1000 PNU of ragweed allergen yielded a maximal response.

For the second part of this thesis work, again the nasal mucosal explant technique was carried out using human nasal mucosa biopsies obtained from the inferior turbinate of 6 allergic rhinitis patients, and 6 normal control subjects outside the ragweed season. Under the same experimental conditions described above in more

detail, the tissue was cultured in the presence or absence of Escherichia coli (*Ecoli*) LPS (Serotype 026:B6) at increasing concentrations (0.001-1 μg/ml). The tissue was then incubated for 24 hours in 5% CO2 /95% air.



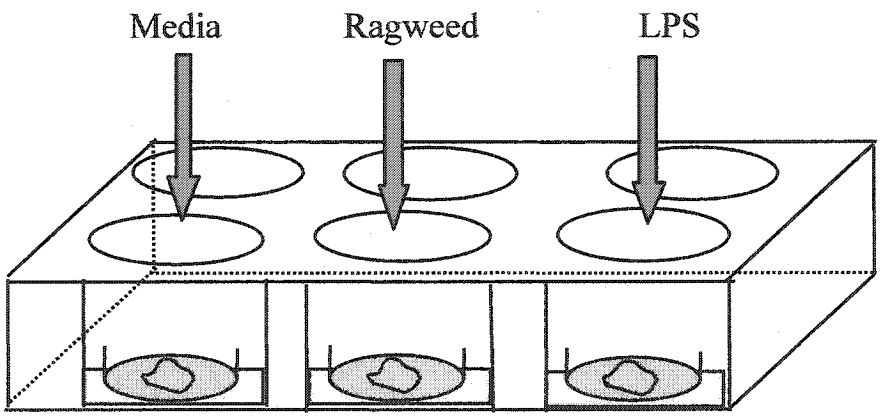


Fig 9. Nasal explant technique. In a 6 well culturing plate, explanted nasal tissue is placed on a milli-pore filter, where it comes in contact with the culturing medium and the appropriate stimulant in each well. The tissue is then incubated at 37° C for 24 hours.

2.3 Basic Principles of Immunocytochemistry

Immunocytochemistry (ICC) is a method used to identify cellular or tissue associated antigens by means of antigen-antibody interaction. The concept of using antibodies raised in the laboratory in order to localize antigen within tissue has been employed as an important tool in biological research since the 1940s (272). Immunocytochemistry has allowed the precise examination of aspects concerning cell function, greatly enhancing our understanding of disease processes. This technique is widely used in the lab as a tool to detect the presence of particular cell types, through phenotypic markers, as well as their products. In this thesis, immunocytochemistry was used to detect the expression of GATA-3, cMaf, and STAT6 protein within human nasal and bronchial tissue. In addition, this technique enabled us to characterize the presence of inflammatory cells such as eosinophils, T cells and macrophages.

2.3.1 Antibodies

In general, antibodies used for immunocytochemical investigations are usually monoclonal or polyclonal, both of which were used in this thesis work. Polyclonal antibodies are generated by immunization of an animal with a small amount of antigen of interest. The animal will then produce antibodies with different specificity and binding affinity for the particular antigen, which can be obtained by routine bleeding (273). The sera of an immunized animal contains a mixture of antibodies recognizing different epitopes of the immunizing antigen.

Monoclonal antibodies are the secreted Ig of the clonal progeny of a single hybrid plasma cell. Their generation is more time consuming and complex than the production of of polyclonal antisera (274). Unlike polyclonal antibodies however, monoclonal antibodies are specific for a single epitope of the antigen. A potential disadvantage of monoclonal antibodies is that they have a lower sensitivity than polyclonal antibodies. One single monoclonal antibody will bind to molecules containing only one copy of the epitope (275).

2.3.2 Different Methods of Immunocytochemistry

In general, three principle methods are available for immunocytochemistry including *a)* the direct method, *b)* indirect method and *c)* unlabelled antibody-enzyme methods. The direct and most simple method involves the application of a labeled primary antibody onto the tissue that binds a specific antigen (276). The indirect method uses an unlabelled secondary antibody raised to the Ig of the species providing the primary antibody (277). The unlabelled antibody enzyme method is a modification of the indirect method whereby an unconjugated bridging antibody between the primary antibody and the label detection reagent is used [Cordell, 1984 #292. Most of the immunostaining for this thesis was done using this approach; more specifically the alkaline phosphatase-anti-alkaline phosphatase (APAAP) enzyme linked technique (Fig. 10). This method is extremely sensitive and valuable for detecting low amounts of antigen.

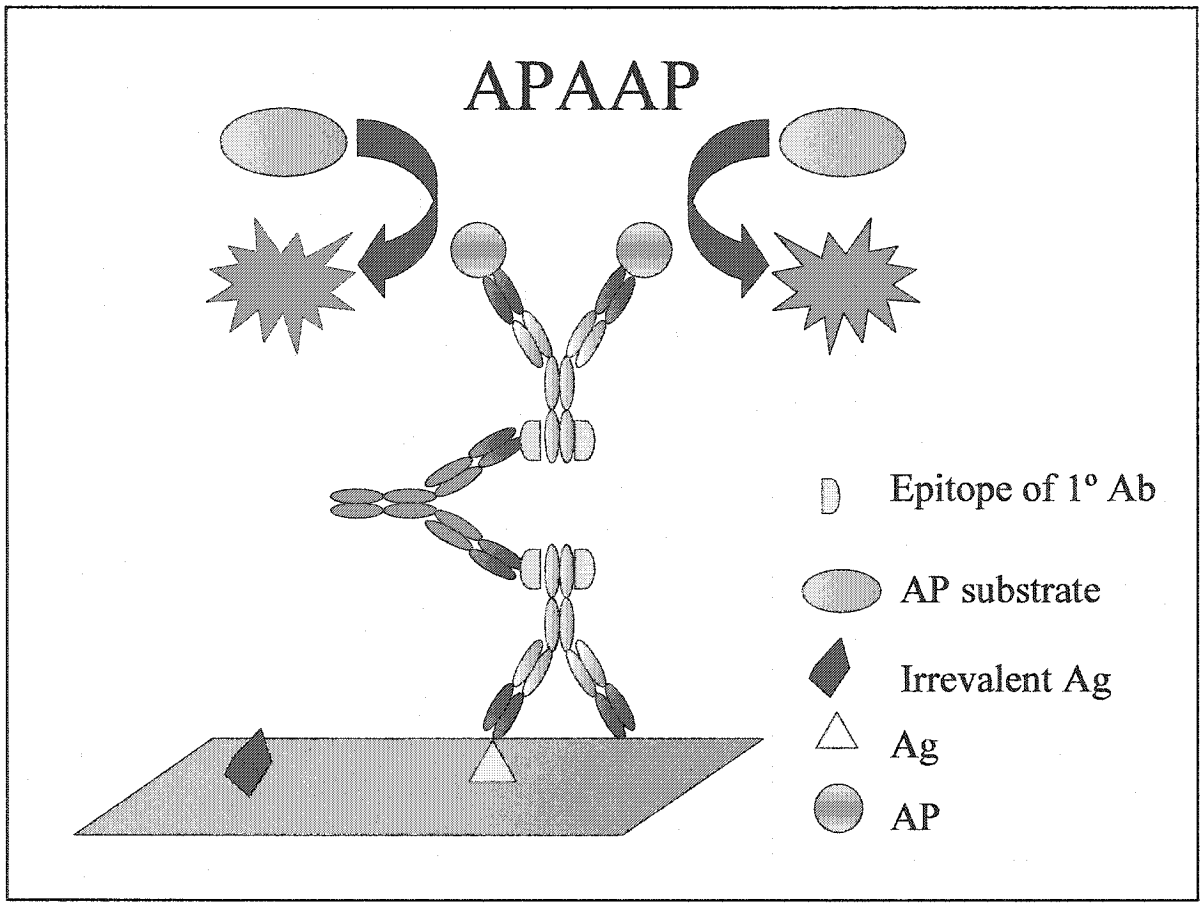


Fig 10. APAAP technique for immunocytochemistry. A primary unlabelled antibody is applied directly to the tissue preparation. An unconjugated, bridging, secondary antibody is used between the primary antibody and an enzyme-anti-enzyme antibody complex. A label detecting reagent, such as fast red, is then applied in order to visualize antigen postivity (alkaline phosphatase-anti-alkaline phosphatase enzyme linked technique).

2.3.3 Immunocytochemistry Protocols

2.3.3.1 Tissue Preparation for ICC

In preparation for immunocytochemistry, the nasal biopsy tissue was immersed in 15% PBS on ice for 15-20 minutes. After the tissue was blocked in OCT medium, cryostat sections were cut at a thickness of 5 μm, mounted on microscope slides, air dried for 1 hour. The sections were subsequently fixed by immersion in a mixture of acetone-methanol (60:40) for 7 minutes at room temperature, air dried for 1 hr, and stored at -20°C until further use.

2.3.3.2 Alkaline Phosphatase anti-alkaline phosphatase technique

Sections were hydrated in Tris buffered saline (TBS) solution and incubated with a mixture of commercially available, non-specific blocking antibodies for 10 minutes. Mouse monoclonal antibodies directed against human CD3, tryptase, MBP, and CD68 phenotypic cell markers; human GATA-3, STAT6, and YY1 transcription factors; and human IL-12 and IFN-γ cytokines were used. A rabbit polyclonal antibody directed against human cMaf was also used. Each antibody was diluted to a specific final concentration with an antibody diluting buffer: CD3 (1:100), tryptase (1:250), MBP (1:30), CD68 (1:10), GATA-3 (1:50), STAT6 (1:50), cMaf (1:500), IL-12 (1:20), IFN-γ (1:50), YY1 (1:50). Sections were incubated with 45-60 μl of primary antibody solution in a humid chamber overnight at 4°C. The following day, slides were washed for 6 minutes (2 washes for 3 minutes each) in TBS and incubated with a secondary layer consisting of rabbit anti-mouse polyclonal IgG (1:60 dilution) at room temperature for 30 minutes. After this, slides were washed in TBS for 6

minutes and the tertiary layer of alkaline phophatase (AP)-conjugated rat anti-rabbit polyclonal IgG (1:60 dilution) was applied for 30 minutes. To visualize the cells binding this antibody-AP complex, sections were incubated with a mixture of AP substrate and Fast Red TR chromogen (0.5mg/ml). Since a polyclonal antibody was used for the detection of cMaf, the secondary Ab used was a swine antirabbit Ig, and the tertiary layer was an alkaline phosphatase-conjugated Streptavidin complex, in which Fast chromogen was dissolved. When the enzyme/substrate reaction takes place, the chromogen precipitates onto the cells linked to AP, resulting in positive cells staining red.

2.4 Basic Principles of *in situ* hybridization

In situ hybridization (ISH) is the cellular localization of specific nucleic acid sequences (DNA or RNA), using a labeled complementary strand. ISH was first introduced in 1969 by Pardue & Gall (278), and was used primarily for the localization of DNA sequences. In more recent years, ISH has been applied to localize mRNA, which is the intermediate molecule in the transfer of genetic information from genomic DNA to a functional polypeptide (279), (280), (106). The general principle of mRNA ISH is based on the fact that labeled single-stranded RNA (riboprobes) are hybridized intracellularly to mRNA, under appropriate conditions, thereby forming stable hybrids. The demonstration of mRNA within a cell provides valuable information about gene expression, and indicates possible synthesis of the corresponding protein. Although a number of techniques are based on the same principle of complementary sequence hybridization, such as Northern blots or

polymerase chain reaction, the advantage of ISH over other techniques is that signal can be localized to a particular cell type within the tissue.

In this thesis, we used *in situ* hybridization in order to detect the mRNA expression of GATA-3, as well as that of IL-4, IL-5 and IL-13 cytokines, within human nasal and bronchial tissue.

2.4.1 Probe Construction

Different types of probes are available to detect mRNA, including double and single-stranded DNA probes, oligonucleotides, and single-stranded RNA probes. Single–stranded RNA probes have been used extensively in recent years for the detection of mRNA by ISH (281), (282). The use of RNA probes has a number of advantages over types of probes including high thermal stability and affinity of RNA-RNA hybrids, and availability of ribonuclease (RNase) to remove unhybridized probe during technical procedure of ISH. Thus, all these factors favor high specificity and sensitivity for RNA probes (283), (284). Generation of single stranded RNA, complementary to mRNA requires the insertion of cDNA into an expression vector with promoter regions that recognize RNA polymerases. Using the appropriate restriction enzymes the vector is linearized immediately downstream of the insert. Bluescript and PGEM vectors contain a multiple cloning site flanked by two promoters, which allow transcription of the insert in alternate directions, thereby producing fragments that are either anti-sense (complementary to mRNA), or sense (with identical sequences to mRNA). Although the instructions for protein production are found within the sequence of DNA, it is the mRNA that conveys this information

to the 'protein synthesis' machinery. To synthesize a single-stranded RNA probe, the cDNA which is attached to the promoter site must be transcribed in the presence of nucleotides and the appropriate RNA polymerase (SP6, T7 or T3 polymerase) (Fig.11).

2.4.2 Probe Labeling

For purposes of *in situ* detection, RNA probes are labeled with either radioactive or non-radioactive agents. Biotin and digoxigenin-11 UTP, are non-isotopic labels used for RNA hybridization, and although their signals are developed in very short time in comparison to radio-labeled probes, their major limitation is the relatively poor sensitivity for detection of low copy numbers of mRNA (285), (286). The use of radio-labeled probes however, has several advantages including the fact that radio-isotopes are readily incorporated into the synthesized RNA, and that autoradiography represents the most sensitive detection system available. There are different radio-isotopes used for labeling RNA including ^{3}H, ^{32}P, ^{35}S, and with these labels, the hybridization signal can be detected with autoradiography (Fig.11). The label used for a part of this thesis work was ^{35}S, which has a half life of 87 days, requires relatively short exposure time of 8-15 days, and gives quite high photographic efficiency allowing for adequate visualization of cellular and subcellular detail.

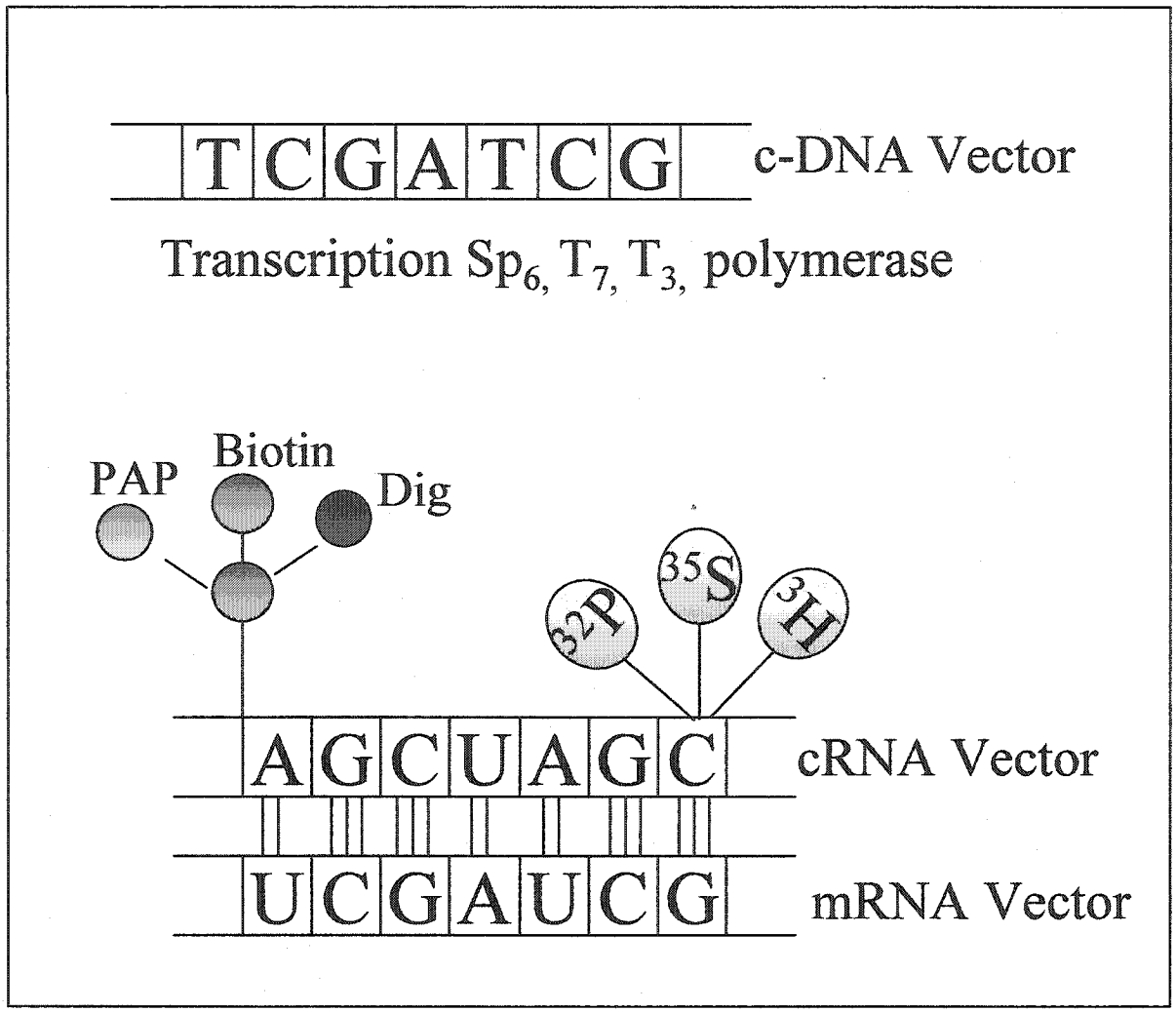


Fig 11. Probe construction and labelling. To synthesize a single stranded RNA probe, the cDNA which is attached to the promoter site must be transcribed in the presence of nucleotides as well as the appropriate RNA polymerase (Sp6, T7 or T3). RNA probes are then labeled with either radioactive (^{3}H, ^{32}P, ^{35}S) or non-radioactive (biotin, Dig, PAP) agents.

