Université de Montréal

Inflammation dans les bronches du modèle d'asthme chez le rat Brown Norway

Inflammation in the Airways of the Brown Norway Rat Model of Asthma

par

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Table of Contents

Index of Figures

Appendix of Abbreviations

AHR : Airway Hyperresponsiveness

Alum : Aluminum

AM : Airway Macrophages

BAL : Bronchoalveolar Lavage

BCG : Bacillus Calmette-Guerin

BSA : Bovine Serum Albumin

CCR : Cysteine-Cysteine Chemokine Receptor

CFC : Chlorofluorocarbons

CFU-Eo : Colony forming units of eosinophils

CyA : Cyclosporin A

Cys-LT_1R: Cysteinyl-leukotriene receptor 1

DC : Dendritic cells

DEP : Diesel Exhaust Particle

DNA : Deoxyribonucleic Acid

ECP : Eosinophil Cationic Protein

$EC_{200}R_L$: Concentration of Agonist causing 200% increase in Lung Resistance

EDN: Eosinophil-derived neurotoxin

ELISA : Enzyme-Linked Immunosorbent Assay

ER : Early Response

EPO : Eosinophil peroxidase

EU : Endotoxin Units

FEV_1 : Forced Expiratory Volume per second

GM-CSF : Granulocyte/Macrophage Colony Stimulating Factor

Hz: Hertz

IgE : Immunoglobulin E

IgG_{2a}: Immunoglobulin G2a

IgM: Immunoglobulin M

ICS: Inhaled Corticosteroids

ICAM-1: Intercellular Adhesion Molecule-1

IFN-γ : Interferon-gamma

IFN-γR: Interferon-gamma receptor

IP10: Interferon-gamma inducible protein

IL-2 : Interleukin-2

IL-3: Interleukin-3

IL-4 : Interleukin-4

IL-4R: Interleukin-4 receptor

IL-5 : Interleukin-5

IL-8: Interleukin-8

IL-13 : Interleukin-13

kD: kilodalton

LAL: Limulus Amebocyte Lysate

LR : Late Airway Response

LT : Leukotrienes

LTC_4: Leukotriene C4

LTD_4: Leukotriene D4

LTE_4: Leukotriene E4

LPS: Lipopolysaccharides

MBP : Major Basic Protein

MCP-4: Major cationic protein-4

MHC : Major Histocomptability Complex

MMP-9: Matrix Metalloproteinase-9

MDI: Metered dose inhaler

Mch: Methacholine

M-MLV: Moloney Murine Leukemia Virus

MDC: Monocyte derived chemokine

MK-0476 : Montelukast

MUC-5: Mucin

NK: Natural Killer

NO: Nitric oxide

ODN: Oligodeoxynucleotide

OCS: Oral Corticosteroids

OA : Ovalbumin

PenH : Enhanced pause

PBMNC: Peripheral blood mononuclear cells

PBS: Phosphate-buffered saline

PAF: Platelet-activating factor

Ptp: Transpulmonary pressure

PGE_2: Prostaglandin 2

RS: Radioactive signal

R_L: Lung Resistance

SQ-PCR: Semi-quantitative polymerase chain reaction

STAT: Signal transducers and activators of transcription

SRS-A: Slow reacting substance of anaphylaxis

SD: Sprague-Dawley

Th0: Type 0 T lymphocyte

Th1 : Type 1 T Lymphocyte

Th2 : Type 2 T Lymphocyte

Th3: Type 3 T lymphocyte

Thp: Precursor T helper lymphocyte

TRFK-5 : Anti-IL-5 Monoclonal Antibody

TXA_2: Thromboxane 2

TGF-β: Transforming growth factor beta

TBS: Tris-Borate Solution

TNF-a: Tumor Necrosis Factor Alpha

UK: United Kingdom

µl: microlitre

VCAM-1 : Vascular cell adhesion molecule 1

VLA-4: Vascular leukocyte antigen-4

Summary

Asthma is the most commonly reported respiratory disease in clinical practice, affecting both children and adults (1). This disease is characterized by reversible airflow obstruction, increased bronchial responsiveness, and airway inflammation (2). It has been shown that persistent inflammation of the airways can lead to chronic and possibly irreversible changes that affect the airway physiological response to different stimuli (3). Over the last twenty years, cysteinyl leukotrienes (cys-LT) have been shown to be potent bronchoconstrictor agents and important inflammatory mediators in the pathophysiology of both acute and chronic asthma (1). Leukotrienes (LT) can induce smooth muscle contraction, increase vascular permeability, stimulate mucus secretion and recruit eosinophils into the lungs (4). A number of activated inflammatory cells (i.e. mast cells and eosinophils) can release cys-LTs (4) and it has been shown that cys-LTs can collaborate with cytokines, namely Interleukin (IL)-5, in the recruitment of eosinophils into the asthmatic airways (5), suggesting an autocrine cys-LT pathway mediating the asthma phenotype. IL-5, alone, is a very important cytokine in asthma. IL-5 is increased in the lungs of allergic and nonallergic asthmatics (6). Intra-tracheal administration of IL-5 to human asthmatics or to sensitized animals with the characteristics of atopic asthmatics increases the airway response (7,8).