2.4.3 *In situ* hybridization Protocols

2.4.3.1 Tissue Preparation

In preparation for *in situ* hybridization, biopsies underwent fixation in order to preserve the tissue in a morphologically stable state while retaining the maximal accessible mRNA within the cells. Tissue was placed immediately in freshly prepared 4% paraformaldehyde for 2 hr, followed by three washes with 15% sucrose in DEPC treated 0.1 M phosphate- buffered saline (PBS), pH 7.4 (first two washes for 1 hr at room temperature and then overnight at 4° C). Biopsies were then placed in OCT (optimal cutting temperature) embedding medium, snap frozen in isopentane precooled in liquid nitrogen, and stored at -80° C until further use.

2.4.3.2 Tissue Sectioning

In preparation for *in situ* hybridization cryostat sections, 10 μm thick, were cut from the each block and mounted on poly-L-Lysine (PLL)-coated microscope slides, in order to maximize tissue retention on the slides throughout the various rigorous treatments involved in *in situ* hybridization. Poly-L-lysine gives the slides a positive charge and provides firm adhesion of the tissue (287). They were then air dried for 1 hr, and left to incubate at 37° C overnight to allow for dehydration and maximum adherence of tissue. Fixation in freshly prepared paraformaldehyde maintains tissue morphology while allowing cellular penetration of the probe and thus efficient hybridization.

2.4.3.3 Probe construction

In this thesis, both anti-sense and sense probes were generated for GATA-3, IL-4, and IL-5 and IL-13 mRNA The IL-4 probe was generated from a human 500bp fragment (gift provided by Dr. J.Y. Bonnefoy, Glaxo Institute for Molecular Biology, Geneva, Switzerland). It was inserted into a pGEM vector, linearized by SPH1 and EcoR1 restriction enzymes and transcribed in the presence of ^{35}S-UTP with T7 or Sp6 polymerase for the antisense and sense probes, respectively. The sequence for IL-13 probe (generously provided by Dr. A. Minty, Sanofi Recherche, Laberge, Cedex, France) corresponded to a 1282bp fragment. It was inserted into a Bluescript vector and linearized with Not 1 and BamH1 restriction enzymes. Sense and antisense probes were generated by *in vitro* transcription using T3 and T7 RNA polymerases and the probe was labeled by ^{35}S-UTP incorporation. The IL-5 probe was generated from a 421bp cDNA fragment (provided by Eric Kawashima, Glaxo Institute for Molecular Biology). It was inserted into a pGEM vector, and linearized with BamH1 and XbaI restriction enzymes. Sense and antisense probes were generated by *in vitro* transcription using T7 and Sp6 RNA polymerases respectively, and labeled by ^{35}S-UTP incorporation. The GATA-3 riboprobe was generated from a human cDNA fragment (gift provided by Dr. Anuradha Ray, Yale University School of Medicine, New Haven). It was inserted into a pGEM vector, linearized by BamHI and XbaI restriction enzymes, and transcribed in the presence of ^{35}S-UTP with T7 or Sp6 polymerase for the antisense and sense probes, respectively.

2.4.3.4 In vitro transcription and probe labeling

To perform *in vitro* transcription of radio-labeled probes, 1.0 μg/ml of linearized plasmid template, (either GATA-3, IL-4. IL-13 or IL-5), was added to 10μl mixture of 5X transcription buffer (200mM Tris-HCl; pH 7.5, 30 mM $MgCl_2$, 10mM spermidine, 5 mM NaCl), 100 mM diethylthreitol, 25 U/μl of ribonuclease inhibitor, nucleotide mixture (2.5 μM of ATP, GTP, CTP), 25 mCu of ^{35}S-UTP and 10 units of RNA polymerase (T7, Sp6 or T3) and heated to 37°C in a waterbath for 60 minutes. The polymerase catalyzes transcription from its corresponding promoter on the vector and the nucleotides bind to the cDNA in complimentary fashion. RNase-free DNase is added to separate the RNA probe from the cDNA template, leaving single-stranded RNA fragments. To extract the probe from unincorporated nucleotides, 10 μg/ml of tRNA, 4M NaCl and phenol-chloroform (1:1, v/v) were added. After microfugation at 12 000g, the aqueous phase was removed and subjected to a second extraction with an equal volume of chloroform. The aqueous phase was treated with 100μl of 7M ammonium acetate (2.5 M final concentration) and 750 μl of cold (stored at –20°C or for two hours at -80°C. This mixture was microfuged at 4°C, the aqueous phase removed, except the last 50μl, which was placed in a speed vacuum centrifuge for dissipation. The purified RNA probe (the remaining pellet), was then dissolved in 20 μl of DEPC-treated H20. Incorporation of the radiolabel was assessed using a β–emissions counter by placing a 1 μl sample of the probe into 5 ml of scintilating fluid. Measurements were made in total counts per million (cpm) where 35S labeled probes were always $\geq 1.0 \times 10^6$ cpm.

2.4.3.5 Prehybridization

In order to increase the efficiency of hybridization and to render target sequences more accessible to the probe, slides underwent prehybridization treatment allowing cell membrane permeabilization. Initially slides were immersed in 0.1M glycine/PBS and then in a 0.3% Triton-X-100/PBS solution, which degrades the cell surface proteins, for 5 minutes. Slides were then placed in a 1μg/ml proteinase K solution, dissolved in 1M Tris-HCl pH 8.0 and 0.5M EDTA and PBS, for 20 minutes at 37°C to degrade intramembrane proteins. Further permeabilization and fixation was performed by placing the slides in a freshly prepared solution of 4% PF/PBS for 5 minutes. Pre-hybridization also includes steps for reducing non-specific binding. These were carried out by immersing the slides in high salt solutions of *1)* 0.25% acetic anhydride and 0.1M triethanolamide, and *2)* 0.1M N-ethylmalamide and 0.1M iodoacetamide, both dissolved in distilled water, and used at a temperature of 37°C for 10 minutes and 20 minutes respectively. Immersion in a mixture of 50% ionizing formamide in 4X standard saline-citrate (SSC) for 15 minutes at 37°C was used to regulate tissue equilibrium and stabilization. Finally, slides were dehydrated in increasing concentrations of ethanol (70%, 90% and 100%) for 5 minutes each and left to dry for a period of at least 2 hours.

2.4.3.6 Hybridization

In order to allow the hybridization of cRNA and cytoplasmic mRNA to take place, the radiolabelled probe was incubated with the pretreated tissue within optimal incubation conditions. A hybridization mixture was prepared consisting of

hybridization buffer (50% deionized formamide, 5X Denhardt's solution, 10% dextran sulphate, 0.5% sodium pyrophosphate, and 0.5% SDS and 100mM dithiothreitol (DTT). To this buffer, 0.75 X 10^6 cpm/section of radiolabelled riboprobe (GATA-3, IL-4, IL-5 and IL-13) was added as well as 10mM of DTT. Each tissue section was incubated with 15µl of this probe mixture prehetaed to 37°C. The sections were then covered with suitably sized dimethyldichlorosilane-coated coverslips. Slides were placed in a humid chamber and incubated overnight at 42°C allowing hybridization to take place.

2.4.3.7 Post-hybridization

Post hybrization washes are important as they determine the stringency of the *in situ* procedure allowing diffusion of material trapped in the section, and selecting for a 'good fit' between the probe and target mRNA. Once the coverslips were removed by gently immersing each slide in 4X SSC, slides were washed in three changes of 4X SSC at 42°C for 20 minutes each with gentle agitation. These are considered stringent conditions since both the temperature and salt concentrations were relatively high thus effective at reducing non-specific binding of the probe. The slides were then treated with 20µg/ml of RNAse, dissolved in 4M NaCl, 1M Tris and 0.5M EDTA, for 30 mins at 42°C. This was done in order to eliminate the excess unhybridized single-stranded cRNA, minimizing non-specific interactions. Subsequently, slides were put through decreasing concentrations of SSC washes of 2X, 1X, 0.5X and 0.1X SSC for 20 minutes at 42°C each, and then dehydrated in 70%, 90%, and 100% ethanol solutions with 0.3% acetic anhydride, for 10 minutes

each at room temperature. The slides were then left to air dry at room temperature for one hour.

2.4.3.8 Autoradiography

To visualize hybridization signals slides were dipped in liquid emulsion in a dark room under red light and left overnight to dry in complete darkness. Exposure took place in a light-proof sealed black box at 4°C for a period of 10-15 days. Following this exposure period, sides were developed under red light using Kodak D-19 developing solution at 16-18°C for 3.5 minutes with gentle agitation. This reaction was arrested by placing the slides in water. Fixation of the development was performed by immersing the slides in Rapid Fixer solution for 5 minutes at 16-18°C and then rinsed for 20 minutes in water. Using a straight razor blade, excess emulsion was removed and special care was taken to clean the underside of the slide beneath the tissue. Tissue sections were then counter-stained in hematoxylin, a blue basic dye that combines with acid substances and therefore stains the nuclei, creating a contrast for histological analysis with light field microscopy. Slides were then dehydrated in increasing concentrations of ethanol (70-100%) and finally immersed in xylene. Subsequently, slides were coverslipped using entellyn which adheres the coverslip to the slide. When analyzing the slide under light microscopy, a positive signal is observed as a cluster of small black silver grains overlying the cell.

2.4.4 Controls for in situ hybridization

Proper controls are necessary in *in situ* hybridization experiments to assess the specificity of the probe and the procedures used. In this study we used negative controls such a sense probe or RNase pretreatment. The sequence of a sense probe is identical to that of mRNA and therefore replacing the anti-sense with a sense probe in the hybridization mixture should not result in a positive signal. Another control performed was to pre-treat the tissue with an 100μg/ml of RNase A at 37°C prior to the prehybridization step, digesting all single stranded RNA. Loss of signal by RNAse pretreatment shows that the probe was interacting with digestable nucleic acids.

2.4.5 Simultaneous *in situ* hybridization

To colocalize GATA-3 and IL-5 mRNA, double in situ hybridization was performed. The GATA-3 antisense riboprobe was labeled with ^{35}S-UTP, and the IL-5 probe was labeled with biotin-11-UTP. Sections were treated similarly to single *in situ* hybridization but hybridized simultaneously with radiolabeled GATA-3 and non-radiolabeled IL-5. Biotin-labeled cRNA-mRNA hybrids were visualized by using an avidin-biotin complex and diaminobenzidine (brown stain). Sections were then dried and covered with emulsion fluid to develop the radiolabeled cRNA-mRNA hybrid. Double-hybridization signals were demonstrated by the presence of silver grains in brown-staining cells.

2.4.6 Combined immunocytochemistry –in situ hybridization

Simultaneous immunocytochemistry and *in situ* hybridization was performed to identify the phenotype of GATA-3 mRNA positive cells. The specimens were immunostained using the APAAP technique as described above in more detail, in order to phenotype cells with monoclonal antibodies directed against human T cells (anti-CD3), mast cells (anti-tryptase), eosinophils (anti-MBP) and neutrophils (anti-elastase) used at the dilutions indicated above. All solutions used were made up in 0.1 % diethylpyrocarbonate-treated double distilled water to avoid RNase contamination, and specimens were processed for in situ with a radiolabelled antisense riboprobe specific for GATA-3 after immunocytochemical staining.

2.5 Quantification

Quantification of results for immunocytochemistry and *in situ* hybridization consists of point counting, where the results are reported as the number of cells expressing the protein and mRNA transcript of interest. To do this, light microscopy was used to analyze coded slides for positive signals with an eye piece graticule of 0.202 mm^2, at 200X magnification. For analysis of nasal and bronchial tissue, the graticule was placed under the basement membrane, and the number of positive cells were counted and expressed as the mean of at least 6 to 8 fields per square millimeter. In BAL fluid, at least 1000 cells were counted, and the results were reported as the number of positive cells per 1000 cells. Counting was done in a blinded fashion by two independent examiners where inter-observer variability was less than 10%.

CHAPTER III: RESULTS

3.1 Gene Expression of GATA-3 in Asthma

3.1.1 Airway Cellular Profile

Bronchoalveolar lavage fluid and bronchial biopsies were taken from atopic asthmatics and nonasthmatic controls. The demographics, allergen reactivity, and indices of lung function for each group are shown in Table 2.

The total cell recovery in BAL fluid did not differ significantly between asthmatic patients and normal control subjects. The numbers of cells expressing the phenotypic markers CD3, MBP, tryptase, and CD68 by immunocytochemistry in bronchial biopsy specimens and BAL was determined. Whereas the absolute numbers of MBP+ eosinophils were significantly elevated in the asthmatic patients as compared with the control subjects ($p<0.001$), numbers of CD3+, tryptase+, and CD68+ cells did not differ significantly. Similarly, in BAL fluid only the percentage of MBP+ eosinophils were significantly higher in asthmatic patients (4.5%) compared with normal control subjects (0.5%).

3.1.2 GATA-3 mRNA expression

GATA-3 gene expression was investigated by *in situ* hybridization with radiolabeled antisense riboprobes. Numbers of cells expressing GATA-3 mRNA were significantly increased in BAL cells from asthmatic patients compared with those from normal control subjects ($p<0.001$) (Fig 12A). Similar results were seen in biopsy specimens from asthmatic subjects compared with those from normal control subjects (Fig 12 B). The numbers of cells expressing GATA-3 mRNA were compared

Table 2. Demographics of asthmatic patients and normal control subjects

	Asthmatic Patients	Controls
Age (y)	25-50	20-33
Sex (M/F)	6/4	7/3
FEV_1 (% predicted)		
Mean	95	103.2
Range	70-128	93-119
Positive skin test responses	10/10	0/10
Total IgE (IU/ml)	920±302	95±37
Methacoline PC_{20} (mg/ml)	2.64±1.22	>16

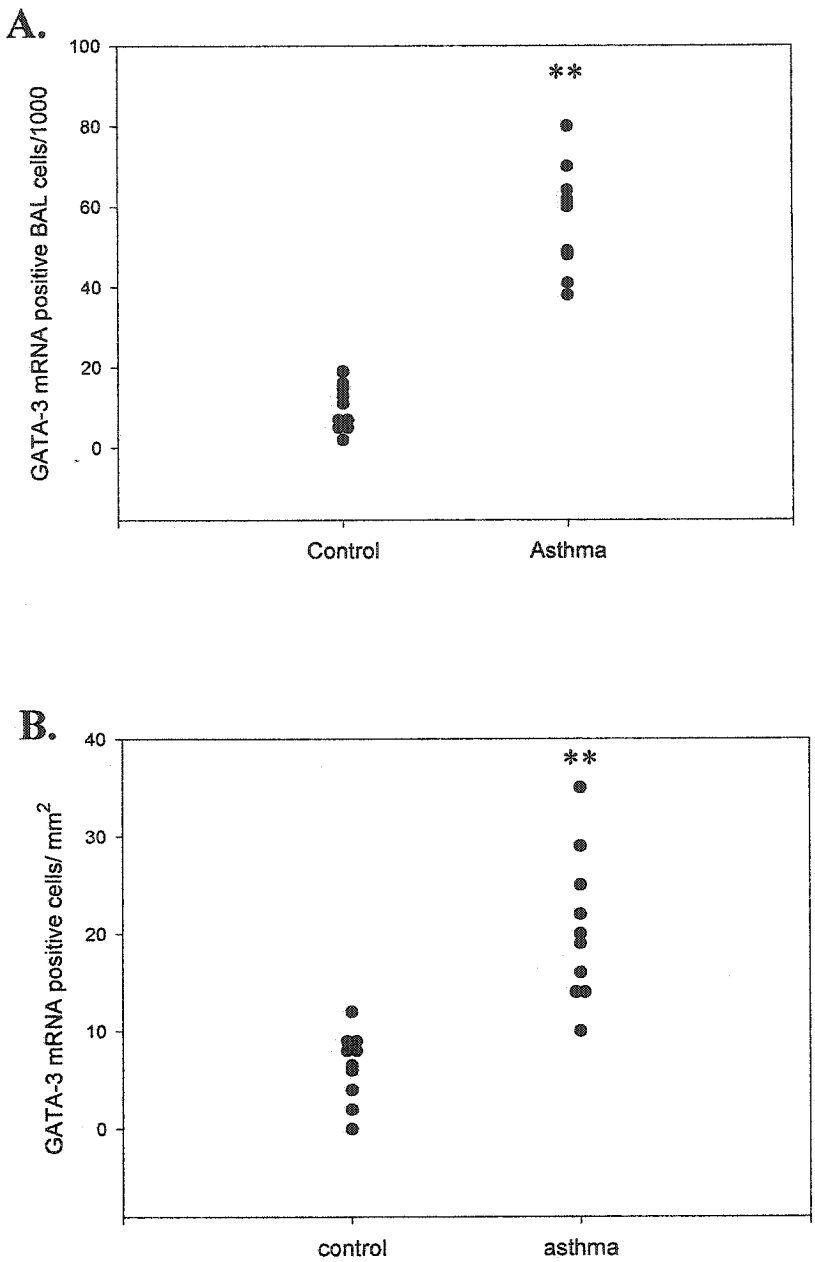


FIG 12. GATA-3 gene expression in BAL fluid **(A)** and bronchial biopsy specimens **(B)** from subjects with atopic asthma and nonasthmatic control subjects. Numbers of GATA-3 mRNA + cells determined by *in situ* hybridization, were significantly higher in asthmatic subjects compared to controls, both in BAL fluid and biopsy specimens ** $P<0.001$. The number of cells expressing GATA-3 mRNA in each group were compared by using a Mann-Whitney U test.

by using the Mann-Whitney U test. Results were considered statistically significant for P values less than .05. Positive GATA-3 mRNA signals localized predominantly to infiltrating inflammatory cells, with no signal appearing in airway structural cells such as epithelial or endothelial cells (Fig 13). Treatment of sections with the antisense probe for GATA-3 subsequent to RNase treatment, or with the sense probe for GATA-3 as control experiments, resulted in an absence of hybridization signal. In asthmatic subjects the numbers of cells expressing GATA-3 mRNA correlated inversely with airway caliber (FEV_1) ($p<0.01$, $r=-0.81$) and directly with bronchial responsiveness ($p<0.05$, $r=0.60$). The coefficiants of determination *(r)* and significance values were calculated by using linear regression analysis.

3.1.3 Phenotype of GATA-3 mRNA+ cells in the airways

Using a combination of immunocytochemistry and radioactive *in situ* hybridization, we phenotyped the cells expressing GATA-3 mRNA on BAL specimens from asthmatic subjects. As shown in Fig. 13, double-positive cells exhibited both a red color revealed by immunocytochemistry, and overlying silver grains by *in situ* hybridization. In BAL cells from asthmatic patients, the majority of cells expressing GATA-3 mRNA were CD3+ cells (73.7% ± 10.5%). Smaller percentages of GATA-3 mRNA expressing cells were accounted for by MBP+ eosinophils (12.4% ± 3.9%) and tryptase + mast cells (7.8% ± 6.3%), but CD68+ cells showed no hybridization with the GATA-3 antisense probe (Table 3). The specificity of the signals was confirmed by a series of controls involving substitution

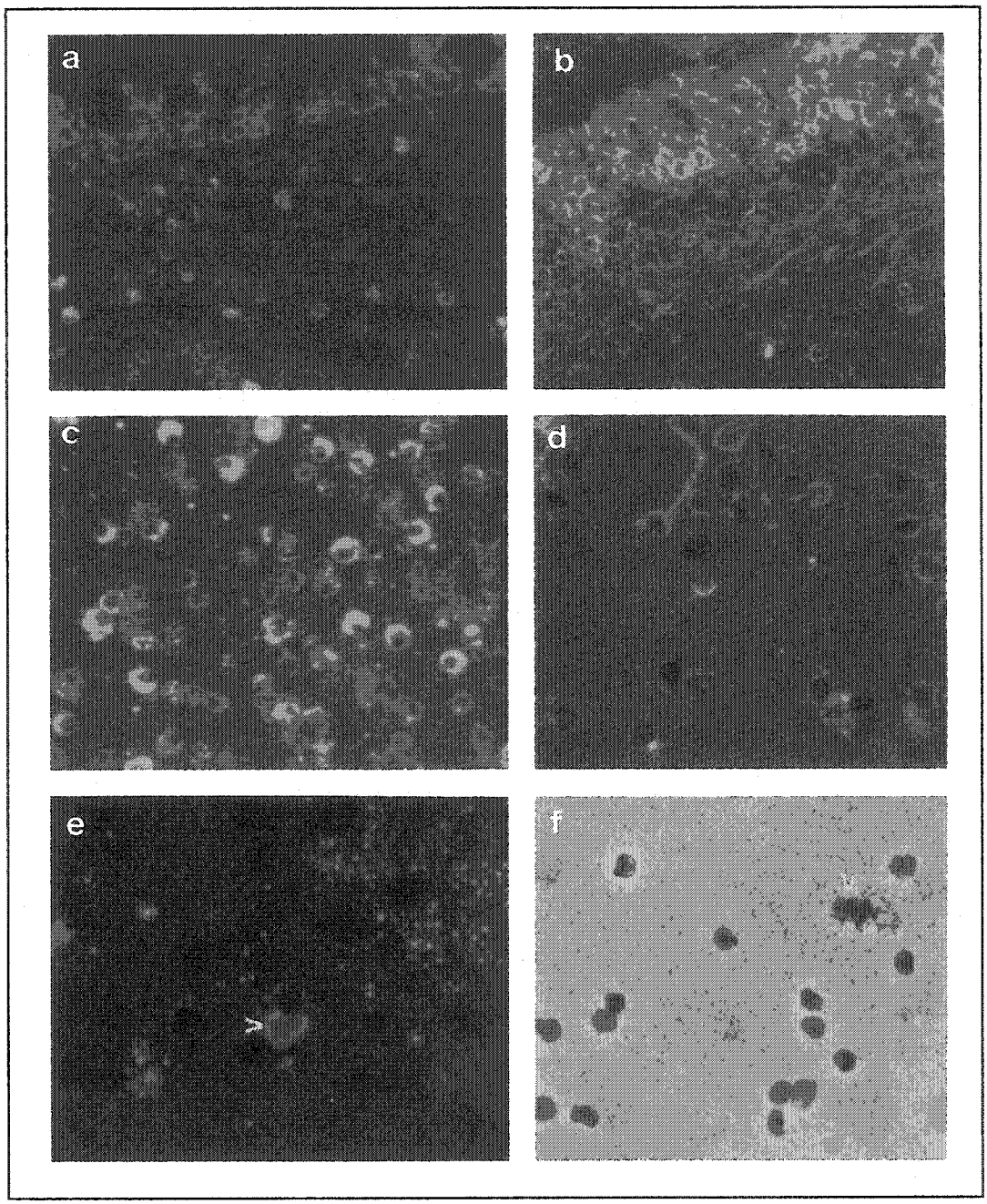


FIG 13. Representative examples of *in situ* hybridization of GATA-3 mRNA in bronchial biopsy specimens and BAL preparations from atopic asthmatic subjects and normal control subjects by using complementary RNA probes. **a)** *in situ* hybridization (dark field illumination) of GATA-3 mRNA in a bronchial biopsy specimen from an asthmatic subject. Note the presence of positive signals within infiltrarting cells but in the epithelial or endothelial cells. **b)** GATA-3 mRNA expression under dark field illumination in a bronchial biopsy specimen from a normal control. **c)** GATA-3 mRNA in BAL cells from a subject with asthma and **d)** a normal control subject. **e)** Combined in situ hybridization –immunocytochemistry localizing GATA-3 mRNA to CD3 immunoreactive cells in BAL fluid from an asthmatic subject (*arrowhead*). **f)** Coexpression of GATA-3 mRNA and IL-5 mRNA in BAL cells from an asthmatic subject by double *in situ* hybridization (arrowhead). GATA-3 probes were labeled with ^{35}S, and IL-5 probes were labeled with biotin. Signals were developed by autoradiography and diaminobenzidine, respectively.

Table 3. Percentage of GATA-3 mRNA + cells coexpressing CD3, MBP, tryptase, and CD68 immunoreactivity in BAL fluid from atopic asthmatic subjects*

Subject	%GATA-3+/CD3+	%GATA-3+/MBP+	%GATA-3+/tryptase+	%GATA-3+/CD68+
1	79	10	5	0
2	72	11	6	0
3	75	9	4	0
4	83	10	3	0
5	62	17	7	0
6	89	10	1	0
7	85	9	4	0
8	56	21	17	0
9	69	13	11	0
10	67	14	20	0
Mean ± SD	73.7 ± 10.5	12.4±3.9	7.8±6.3	0

* Results of combined in situ hybridization-immunocytochemistry of BAL fluid obtained from asthmatic subjects

of the primary antibody with an irrelevant species and isotype-matched antibody and/or treatment with the GATA-3 sense riboprobe.

3.1.4 Colocalization of GATA-3 mRNA to cells expressing IL-5 transcripts

Because GATA-3 has been shown to be crucial for IL-5 gene expression in vitro, we examined the expression of IL-5 in the same asthmatic and control subjects. The numbers of IL-5 mRNA + cells were substantially increased in BAL fluid from atopic asthmatic subjects ($P<0.001$) compared with BAL fluid from normal control subjects. Similar results were seen in bronchial biopsies from asthmatic subjects ($P<0.001$) compared with biopsy specimens from control subjects (Fig14A and B). Numbers of cells expressing GATA-3 mRNA correlated significantly with IL-5 mRNA+ cell numbers in BAL fluid and biopsy specimens from asthmatic subjects ($p<0.001$, $r=0.879$ in BAL fluid; $p<0.05$, $r=0.721$ in biopsy specimens) (Fig. 15A and B). Correlations were determined by linear regression analysis. To determine whether GATA-3 and IL-5 transcripts are coexpressed by the same cells, double *in situ* hybridization analyses was performed on BAL cells from asthmatic patients by using a radiolabelled GATA-3 probe and a nonradiolabelled IL-5 probe. The mean percentage of IL-5 mRNA positive cells coexpressing GATA-3 mRNA was 76%. In contrast, 91% (mean percentage) of the GATA-3 mRNA positive cells expressed IL-5 transcripts in BAL fluid. An example of the coexpression of GATA-3 and IL-5 mRNA is shown in Fig. 13.

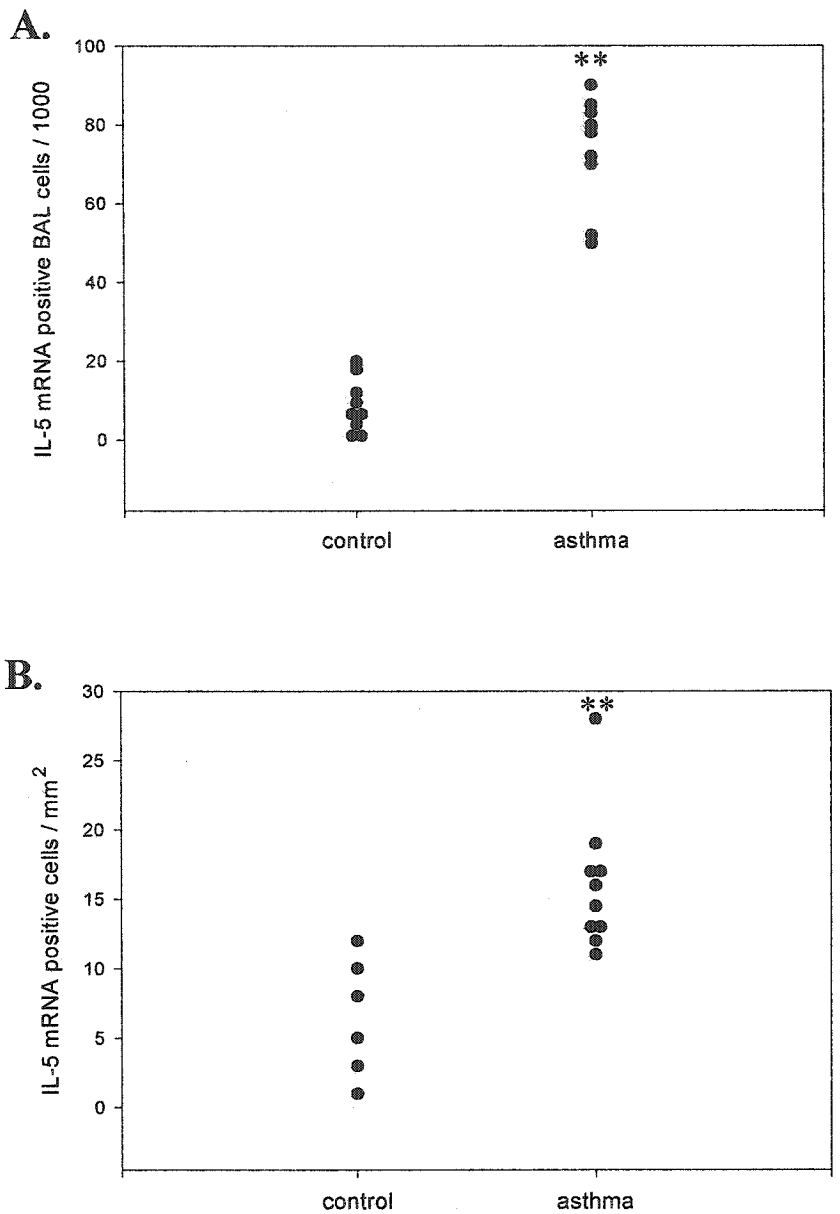


FIG 14. Expression of IL-5 mRNA in BAL fluid **(A)** and bronchial biopsy specimens **(B)** from subjects with atopic asthma and normal control subjects. Numbers of IL-5 mRNA + cells were significantly higher in asthmatic subjects compared to controls, both in BAL fluid and biopsy specimens ** *P<0.001*. The number of cells expressing IL-5 mRNA in each group were compared by using a Mann-Whitney U test.

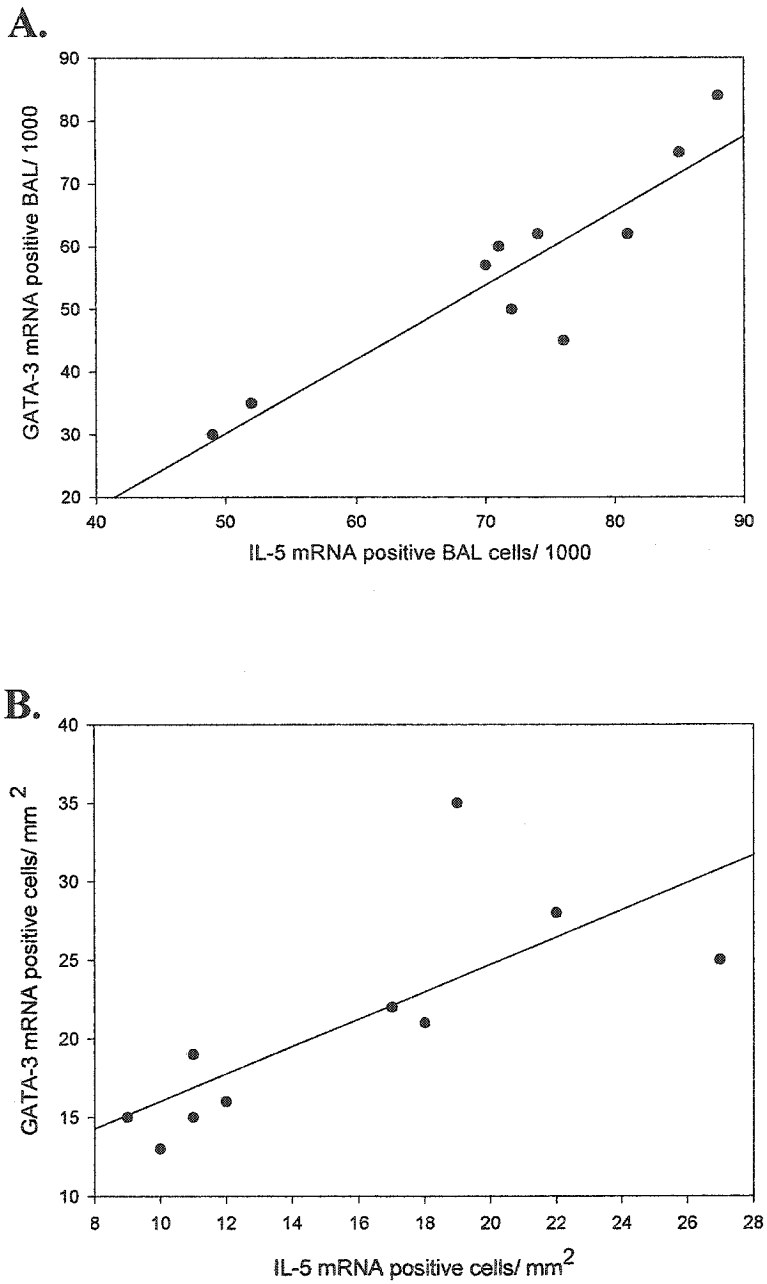


FIG 15. Correlation of GATA-3 mRNA + cells and IL-5 mRNA + cells in BAL fluid **(A)** and bronchial biopsy specimens **(B)** from atopic asthmatic subjects. Correlations were determined by linear regression analysis. Numbers of cells expressing GATA-3 mRNA correlated significantly with IL-5 mRNA + cell numbers in BAL fluid and biopsy specimens from asthmatic subjects ($P<0.001$, $r=0.879$ for BAL fluid; $P<0.05$, $r=0.721$ for bronchial biopsy specimens)

3.2 GATA-3 expression in the upper airway mucosa following in vivo allergen challenge

3.2.1 Clinical response following in vivo allergen provocation

Nasal provocation with allergen resulted in immediate nasal symptoms with increases in sneezes, weight of nasal secretions, and blocking of the nostril in all subjects tested. Symptoms of nasal blocking, sneezing or both occurred at 3 to 10 hours after allergen challenge in every subject. The presence of symptoms confirmed the adequacy of our in vivo challenge protocol.

3.2.2 Expression of GATA-3 and cytokine mRNA after in vivo allergen provocation

There were greater numbers of GATA-3 and IL-5, IL-4 and IL-13 mRNA+ cells in the nasal biopsy specimens taken from patients with allergic rhinitis compared to control subjects. In contrast, there was no difference in the numbers of IFN-γ mRNA + cells between the two groups (subjects with allergic rhinitis, 4.6 ± 1.1 cells/mm^2; control subjects, 2.5 ± 0.7 cells/mm^2).

In biopsy specimens obtained from control individuals, there was no observable increase in the numbers of cells exhibiting positive mRNA signals for GATA-3, IL-4, IL-5, and IL-13, 24 hours after allergen challenge. In contrast, there was a significant increase in the numbers of GATA-3 and IL-5mRNA+ cells 24 hours after allergen challenge compared to baseline, in nasal biopsy specimens from patients with allergic rhinitis ($p<0.05$) (Fig. 16A and B). In this same group of patients, the numbers of IL-4 and IL-13 mRNA+ cells also significantly increased after allergen challenge ($p<0.0.5$) (Fig. 17A and B). There were no changes in the

numbers of IFN-γ mRNA+ cells after allergen challenge in the patients with allergic rhinitis (4.6 ± 1.1 cells/mm^2 vs 4.4 ± 0.6 cells/mm^2). The Student paired t test was used to examine changes within the allergic rhinitis and control groups, before and after allergen challenge. The Student unpaired t test was used on the Δ values (difference before and after challenge) between the control and allergic rhinitis groups.

3.2.3 Correlation between the expression of GATA-3 and IL-5 mRNA

After *in vivo* allergen challenge, there was a significant correlation between the numbers of GATA-3 and IL-5 mRNA+ cells in the nasal biopsy specimens from patients with allergic rhinitis before and after allergen challenge ($r=0.87$; $p<0.01$; $n=12$). To assess this relationship, the correlation coefficiant (*r)* was evaluated by using the Fisher Z transformation method. Probability values less than .05 were considered statistically significant.

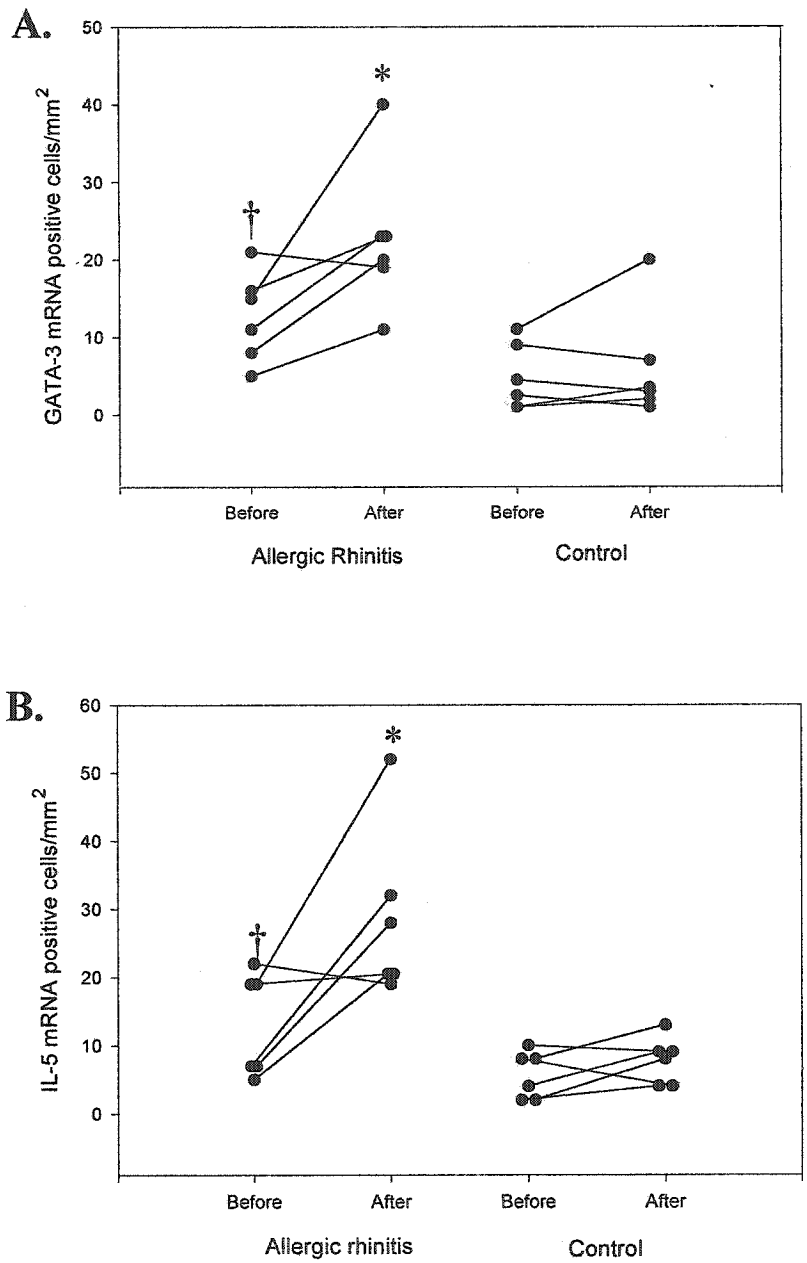


FIG 16. Expression of GATA-3 **(A)** and IL-5 mRNA **(B)** within the submucosa of nasal biopsy specimens taken from patients with allergic rhinitis and normal control subjects. There was a significant increase in the number of cells expressing GATA-3 and IL-5 mRNA after allergen challenge only in nasal biopsy specimens from patients with allergic rhinitis *(*P<0.05, Student paired t test)*. There was also a significant difference in the numbers of GATA-3 and IL-5 mRNA + cells between patients with allergic rhinitis and normal control subjects, before allergen provocation *(† P<0.05, Student unpaired t test)*.

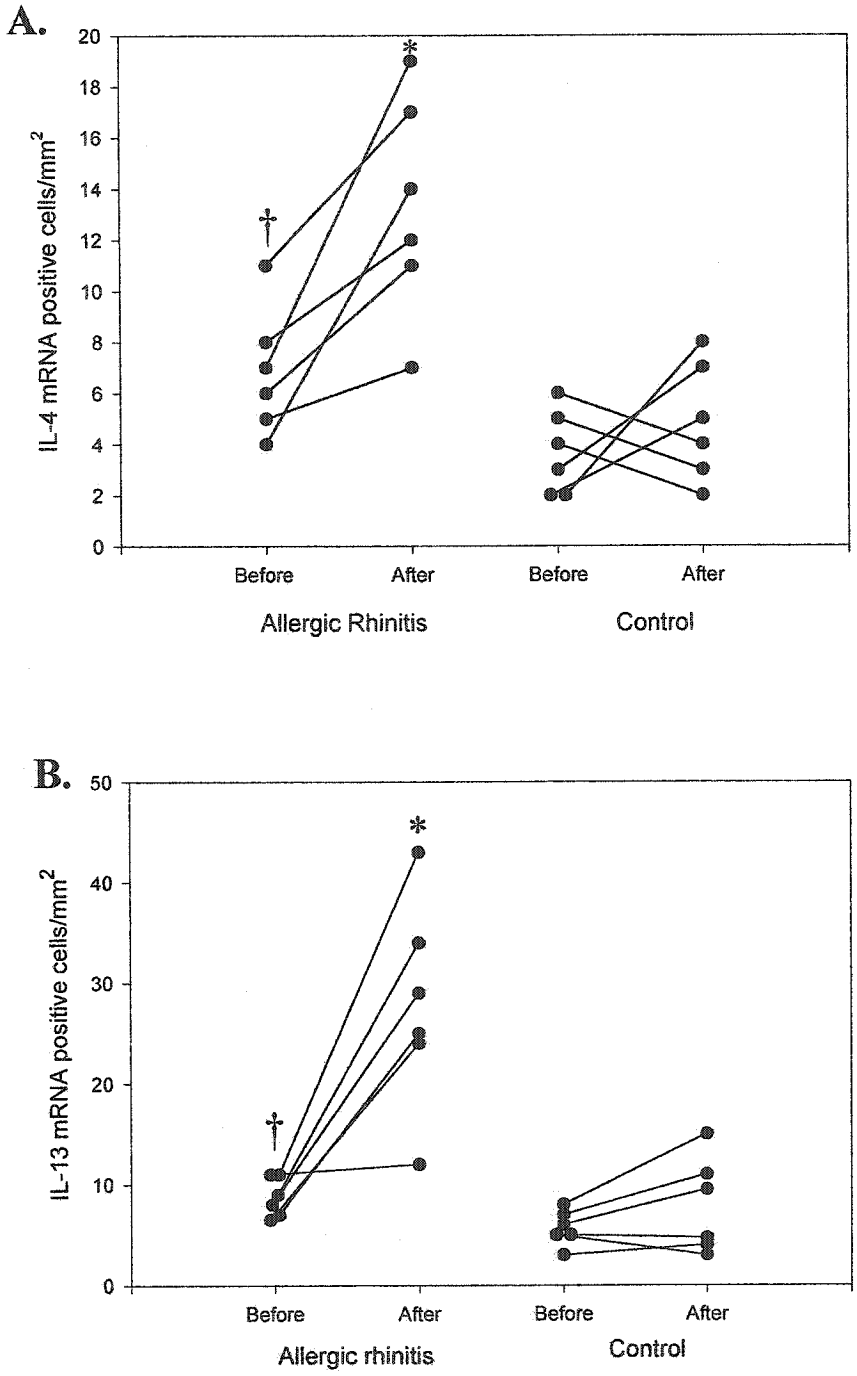


FIG 17. Expression of IL-4 **(A)** and IL-13 **(B)** mRNA within the submucosa of nasal biopsy specimens taken from allergic rhinitis and normal control subjects before and after allergen challenge. There was an increase in the numbers of cells expressing IL-4 and IL-13 mRNA after allergen challenge, only in nasal biopsy specimens from patients with allergic rhinitis (*$*P<0.05$, Student paired t test*). There was also a significant difference in the numbers of IL-4 and IL-13 mRNA+ cells between the patients with allergic rhinitis and the normal control subjects at baseline, before allergen provocation (*$\dagger P<0.05$, Student unpaired t test*).

3.3 Expression of GATA-3 and cytokine mRNA after *in vitro* allergen provocation in Nasal explants

Within nasal tissue cultured in medium alone, there were significant differences in the numbers of GATA-3 and IL-5 mRNA+ cells as well as IL-4 and IL-13 mRNA + cells within tissue obtained from control subjects compared with tissue from individuals with allergic rhinitis ($p<0.05$). There was no difference however in the number of IFN-γ mRNA + cells in tissue from control subjects with allergic rhinitis after culture in medium alone. In the control group, there was no difference in the numbers of GATA-3 or cytokine mRNA + cells in tissue explants challenged with medium alone or ragweed allergen. In comparison, there were significant differences in the numbers of GATA-3 (Fig.18A) and IL-5 (Fig.18B) mRNA+ cells, in the nasal explants from the patients with allergic rhinitis ($p<0.05$) after allergen challenge, represented in Fig.19. Similar results were seen with IL-4 and IL-13 mRNA+ cells, which significantly increased in nasal explants from allergic rhinitic patients ($P<0.01$ and $P<0.05$ respectively) compared to controls, following *in vitro* allergen challenge (Fig.20A and B). There was no difference however in the numbers of IFN-γ mRNA + cells after 24 hours of culture with allergen compared with medium alone in the explants from these subjects.

3.3.1 Correlation between the expression of GATA-3 and IL-5 mRNA

Consistent with *in vivo* allergen challenge data, in the nasal explants there was a significant correlation between the numbers of GATA-3 and IL-5 mRNA+ cells following incubation with either medium or allergen in tissues from patients with allergic rhinitis ($r=0.79$; $p<0.01$).

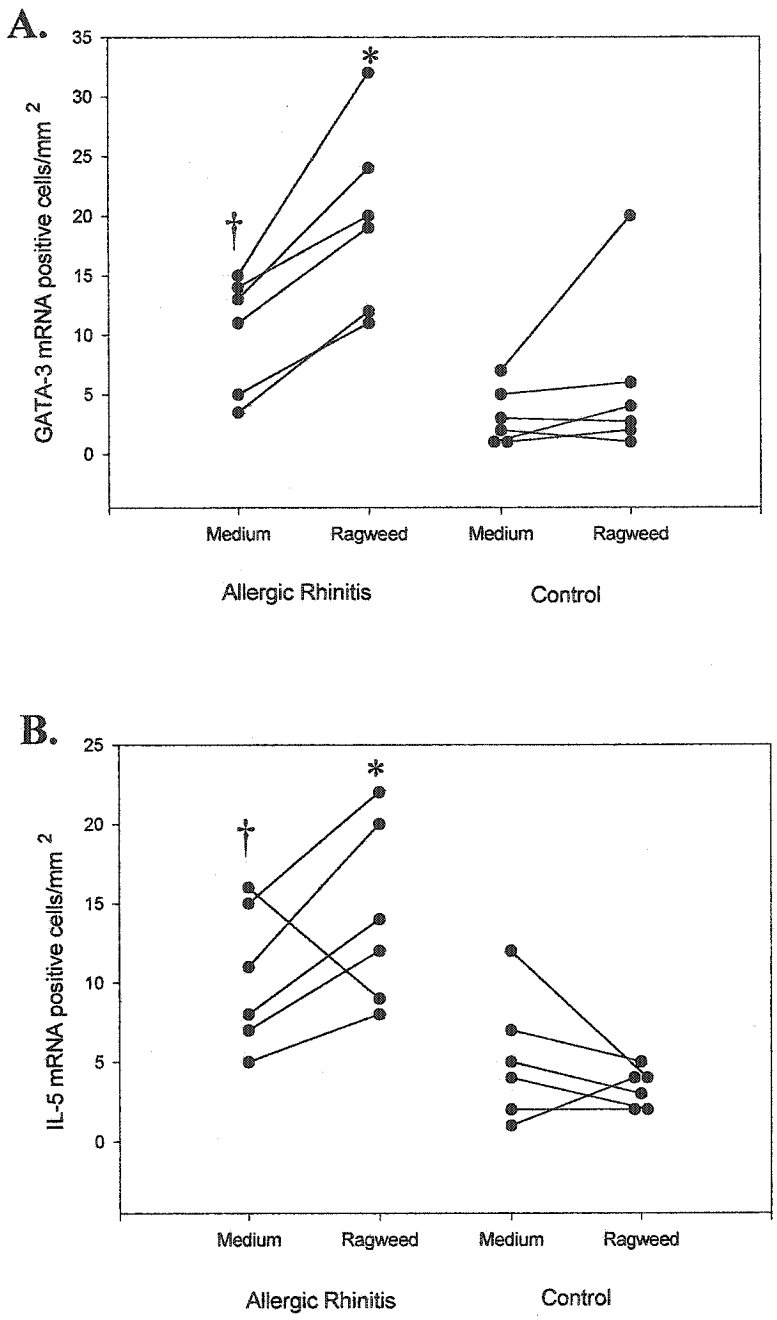


FIG 18. Expression of GATA-3 **(A)** and IL-5 mRNA + cells **(B)** within the nasal mucosa of tissue explants taken from allergic rhinitis and normal control subjects. The numbers of GATA-3 and IL-5 mRNA were evaluated 24 hours after in vitro ragweed challenge or exposure to medium alone. There were increased numbers of cells expressing GATA-3 and IL-5 mRNA after allergen challenge only in nasal explants from patients with allergic rhinitis (*$P<0.05$, *Student paired t test*). There was also a significant difference in the numbers of GATA-3 and IL-5 mRNA + cells between the patients with allergic rhinitis and the normal control subjects after incubation with medium alone (†$P<0.05$, *Student unpaired t test*).

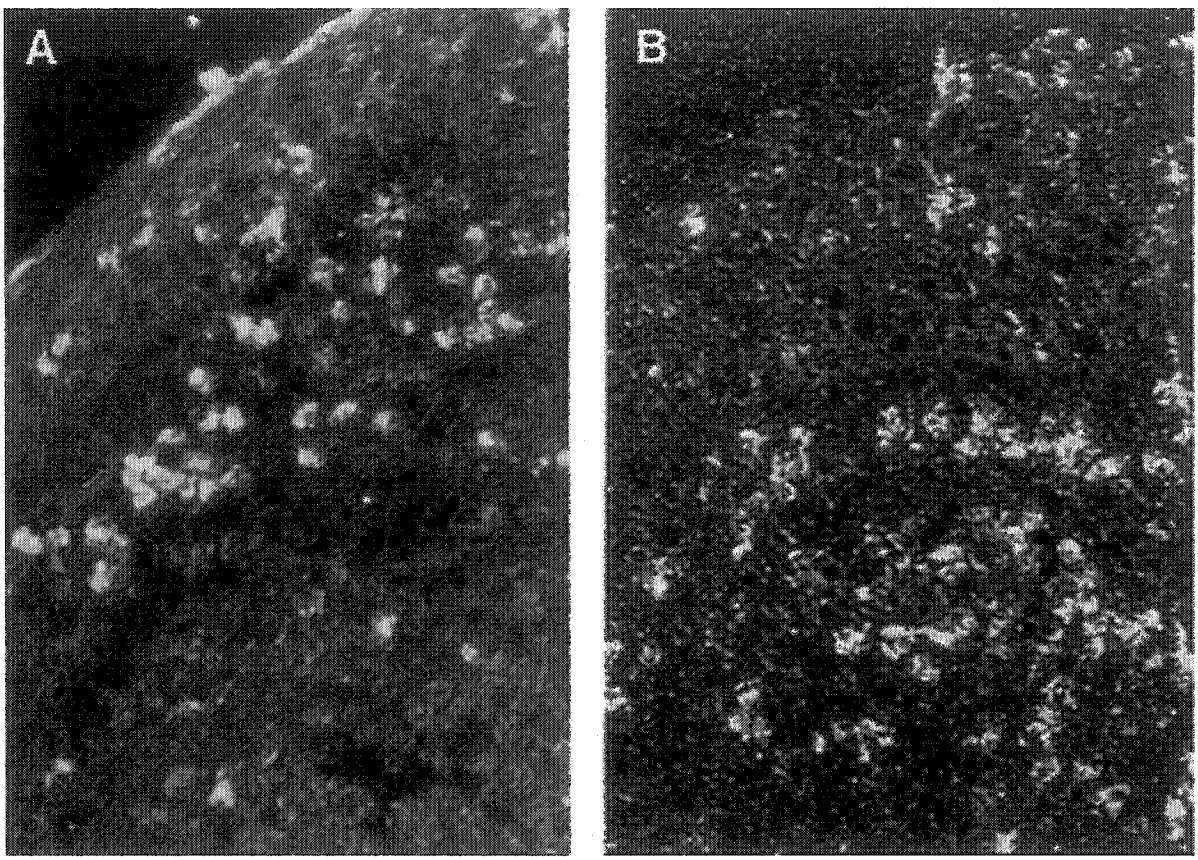


FIG 19. Representative photomicrographs of in situ hybridization under dark field illumination with ^{35}S labeled riboprobes for GATA-3 **(A)**, and IL-5 mRNA **(B)** in tissue explants cultured for 24 hours with ragweed allergen. Note the scattering of illuminated cells throughout the submucosal layer.

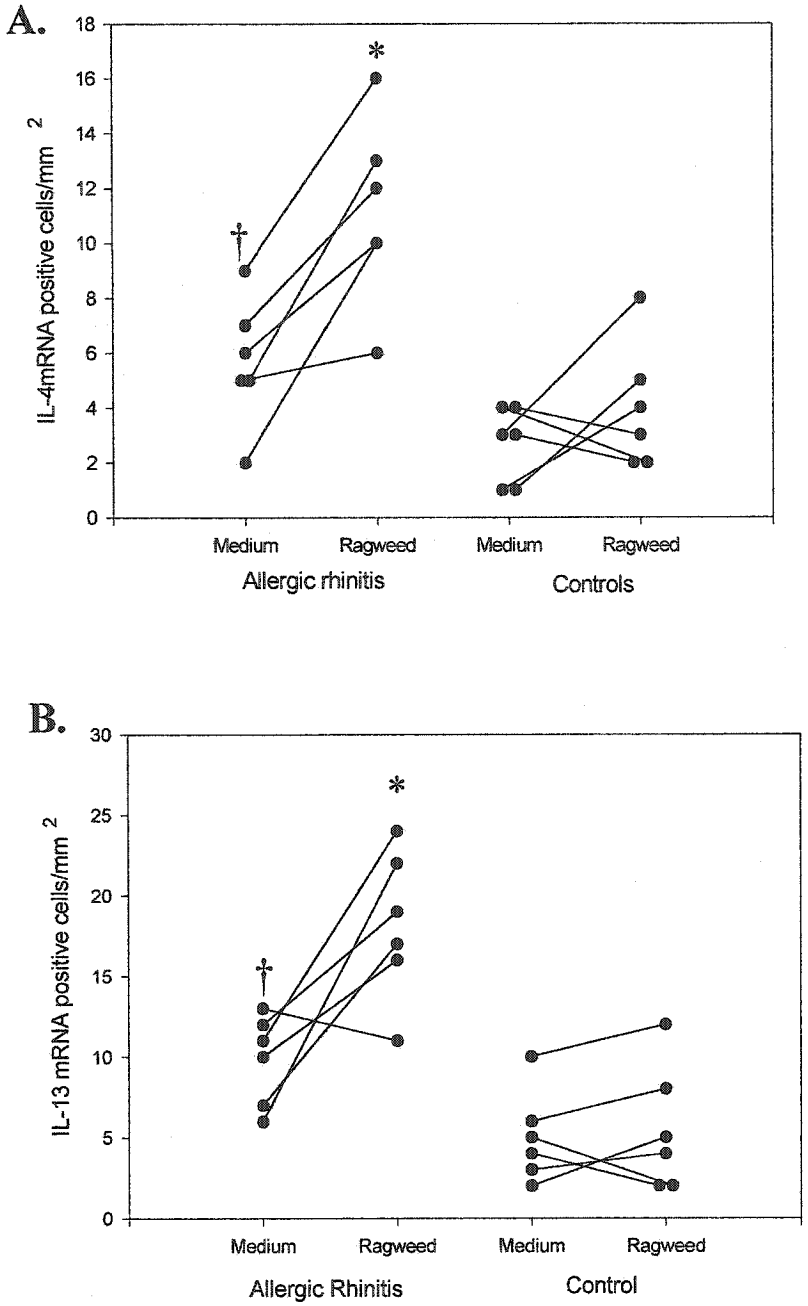


FIG 20. Expression of IL-4 **(A)** and IL-13 **(B)** mRNA + cells within the nasal mucosa of tissue explants taken from patients with allergic rhinitis and normal control subjects. There was an increased number of cells expressing IL-4 and IL-13 mRNA after allergen challenge in nasal explants only from patients with allergic rhinitis (**P<0.05, Student paired t test*). There was also a significant difference in the numbers of these mRNA + cells in the nasal biopsies between patients with allergic rhinitis versus normal control subjects, following culture with medium alone (*†P<0.05, Student unpaired t test*).

3.4 GATA-3, STAT6 and cMaf expression in atopic and non-atopic asthma

Patient demographics are represented in Table 4. Briefly, all asthmatics were mild to moderate in disease severity. Characteristically, the non-atopic group was older, had lower IgE levels, and tended to have better FEV_1 values than atopic asthmatics.

3.4.1 GATA-3 and cMAF expression

The number of cells expressing immunoreactivity for GATA-3 and cMAF were higher in biopsies obtained from atopic (15.6 ± 5.2, 19.11 ± 6.5) as well as non-atopic (12.0 ± 4.6, 14.6 ± 4.9) asthmatics, compared to normal controls (3.6 ± 1.6, 7.1 ± 3.0), and to patients with tuberculosis (1.8 ± 0.5, 2.3 ± 0.8) ($p<0.0001$). Statistical significance was determined using the unpaired student *t* test where values less than .05 were considered statistically significant. The density of cells expressing either GATA-3 or cMAF was not different between the two asthma phenotypes (Fig 21).

3.4.2 STAT-6 expression

There were also higher numbers of cells expressing STAT-6 in atopic (17.5 ± 6.0, $p<0.0001$) and non-atopic (6.2 ± 2.2, $p<0.05$) asthmatics compared to normal controls (2.4 ± 1.2). However, unlike GATA-3 and cMAF, fewer cells expressing STAT-6 were observed in non-atopic compared to atopic asthmatics ($p<0.0001$) (Fig.21C and Fig.22). Statistical significance was determined using the unpaired student *t* test.

Table 4. Patient characteristics

	Control	Nonatopic asthma	Atopic asthma
n	8	7	8
Sex (F/M)	6/2	5/2	4/4
Age (y)*	28.8 ± 7.24	42.9 ± 13.4	29.4 ± 13.2
FEV_1 (L)*	3.34 ± 0.84	2.96 ± 0.83	3.16 ± 1.03
FEV_1 (% predicted)*	97.7 ± 9.35	85.3 ± 19.3	87.9 ± 21.06
PC20 range (μg/ml)	>16	0.11-4.0	0.52-4.0
IgE (IU/ml)*	29 ± 12.3	33.8 ± 24.7	643.75 ± 425.1
Inhaled steroid BDP (μg)*	0	1100 ± 547.7	1125 ± 353.6

* Data expressed as mean ± SD

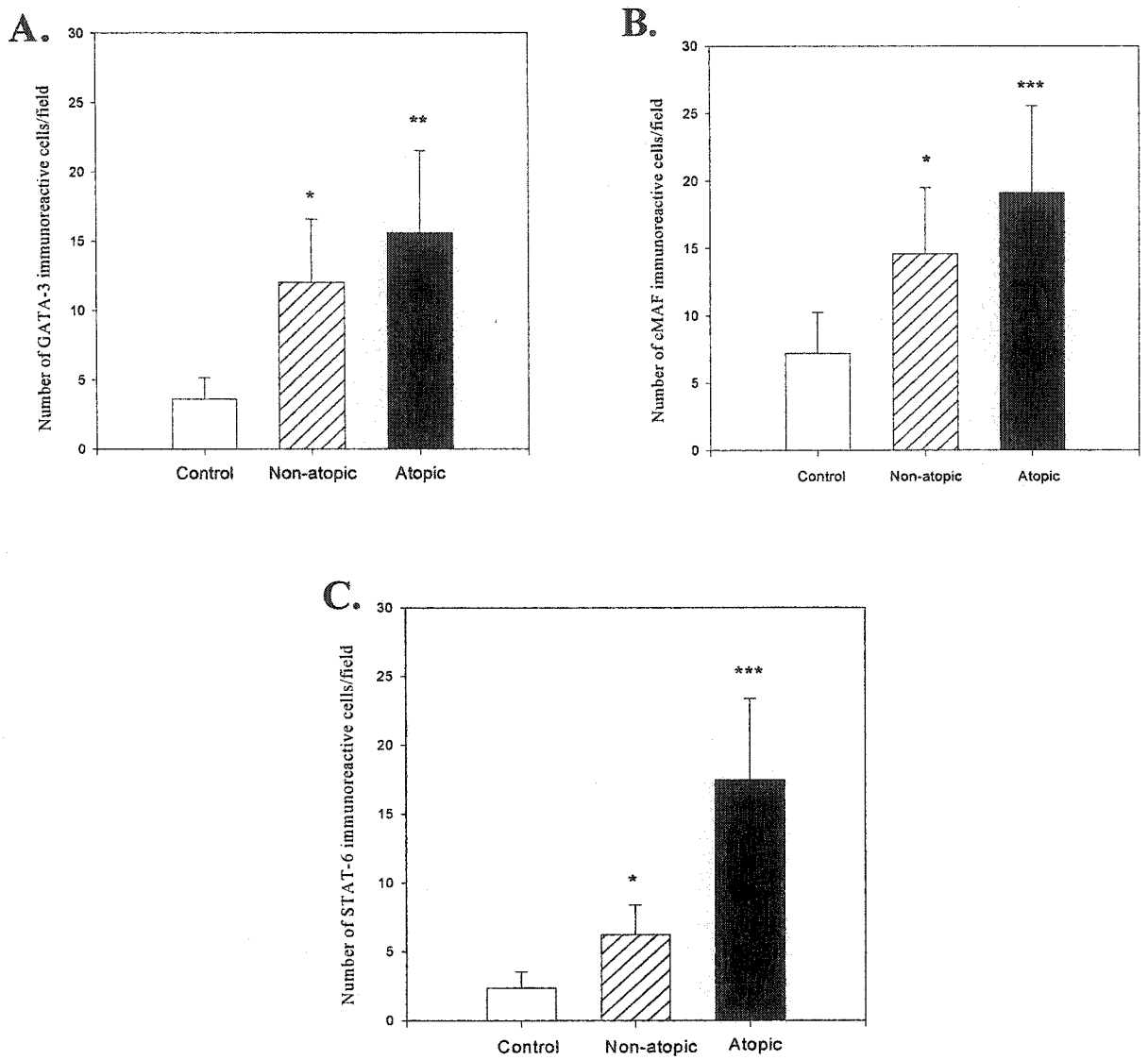


FIG 21. GATA-3, cMAF and STAT6 expression in bronchial biopsies from control subjects (open bars), patients with nonatopic asthma (hatched bars), and patients with atopic asthma (filled bars). There were significantly more cells expressing GATA-3 **(A)** and cMAF **(B)** in both atopic and nonatopic asthmatics compared to controls. The number of STAT6 immunoreactive cells **(C)** was also increased in patients with asthma compared with control subjects; however, there were statistically more STAT6 cells in patients with atopic versus nonatopic asthma. (* *P<0.05*, ** *P<0.001*, *** *P<0.0001*, *Student unpaired t test*)

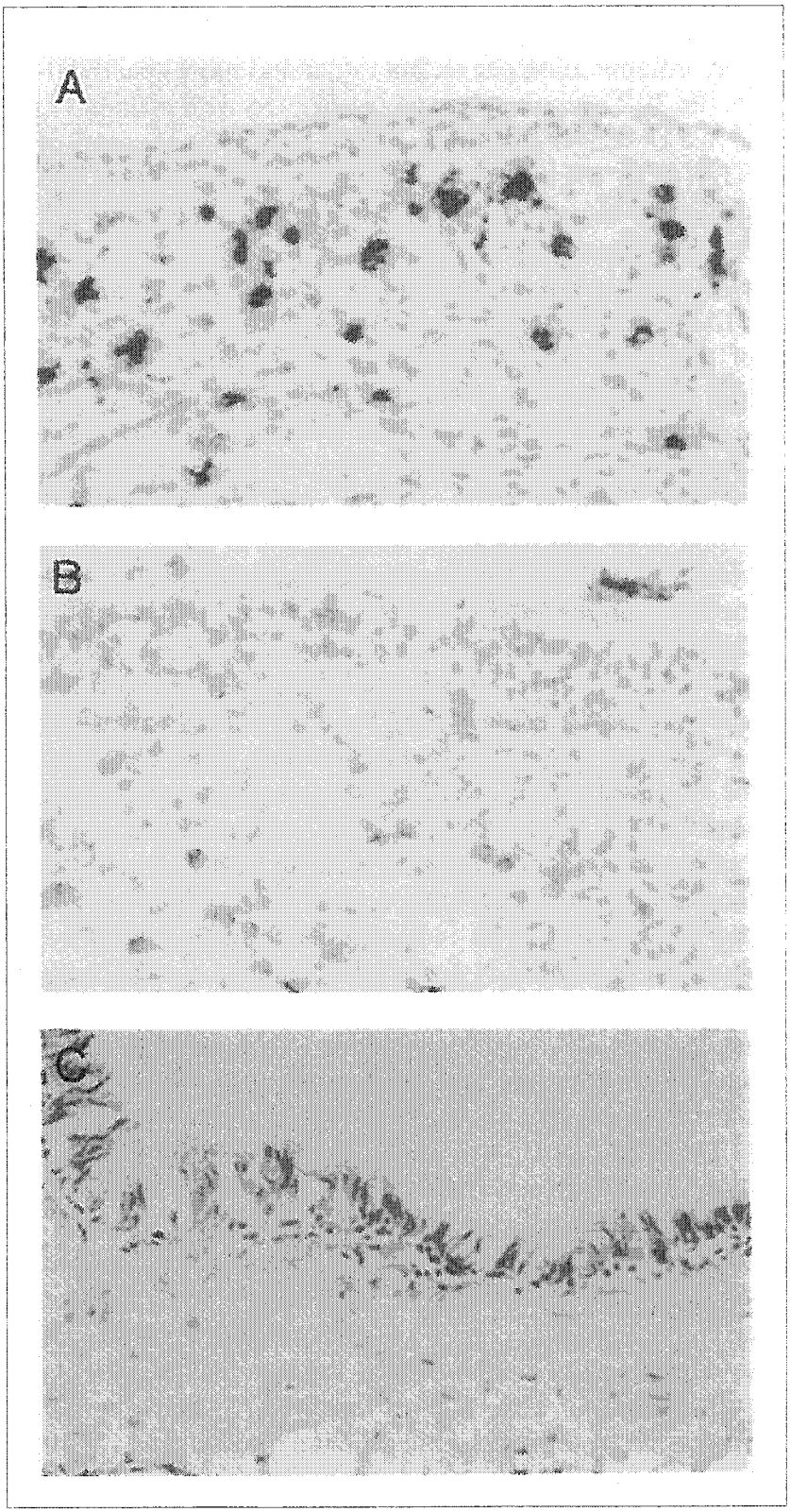


Fig 22. Representative example of STAT6 immunoreactivity in bronchial biopsy specimens from a patient with atopic asthma (A), a patient with nonatopic asthma (B), and a healthy control subject (C).

3.5 YY1 immunoreactivity in tuberculosis

Immunocytochemistry using a YY1 mAb demonstrated a significantly higher number of YY1 positive cells within bronchial biopsies from subjects with tuberculosis (6.08 ± 1.77, $p<0.05$) when compared to that of asthmatics (2.19 ± 1.2) and normal controls (1.37 ± 0.7) (Fig.23). Statistical significance was determined using an unpaired student *t* test. Morphological analysis revealed that the majority of YY1 positive cells were T cells located within subepithelial layers.

3.6 LPS stimulation in the explant system

3.6.1 Response of allergic tissue to LPS stimulation

Although previous work in animal models have used 50μg/ml of LPS to induce an inflammatory response, the conditions for *ex vivo* challenge of nasal mucosal tissue with LPS had not been determined. We therefore performed a dose response with increasing concentrations of LPS (0.001, 0.01, 0.1 and 1μg/ml) to obtain an optimal dose capable of inducing a Th1 immune response in tissue culture. Under basal conditions, in unstimulated tissue, IFN-γ and IL-12 were expressed at detectable levels. There was however a significantly higher expression of IL-12 (9.1± 4.5) (Fig.24A) and IFN-γ protein (8.02 ± 4.5; $p<0.05$) (Fig.24B), following LPS stimulation at a concentration of 0.1μg/ml, compared to that cultured in medium alone (2.75±1.5; 2.5±1.8, respectively). The data was analyzed using analysis of variance (ANOVA) with a Dunnett's correction, to test for significance. Differences were accepted as statistically significant at $p<0.05$.

3.6.2 *Expression of YY1 in LPS stimulated tissue*

YY1 immunoreactivity was detected in the submucosal layer of all tissue sections cultured in all three different conditions; medium alone, ragweed and LPS stimulation, for 24 hours. There was, however, a significantly higher number of YY1 positive cells in nasal tissue cultured with LPS at 0.1μg/ml (7.0± 2.1;$p< 0.05$) when compared to tissue cultured with ragweed (2.2±1.5) or medium alone (1.8±0.5) (Fig.25). Statistical significance was determined using an unpaired student *t* test.

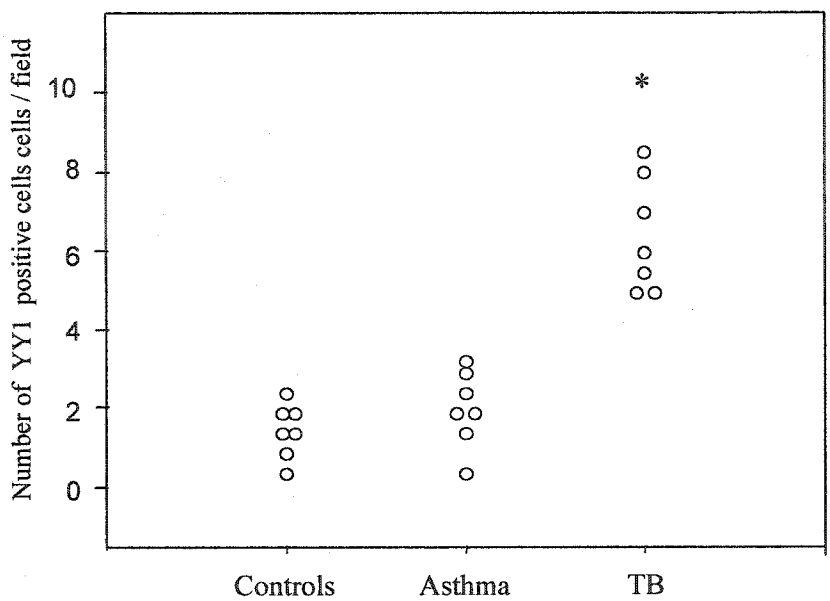


FIG 23. YY1 immunoreactive cells in bronchial biopsies from control, asthmatic, and tuberculosis subjects. There were significantly higher numbers of YY1 immunoreactive cells in bronchial biopsies from subjects with tuberculosis compared to asthmatic and control subjects. *(* $P<0.05$, Student unpaired t test)*

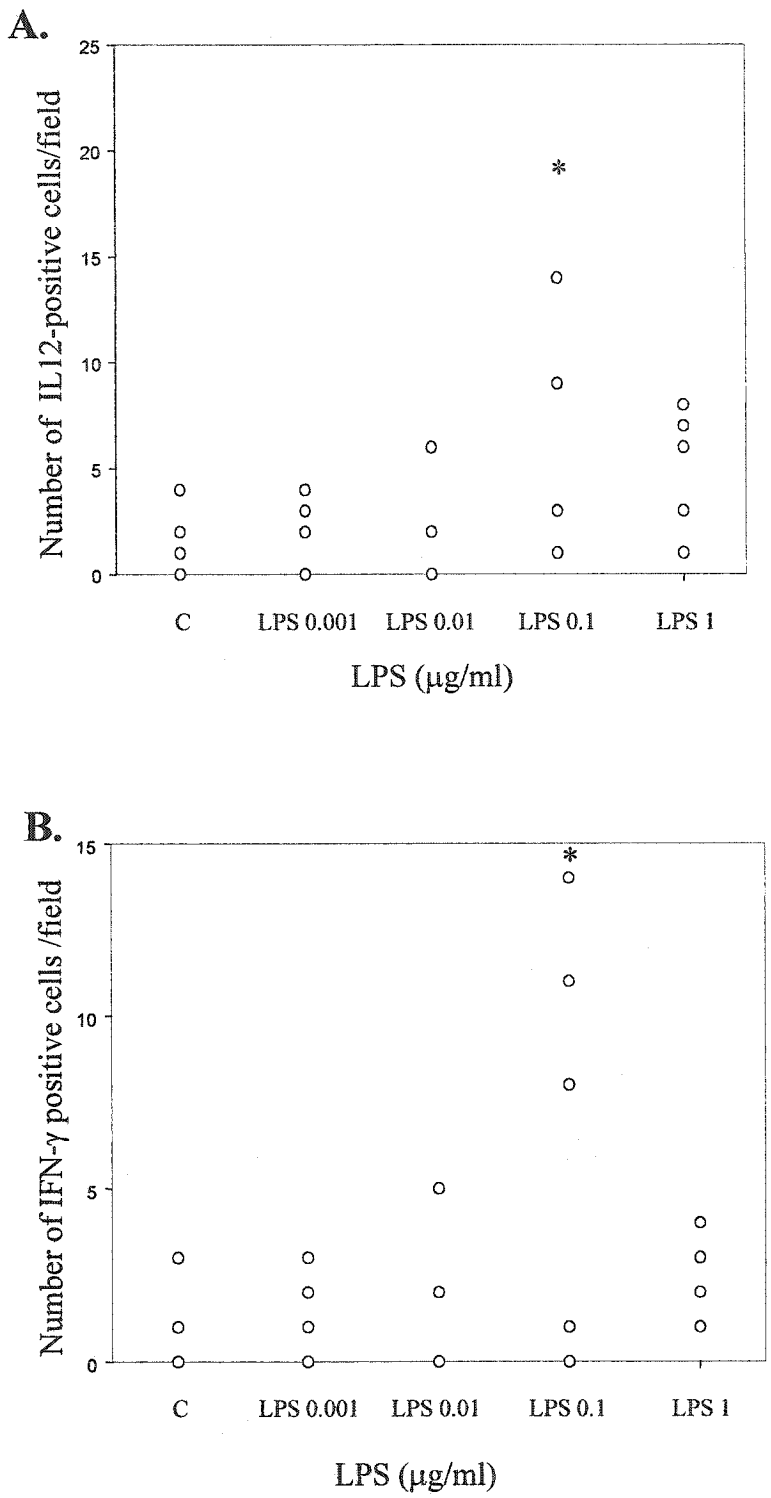


FIG 24. Number of IL-12 (A) and IFN-γ (B) immunoreactive cells in explanted allergic nasal mucosa following LPS stimulation at increasing doses (0.001-1μg/ml). There were significantly higher numbers of IL-12 and IFN-γ immunoreactive cells following LPS stimulation (0.1μg/ml) compared medium alone. (*$P<0.05$, *ANOVA)*

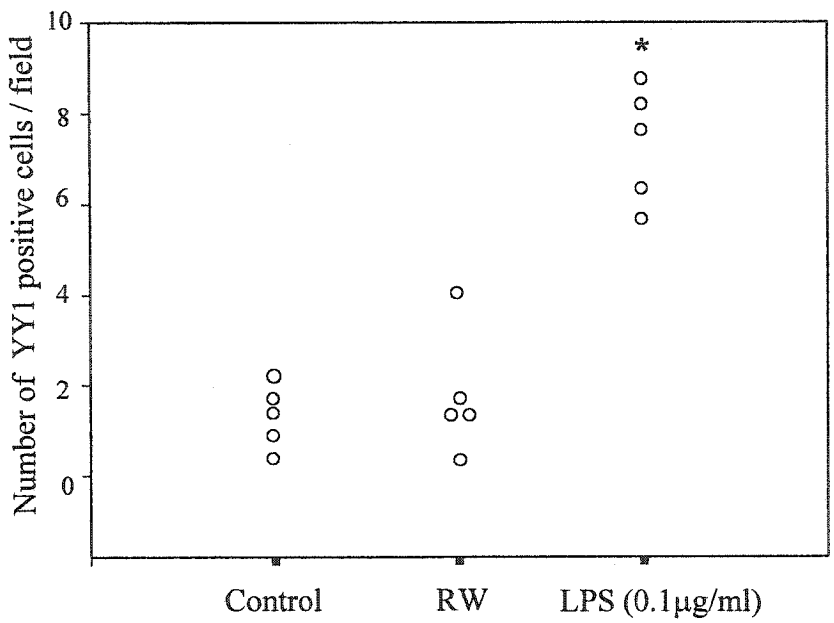


FIG 25. YY1 immunoreactivity in explanted allergic nasal mucosa following LPS stimulation at increasing doses (0.001-1 μg/ml). There were significantly increased numbers of YY1 + cells in the nasal tissue following incubation with LPS (0.1μg/ml) compared to that cultured with medium alone. * *P<0.05, unpaired student t test*

CHAPTER IV: DISCUSSION

4.1 General Overview

Atopy, characterized by raised IgE levels, underlies allergic diseases such as asthma and rhinitis. The interaction of the an allergen with the innate immune system, leads to the stimulation and overexpression of Th2 cytokines, particularly IL-4 and IL-5, which are essentially responsible for orchestrating airway inflammation in allergic disease. As such, Th2 cytokines have become attractive therapeutic targets. Although corticosteroids are the most effective anti-inflammatory drugs currently available for the treatment of allergic disease, they are not specific for Th2 cytokines, and may lead to general immunosuppression. Alternative methods aimed at targeting specifically Th2 cytokines are being developed. To date, human clinical trials using blocking antibodies for IL-4 or IL-5 have not been proven effective at suppressing allergic responses, since a number of cytokines work together to perpetuate this process. Therefore, finding a common factor that controls the expression of all, or several Th2 cytokines, would provide an ideal therapeutic target. Transcription factors have been shown to regulate the expression of multiple inflammatory genes, including cytokines. GATA-3, cMaf, and STAT6 have all recently been implicated in allergic diseases. Most of the data reporting that these transcription factors are responsible for the gene expression of Th2 cytokines however, is derived from *in vitro* studies and animal models. To date, the expression of these transcription factors had not been investigated in human allergic disease. These factors may be potential therapeutic targets in the treatment of allergic disease. Although a number of studies have focused on Th2 cytokines and the factors that regulate their expression, little is

know about Th1 regulating factors. Studies have recently begun to investigate this area, since it has been proposed that immune deviation toward Th1 can protect against allergies. It has been argued that bacterial and viral infections during early life direct the maturing immune system toward Th1 counterbalancing proallergic responses of Th2 cells. Thus, a reduction in the overall microbial burden will result in weak Th1 imprinting, and unrestrained Th2 responses, allowing for an increase in risk of developing allergy. Immunomodulation of the immune system towards Th1 has become a potential approach to dissolve the allergen-induced airway inflammation. Although allergen immunotherapy appears to induce a Th1 cytokine expression (288), (289), and has been proven highly effective in some allergic patients, not all patients respond to this form of treatment. Furthermore, a number of disadvantages and risks are associated with its use. Alternative strategies aimed at shifting the Th2 response towards Th1, are currently being investigated. Since endotoxin (LPS) exposure early on in life has been shown to deviate the immune response towards a Th1 phenotype (256), (161), microbial agents have become potential therapeutic agents. Taken together, these observations set the basis for the work in this thesis.

This thesis provides first time evidence for an increased expression of GATA-3, STAT6 and cMaf transcription factors in human allergic airways. The results shown here were acquired using human asthmatic bronchial biopsies, as well as nasal mucosal biopsies, following *in vivo* and *in vitro* allergen challenge. We also demonstrated that *in vitro*, LPS is capable of inducing a Th1 immune response in nasal mucosa tissue from allergic rhinitics, demonstrated by an increase in IL-12 and IFN-γ immunoreactivity, as well as the YY1 transcription factor.

4.2 Discussion of Results

Despite advances in our understanding of Th2 type cytokines and their critical role in allergic disease, the mechanisms that regulate selective Th2 type cytokine expression *in vivo,* have just recently been characterized in the last few years. There is evidence to suggest that central to the control of gene expression is transcription factors. The GATA-3 transcription factor has recently been shown to be selectively expressed in Th2-type cytokine producing cells. To date, most of the data demonstrating the role of GATA-3 in Th2 cytokine gene expression have been derived *in vitro*, and from animal models.

In this thesis we sought out to determine whether GATA-3 was expressed in human allergic tissue. Using GATA-3 mRNA riboprobes, we demonstrated that the gene expression of GATA-3 is significantly higher in the airways of asthmatic subjects compared to that of controls. Furthermore, the majority of GATA-3 mRNA+ cells were phenotypically identified as T lymphocytes, with lesser contributions from eosinophils and mast cells. Consistent with the *in vitro* activity of GATA-3 as a transactivator of IL-5 gene transcription, a large proportion of cells expressing IL-5 transcripts were found to coexpress GATA-3 mRNA in asthmatic airways. Taken together, the results of this study provide the first evidence implicating a specific transcription factor in the dysregulated IL-5 gene expression *in vivo* in asthmatic airways. In agreement with previous studies of mild asthma, the numbers of T lymphocytes in the bronchial mucosa did not differ significantly between asthmatic and normal subject's (117). Augmented GATA-3 expression therefore does not appear to be a consequence of T-cell recruitment. However, GATA-3 mRNA was

found to be expressed in small numbers of cells in BAL fluid and biopsy specimens from normal control subjects, in parallel with the low basal expression of IL-5 transcripts. Although the majority of GATA-3 was localized to T cells, as well as eosinophils and mast cells, a small number of GATA-3 mRNA-positive signals in asthmatic airways did not colocalize with any of the cells identified by immunocytochemistry. Basophils, which are present in asthmatic airways but lack a reliable cell surface marker, have been shown to express GATA-3 and may therefore comprise some of the unidentified GATA-3 mRNA+ cells (290).

The numbers of cells expressing GATA-3 mRNA correlated significantly with reduced airway caliber and bronchial hyperresponsiveness. The biologic function of GATA-3 *in vitro* (182) indicates that its effect on pulmonary function is likely a consequence of IL-5–mediated upregulation of eosinophil activity. In agreement with prior studies, IL-5 gene expression was found to be significantly increased in asthmatic airways compared with normal airways (106). Numbers of IL-5 mRNA+ cells both in BAL fluid and bronchial biopsy specimens correlated significantly with the numbers of cells expressing GATA-3 mRNA. To more precisely examine the nature of the association of GATA-3 with IL-5, double in situ hybridization was carried out to colocalize transcription factor and cytokine gene transcripts. Seventy-six percent of GATA-3 mRNA+ cells were found to express IL-5 mRNA, and the remainder (GATA-3 mRNA+/IL-5 mRNA– cells, 24%), might be indicative of cells that express GATA-3, but lack other transcription factors that are also necessary to induce IL-5 gene expression. IL-5 promoter activation also requires binding of proteins to the AP-1 site, which is achieved once the cells are activated (182). A

number of factors could be related to the presence of small numbers (<10%) of IL-5 mRNA +/ GATA-3 mRNA– cells. GATA-3 mRNA may be present, albeit at an undetectable copy number, in IL-5 mRNA+/GATA-3 mRNA– cells. This hypothesis is attractive in light of a recent study demonstrating that IL-5 mRNA is highly stable compared with mRNA for other cytokine genes (291), although the relative stability of IL-5 versus GATA-3 mRNA is not known. It is also possible that certain stimuli in asthmatic airways could increase IL-5 mRNA expression in these minority of cells by a mechanism that bypasses the requirement for GATA-3. However, such a mechanism would likely not be dependent on direct stimulation with antigen or agents that induce an increase in intracellular levels of cAMP (182).

The absence of significant levels of GATA-3 mRNA in BAL cells recovered from patients with tuberculosis, representative of a TH1-mediated disease with low expression of IL-5, support the concept that GATA-3 is mainly involved in the regulation of TH2-type cytokines. The results of this part of the study provide the first *in vivo* evidence of increased GATA-3 gene expression in human airways, associated with the increase in IL-5 mRNA+ cells. These findings support a causal association between augmented GATA-3 expression and dysregulated IL-5 expression in asthma.

We then went further to determine whether the expression of GATA-3 would increase in response to specific allergen. Using the model of allergen-induced rhinitis, we examined the mRNA expression of GATA-3 within the nasal mucosa of ragweed-sensitive subjects prior to, and 24 hours following ragweed allergen challenge. Our results demonstrate that allergen challenge of the nasal mucosa produced an increase in the numbers of cells positive for GATA-3 in subjects with allergic rhinitis. The

increase in GATA-3mRNA was paralleled by an increase in Th2 cytokine, IL-4, IL-5, and IL-13 mRNA, suggesting that GATA-3 may be responsible for the increase in TH2 cytokine gene expression. To determine whether the increase in GATA-3 expression was dependent on infiltrating cells, we used the nasal explant technique, which was recently developed, in our laboratory. This explant system provided us with a means to isolate the nasal tissue from systemic circulation and to determine the contribution of cells residing within the tissue to the increase in GATA-3, irrespective of those cells that may infiltrate in response to allergen exposure *in vivo*. *In vitro* allergen challenge of explanted nasal tissue also increased the numbers of cells expressing GATA-3 mRNA and IL-4, IL-5, and IL-13 mRNA + cells, consistent with the *in vivo* data. This finding suggests that there is a local activation of resident cells in the allergic nasal mucosa in response to allergen, and that the increase in GATA-3 following *in vivo* allergen provocation, was a consequence of both the influx of cells, as well as the local induction of GATA-3mRNA. Furthermore, the numbers of cells positive for GATA-3 mRNA correlated to the numbers of IL-5 mRNA+ cells both *in vivo* and *in vitro*, suggesting a coordinate regulation of GATA-3 and IL-5 mRNA within allergic airways.

At baseline, prior to allergen challenge, there were increased numbers of GATA-3, as well as IL-4, IL-5, and IL-13 mRNA+ cells in the nasal mucosa of allergic rhinitics, even in the absence of seasonal allergen exposure or any clinical symptoms. These results agree with previous data suggesting that the presence of an increased number of cells expressing IL-4 and IL-5 (292), (286), (148), (293) within the nasal mucosa in the absence of allergen is not associated with symptoms of

allergic rhinitis but rather the baseline mucosal inflammation in patients with allergic rhinitis. Our results also confirm previous observations in showing an increase in IL-4, IL-5, and IL-13 but not IFN-γ mRNA expression as a result of deliberate allergen provocation both *in vivo* and *in vitro* (148), (110), (269), (77). The increase in IL-4, IL-5, and IL-13, but not IFN-γ, also the efficacy of both the allergen-induced rhinitis model, as well as that of the nasal explant system since similar trends in cytokine dysregulation are observed during natural allergen exposure in patients with seasonal (294) and perennial (295) rhinitis. The presence of Th2-type cytokines within the nasal mucosa after *in vivo* allergen exposure may be attributed either to migration of cells from the surrounding tissues, or the local activation of resident inflammatory cells. The data we obtained using the tissue explant technique suggest that the latter is at least partially responsible for the observed increase in the expression of GATA-3 and Th2-type cytokine mRNA. However, local activation and migration of cytokine-producing cells cannot explain the *in vivo* data in its entirety. We observed fewer IL-5 and IL-13 mRNA+ cells after ragweed challenge in the explant when compared with allergen challenge *in vivo*, which would indicate that some component of the inflammatory response at 24 hours after allergen exposure was attributed to cellular infiltration.

Furthermore, our data show a positive correlation between the numbers of GATA-3 and IL-5 mRNA+ cells which is consistent with our previous findings in asthmatic bronchial biopsies, and previous *in vitro* data in the literature reporting that GATA-3 is responsible for stimulating the production of IL-5. GATA-3 has been shown to be necessary and sufficient for Th2-type gene expression in CD4+ cells

(182). Moreover, its ectopic expression in developing TH1-type cells has been shown to induce the expression of IL-4 and IL-5 (296). Although the number of GATA-3 mRNA positive cells did not correlate with the number of IL-4 and IL-13mRNA positive cells in our study, we cannot rule out the fact that GATA-3 plays a role in IL-4 and IL-13 gene expression, since a number of *in vitro* studies have shown otherwise (182), (185). A possible reason for our findings may be that counting the number of cells expressing mRNA of interest (point counting), as done in this thesis, does not give any information on copy number. As such, whether individual cells were making more cytokine after allergen challenge exposure than before, could not be determined, and may be the explanation for the lack of direct correlation between IL-4 and IL-13, with GATA-3 expression. Interestingly, these data may also suggest that Th2-type cytokines are differentially regulated in allergic rhinitis (297), a notion that is consistent with the ability of different transcription factors to selectively stimulate the expression of one or more of these cytokines.

In summary our results demonstrate a high expression of GATA-3 mRNA in human allergic airways, which is increased in response to allergen, and paralleled by an increase in Th2 cytokine mRNA expression. Although our findings suggest that the increased expression of GATA-3 is closely associated with that of Th2 cytokines, and may be responsible for their gene regulation, based on our results we cannot conclude that GATA-3 directly stimulates Th2 cytokine gene regulation. Future work using the human nasal explant system to investigate the effects of GATA-3 antisense oligonucleotides on Th2 cytokine expression would provide us with supporting evidence for our findings. Although GATA-3 antisense oligos have been shown to

effectively reduce airway inflammation and hyperresponsiveness in a murine model of asthma, the use of GATA-3 antisense oligos in the explant model using human tissue would be a step closer to human clinical clinical trials.

Recently, other transcription factors associated with Th2 gene expression have been identified. STAT6 and cMaf transcription factors have been implicated in Th2 responses and described to be closely associated with IL-4 gene regulation (211), (298). Using bronchial biopsies from human asthmatics and control subjects, we investigated the expression of cMaf and STAT6 *in vivo* for the first time. Our results demonstrate the constitutive expression of STAT6 and cMaf in human airways, which was significantly higher in that of asthmatic subjects. Our findings of a high expression of cMaf and STAT6 in a Th2 mediated disorder, support data in the literature identifying cMaf as a Th2 specific factor, crucial for the gene expression of IL-4 (207), and STAT6 as a factor responsible for the activation of several IL-4 regulated genes (202). The increase in cMaf and STAT6 which we observed, may be responsible for the increase in IL-4mRNA and immunoreactivity within bronchial biopsies of asthmatics, which we, and others have previously demonstrated (107), (117).

To address the issue of whether GATA-3, cMAF and STAT6 transcription factors are differentially expressed according to atopy status, we investigated their expression in both atopic and nonatopic asthmatics. Asthma can be divided clinically into two variants according to atopic status (36), however, the underlying pathological differences between atopic and non-atopic asthma remain elusive. Histologically, biopsy material from these two groups appears highly similar and

immunocytochemical analyses reveal that the inflammatory cell infiltrate does not seem to differ. There is evidence indicating that CD4+ T lymphocytes and eosinophils accumulate in the airways of non-atopic asthmatics, in a manner analogous to atopic asthmatics (299). While there are varying reports on cytokine expression patterns, at least one group has observed increased levels of IL-4 in non-atopic asthma and that this expression fails to correlate with pulmonary function (300). Furthermore, significantly fewer IL-4R+ cells in non-atopic compared to atopic asthma has recently been observed (301). This suggests that the IL-4 signaling pathway may be impaired in non-atopic asthma. As such, in this thesis we examined the expression of transcription factors GATA-3, cMAF and STAT-6, implicated in Th2 cytokine expression and function, and aimed to determine if their level of expression is dependent upon atopic status of human asthmatic patients. Our findings demonstrate that all three transcription factors, GATA-3, cMaf and STAT6 are constitutively expressed in normal subjects and are upregulated in atopic as well as non-atopic asthma. Interestingly, we also demonstrate that the density of cells expressing STAT-6, contrary to GATA-3 and cMAF, is significantly lower in non-atopic compared to atopic asthma. We reported above, that compared to normal controls, there was an increase in GATA-3 expression within the bronchial mucosa of asthmatics. Here, we indicate that regulation of GATA-3 is not dependent on atopy, since it is similarly increased in non-atopic asthmatics as well.

This study is the first to investigate cMAF in humans where we demonstrate comparable expression within the bronchial mucosa of both atopic and non-atopic asthmatics, at levels significantly higher than controls. GATA-3 and cMAF mediate

activity of both the IL-4 and IL-5 promoters (207), (177). We have not investigated IL-4 or IL-5 levels in these patients, however, the fact that GATA-3 and cMAF expression is in non-atopic and atopic asthma may suggest that they are elevated. Our observation of the absence of significant levels of GATA-3, cMAF, and STAT6 in tissue from patients with tuberculosis, a Th1 mediated disease, supports the concept that these transcription factors are mainly involved in the regulation of Th2 type cytokines. In addition, this suggests that the increased expression of these transcription factors in asthmatic airways is not the consequence of airway inflammation per se, but rather and more specifically, the Th2 immune response associated with asthma.

We observed constitutive and elevated expression of STAT-6 in bronchial biopsies of normal controls and asthmatics, respectively, which agrees with our previous report of STAT-6 expression in the nasal mucosa of control subjects and patients with allergic rhinitics (297). Interestingly however, we observed fewer cells expressing STAT6, in patients with non-atopic asthma, compared to that in atopic asthma. This may be explained by the well known fact that the most striking difference between atopic and non-atopic asthmatics is the lack of demonstrable specific IgE to an allergen in the latter group, with normal or only slightly elevated serum IgE levels (299). Our finding of significantly fewer cells expressing STAT-6 in non-atopic asthma corresponds with the low levels of IgE observed in these patients. In fact, STAT-6 has been shown to play a critical role in regulating allergen-specific IgE production (203), (191), and disruption of the STAT-6 gene results in a loss of IgE (201), (202). Although the numbers of cells expressing STAT-6 expressing cells

were higher in atopic than non-atopic asthma, there were significantly more STAT6 positive cells in the non-atopic asthmatic group than in controls. These findings parallel that of IL-4Rα expression in non-atopic asthmatics and in normal controls (301). This difference could be ascribed merely to a 'more active' response by atopic asthmatics; alternatively, real genetic differences may be involved. Mitsuyasu et al showed that amino acid substitution of Ile for Val in the IL-4R augments STAT6 activation and transcriptional activity of the Iε promoter by IL-4 (302). Rosa-Rosa et al. demonstrate that homozygosity for an IL-4R variant was significantly increased in individuals with atopic asthma and suggested it as an indicator of atopy susceptibility (303). From these studies, it could be postulated that atopic asthmatics may express a more highly active variant of the IL-4R, possibly resulting in a *super*-increase in STAT-6 expression and subsequently in IL-4/STAT-6 responsive genes such as IgE and the IL-4Rα (304), (305).

The ability of IL-4 to inhibit the production of INF-γ, and subsequent Th1 cell differentiation has been shown to involve STAT-6. Goenka et al. demonstrated that STAT-6 binds and inhibits the IFN-γ regulatory factor-1 (IRF-1) site in the IFN-γ promoter (306), whereas Kubo et al observed that in Th2 but not Th1 cells, STAT-6 inactivates a silencer of IL-4 gene activity (307). This would suggest that in non-atopic asthmatics with lower STAT-6 levels, the expression of IFN-γ may be elevated. Indeed, one report indicates that IFN-γ levels are higher in non-atopic than in atopic asthma (307). IFN-γ is a potent activator of eosinophils and induces their release of mediators such as RANTES (308). Furthermore, antibodies against this cytokine are reported to completely abolish airway hyperresponsiveness in allergen

challenged mice (309). As such, it is conceivable that IFN- γ may also be involved in the development of asthma symptoms in non-atopic asthmatics with lowered STAT-6 levels.

In summary, this study is the first to characterize and compare the expression of the Th2 cytokine-associated transcription factors in atopic and non-atopic asthma. GATA-3 and cMAF are upregulated in asthma, regardless of atopic status. While STAT-6 is also increased in both asthma subtypes, it was substantially lower in non-atopic compared to atopic asthma. This work supports the previously ventured notion that the IL-5 pathway, mediated by GATA-3, coincides with asthma, while IL-4 and STAT-6 are more tightly associated with atopy. Furthermore, these findings indicate that a fundamental difference between these two phenotypes of asthma may be the degree of IL-4R signaling, attributed to varying expression of STAT-6, and that the heterogeneity between atopic and non-atopic asthma might be manifested at the level of local transcription factors.

Based on the results of the first part of this thesis, we can conclude that GATA-3 is a critical transcription factor potentially responsible for the regulation of all Th2 cytokines within the airways, both upper and lower, of human allergic patients. Furthermore, cMaf and STAT6 are also upregulated in human allergic airways, however these transcription factors appear to be more closely associated with IL-4 gene expression. Since it appears that blocking individual cytokines, is not sufficient to inhibit the allergic response, transcription factors controlling the expression of all TH2 cytokines, particularly GATA-3, may provide ideal therapeutic targets for the control of allergic disease.

In the second half of this thesis we geared away from the study of Th2 cytokine regulation, and we focused more on Th1 responses and the associated regulating factors. Although much is known about Th2 cytokines and the factors regulating their gene expression, Th1 cytokine gene regulation has not been well studied. The idea that allergic disorders arise from an imbalance between Th1 and Th2 cytokines in the immune system has recently focused attention on Th1 cytokines. The 'Th2 hypothesis' for asthma and allergic rhinitis describes that these conditions are a consequence of an alteration in cytokine milieu with excess in Th2 cytokines, in concert with a decrease in Th1 cytokines, a phenomenon predicted to drive the allergic phenotype (111). Based on this, it is thought that an alternative method to reduce Th2 cell generation in the respiratory tract is to increase levels of IL-12 and IFN-γ in the environment, thus shifting the Th1/Th2 balance toward Th1. Since Th1 cells and cytokines have been shown to antagonize Th2 cell functions, it is thought that immune deviation toward Th1 can protect against allergic disease.

It has recently been proposed that bacterial endotoxin drives the response of the immune system in a Th1 direction, away from its tendency to develop atopic immune responses (22). According to the hygiene hypothesis, reduced exposure to bacteria or their products during early infancy is a key factor in programming the immune response toward an allergic phenotype (310), (311). The results of recent epidemiological studies strongly support the validity of this hypothesis, where the prevalence of atopy is lower in children living in farms who are in direct contact with livestock and poultry (312), (313). This observation has been explained on the basis of a higher exposure of farm children to products of bacteria, such as LPS. In fact, *in*

vitro, LPS has been shown to stimulate the production of IL-12 by antigen presenting cells, thereby providing a negative signal for Th2 polarization by enhanced IFN-γ production (314). The immunological explanation for the hygiene hypothesis has been very influential in directing therapeutic strategies for the control of Th2 mediated disease, involving the use of infectious agents that promote Th1 development, and IFN-γ production. As such, studying Th1 cytokines and in particular the factors that regulate their expression may provide us with further insight and alternative therapeutic strategies for allergic disease.

To date, the few Th1 associated transcription factors, which have been identified, such as NFAT, BCL6, and STAT4 are not specific for Th1 cytokines. Recently, YY1 was identified and described to be involved in the gene regulation of IFN-γ (224). To date, the minimal data available for YY1 in the literature, is mainly derived from *in vitro* studies. In this thesis, we provide first time evidence for the expression of YY1 in human tissue. Our results demonstrate YY1 immunoreactivity within human bronchial biopsies, that was significantly higher from patients with tuberculosis, compared to that of asthmatic and control subjects. We have previously reported that tuberculosis is a Th1 mediated disorder characterized by an increased expression of IL-12 and IFN-γ, and by the infiltration of T lymphocytes and macrophages (155). For this reason, we chose tuberculosis as our model of a human Th1 immune response. Our observation of an increased expression of YY1 in tuberculosis suggests that YY1 may be a Th1 associated transcription factor (155), (315). Although the precise role of YY1 in Th1 responses is not yet clear, i*n vitro* studies have demonstrated that this transcription factor plays a positive role in the

initiating IFN-γ gene expression murine splenocytes (224), and in the negative regulation of hIL-5 gene expression in human T cells (316), (221). As such, the high expression of IFN-γ, and low IL-5 expression in pulmonary tuberculosis, as we've previously reported (155), may be explained in part by the increase in YY1. The source of YY1 was not determined by colocalization studies, however morphological analysis the bronchial tissue reveals that T cells are mainly expressing YY1.

Since it is thought that exposure to bacterial endotoxins can shift the Th2 immune response to Th1 we investigated whether LPS could have this effect on human allergic nasal tissue. In this thesis, we therefore sought to determine whether LPS exposure in explanted allergic nasal tissue induces a change in cytokine production, and whether this would result in a shift in the Th1/Th2 balance as indicated by the production of Th1 cytokines. We demonstrated that following incubation with increasing doses of LPS, allergic nasal tissue expressed higher numbers of IFN-γ and IL-12 positive cells compared to that seen following incubation in medium alone. The majority of cells expressing IFN-γ appear to be T cells, whereas macrophages expressed IL-12.

In animal models, it has been shown that a single *in vivo* challenge of LPS prior to sensitization prevents their development of IgE and as such the development of an allergic response to inhaled allergen (256). Our results demonstrate that in the absence of cellular recruitment, LPS stimulates Th1 cytokine immunoreactivity. This increased expression of IL-12 and IFN-γ within allergic nasal tissue provides evidence that LPS may be effective in shifting the Th2 response towards Th1. Furthermore, we showed that LPS induced the expression of YY1 transcription factor

in allergic nasal tissue, which may responsible for the upregulation of IFN-γ which we previously observed. Our findings suggest that LPS may have the potential to induce a Th1 response in allergic tissue through the induction of Th1 transcription factors. However, as to whether or not LPS can suppress Th2 cytokine expression has not been investigated. Evidence for this could be provided in future experiments investigating the expression of IL-4 or IL-5 cytokines within explanted allergic nasal tissue following LPS incubation.

The immune response to endotoxin varies according to dosage, suggesting that the dose of exposure is a critical factor for the effects of endotoxin. Low level LPS exposure has been shown to preferentially prime macrophages to release cytokines such as TNF-α and IL-12, whereas greater LPS exposure was shown to prime toxic radical production (317), (318). These observations support our findings where a 24 hour incubation of human nasal tissue with a high doses of LPS (1 μg/ml), had toxic effects, demonstrated by a loss of tissue integrity and morphology. Interestingly, there is convincing evidence that high levels of endotoxin exacerbate airway inflammation in allergic asthmatics. In fact, it has been shown that in asthmatics, the concentration of endotoxin in the home environment, was significantly associated with the severity of asthma (319), and that at high levels LPS increased the severity of the disease (320). Therefore Th1 promoting immune responses may occur with lower endotoxin doses, avoiding the inflammatory or toxic immune responses seen at higher doses.

Another critical factor for the effect of endotoxin on the immune system, is the timing of exposure. Emerging evidence suggests that it is exposure to endotoxin

early on in life that may minimize the risk of allergen sensitization, which could have profound effects on the development of allergic asthma. (321). In a rat model of asthma, early exposure to LPS inhibits allergic sensitization, whereas LPS administration in already sensitized animals exacerbated allergic inflammation (256). Although most studies have emphasized the importance of early life in the programming of the appropriate T cell response and memory, alterations in T cell responses may occur even in adulthood. Studies have shown that the reduction of exposure to infections even in adulthood could induce atopic responses in susceptible individuals (322). This is consistent with our findings in which LPS induced a Th1 immune response in allergic nasal tissue from adult subjects with a mature Th2 phenotype.

Although the findings of this study are simply suggestive, and do not provide direct evidence for a protective effect of LPS against allergies, they are additional proof that the hygiene hypothesis may have validity. Furthermore, the use of the explant technique to test the effects of LPS on human tissue holds the potential for the evaluation of pharmacological agents, including innocuous infectious agents such as LPS, as it provides an intermediate system between *in vivo* animal studies and human clinical trials. The advantage of the explant technique is that it avoids the difficulty of re-creating the *in vivo*-like environment, as factors that are potentially important to cell function such as co-stimulatory molecules, and cell-cell interactions remain intact, mimicking physiologic conditions.

Taken together, the results of the second part of this thesis demonstrating that YY1 is increased in bronchial biopsies from patients with tuberculosis, as well as in a

Th1 milieu induced by LPS, confirm that YY1 is a Th1 associated transcription factor, and that it may be contributing to the increased expression of IFN-γ and IL-12, observed in response to LPS. These results suggest that LPS may have protective effects against allergy by shifting the Th2 response towards Th1.

Rigorous studies are still needed to clarify these findings, and to support this idea. Certainly, the associations between endotoxin exposure, and a lower prevalence of allergy do not yet demonstrate a cause-and-effect relationship, and it is currently unclear whether endotoxin is actually responsible for this allergic protective effect. The identification of biomarkers of response to endotoxin exposure, are needed, as well as genetic polymorphisms and mutations that effect endotoxin sensitivity, in order to strengthen endotoxin's link to allergic disease. With microbe-derived immune modulatory therapies already in clinical trials, investigators seeking to optimize therapeutic efficacy and safety with these products can expect to find some guidance with endotoxin.

4.3 Therapeutic Implications

The findings in this thesis suggest that transcription factors, particularly GATA-3, are responsible for the redundant expression of Th2 cytokines observed within human allergic airways. These data also suggest the potential therapeutic utility of agents that can block these factors, as a novel molecular approach for the treatment of allergic disease. Targeting Th2 specific factors such as GATA-3, by using pharmacological or antisense approaches, may inhibit Th2 responses while sparing Th1 cells, avoiding general immunosuppression, as seen with the use of

corticosteroids. Furthermore, this approach has the potential advantage of blocking the production of several Th2 cytokines simultaneously, rather than suppressing the activity of a single cytokine, which has not been proven clinically effective. Treatment with these agents however, should not attempt to totally eliminate the Th2 cytokine response, but rather should try to shift the Th1/Th2 cytokine balance back toward a normal range. As such, consideration should be given to the use of local, rather than systemic drug delivery in both asthma and allergic rhinitis.

The recent focus that microbial agents may provide protective effects against allergies by the induction of Th1 immune responses has led to alternative approaches in switching off Th2 inflammatory responses. Epidemiological evidence, as well as data from *in vitro* and animal studies, has been very influential in directing therapeutic strategies aimed at restoring the Th1/Th2 cytokine balance through the use of infectious agents. The results in this thesis demonstrating that LPS exposure is able to induce the expression of YY1, as well as that of Th1 cytokines, suggests that, with further investigation, endotoxin exposure at low doses may offer a novel strategy for the management of allergies. This form of treatment may avoid global and nonspecific suppression of allergic and Th2 type inflammatory mechanisms, that may be of benefit to the atopic individual when faced with infections. Although Th2 cytokines have harmful effects in terms of allergy, the Th2 cytokine response is required both for host protection, and for suppression of Th1 cytokine-induced immunopathology (323). Again, to avoid any toxic or harmful effects, LPS would be more suitable as local, rather than systemic form of treatment. In support of this would be our findings of a local response to LPS within explanted nasal tissue.

4.4 Future Directions

In order to further support the findings in this thesis, and to answer several questions raised here within, future studies investigating the role of transcription factors in allergic disease need to be conducted. The use of antisense oligonucleotides would provide a more selective experimental approach in determining a functional role of transcription factors in regulating cytokine gene expression. Although antisense-induced local blockade of GATA-3 expression has been investigated in mice, its effects have not been examined in human tissue. Using the nasal explant technique, the actions of GATA-3, STAT6, and cMaf on cytokine gene regulation by the use of antisense treatment could be carefully examined *in vitro*. This would provide useful information that could be extended into the clinic as a novel molecular approach for the treatment of allergic disease. Antisense technology provides unique specificity, and if given locally, in small doses, can enhance safety.

The evidence presented in this thesis suggesting that endotoxins can deviate the dominant Th2 type towards a protective Th1 type profile, supports the idea that a relative lack of infections may be a predisposing factor to the development of atopy. This provides further insight into alternative therapeutic strategies, such as the concept of immunomodulatory vaccination, consisting of inoculating bacterial products which can induce a protective Th1 response preventing allergen sensitization, or may potentially restore the Th1/Th2 balance. Evidence of this awaits human studies, which are currently in progress.

4.5 Conclusion

In this thesis, several important questions have been addressed regarding transcription factors and their role in human disease. The increased understanding, as well as the identification of these transcription factors within human airways, provide new insights into the pathophysiology of allergic diseases. At the same time, it has opened an opportunity for the development of new anti-allergy treatments. With further investigation, targeting Th2 specific transcription factors such as GATA-3, STAT6, or cMAF, by local pharmacological or antisense approaches in human allergic airways, hold potential as a form of treatment in management of allergic diseases. This thesis has also provided us with important insight on the effects of endotoxin exposure on human allergic tissue. Based on our findings, LPS exposure at a low dose may serve as a potential therapeutic agent for the control of allergic disease, through its ability to inhibit or to shift the differentiation of allergen-specific Th2 responses. What needs to be kept in mind however, is that a balance of Th1 and Th2 cytokines in the immune system is required for a normal healthy state. As indicated throughout this thesis, many different therapeutic approaches are possible for the treatment and control of allergic symptoms. The chances of developing a cure for atopy is remote, but strategies to inhibit the development of sensitization in early childhood offer such a prospect in the future.

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