It is clear that enhanced cell-mediated immunity is an important characteristic of asthmatics. This immunity is centralized around the activity of activated T lymphocytes. Activated lymphocytes are present and increased in the airways of patients with asthma (9). The first part of my project studied the relationship between LTs and cell-mediated immunity. By pre-treating sensitized BN rats with interleukin (IL)-2, a T cell growth factor, these animals will have upregulated cellular immunity with increased inflammatory cells in the lung lavage and increased airway response to antigen (10), but bile LT production after antigen challenge in these animals has been previously shown to be comparable to controls (11). My hypothesis is that upregulated cellular immunity creates a state of heightened sensitivity to leukotrienes, which can be measured by assessing the response of the airways to leukotriene D_4. As well, I hypothesize that blocking the cys-LT_1 receptor with Montelukast will alter the effects of IL-2 on the late airway response and possibly affect cytokine production in the lungs.

The second part of my thesis examined whether IL-5 can cause the characteristics of asthma in animals that are considered normal. For these experiments I studied rats that do not develop the early or late airway response after sensitization and antigen challenge. I hypothesize that intra-tracheal administration of IL-5 can affect airway cholinergic responsiveness, the early and late airway response, lung resistance, and airway inflammation in these rats. At the same time, it would be important to see if these effects caused by IL-5 could be reversed by the anti-IL-5 monoclonal antibody (TRFK5).

My results show that there is a link between cell-mediated immunity and the leukotriene pathway. Upregulation of the immune response with IL-2 increases the sensitivity of the airways to LTs. As well, Montelukast inhibits the IL-2-mediated late airway response and modulates cytokine mRNA production after antigen challenge, namely a decrease in Th2 cytokines (IL-4 and IL-5) and an increase in Th1 cytokine (IFN-γ). However, IL-5 administered to normal controls is unable to cause many of the physiologic and inflammatory characteristics of asthma (i.e early and late airway response and lung eosinophilia after challnge, respectively), but the cytokine can trigger airway hyperresponsiveness to methacholine, as well as increase baseline lung resistance 20 hours after antigen challenge. Moreover, Th2 cytokine mRNA expression is increased in the lungs of IL-5 treated animals. In conclusion, the information that is presented in this thesis establishes a link between cell mediated immunity and the leukotriene pathway. I also show that one mediator is not sufficient to induce all the physiological changes that are present in asthma. (Keywords: Asthma, Cysteinyl-Leukotrienes, Interleukin-5)

Résumé

L'asthme est la maladie respiratoire la plus rencontrée en pratique médicale clinique aussi bien chez l'enfant que chez l'adulte (1). Cette maladie est associée à une obstruction réversible du flux d'air, à une augmentation de la réactivité bronchique, ainsi qu'à une inflammation des voies respiratoires (2). Il a été démontré qu'une inflammation persistante des voies respiratoires peut devenir chronique et entraîner des changements irréversibles affectant la réponse physiologique des voies respiratoires face à différents stimuli (3). Durant les deux dernières décennies, il a été démontré que les leukotriènes cysteinyleês (cys-LT) sont des agents broncho-constricteurs ainsi que d'importants médiateurs inflammatoires impliqués dans la physiopathologie de l'asthme chronique et de l'asthme aigu (1). Les leucotriènes (LT) peuvent induire la contraction des fibres musculaires lisses, l'augmentation de la perméabilité vasculaire, la stimulation de la sécrétion de mucus, ainsi que le recrutement des éosinophiles dans les poumons (4). Plusieurs cellules inflammatoires activées (ex. mastocytes et éosinophiles) peuvent libérer les cys-LT (4). Il a d'ailleurs été démontré que les cys-LT peuvent interagir avec les cytokines, notamment l'interleukine (IL)-5, pour recruter les éosinophiles aux voies respiratoires des asthmatiques (5). Il existe donc potentiellement une voie autocrine de la cys-LT promouvant le phénotype de l'asthme. A elle seule, IL-5 est une cytokine très importante dans l'asthme. Ainsi, la concentration d'IL-5 est augmentée dans les poumons des asthmatiques allergiques et non-allergiques (6). L'administration intra-trachéale d'IL-5 augmente la réactivité des voies respiratoires et ce tant chez les humains asthmatiques que chez les animaux sensibilisés servant de modèles de l'asthme atopique (7,8).

Il est maintenant clair que l'augmentation de l'immunité cellulaire est une caractéristique importante chez les patients asthmatiques. Cette immunité est centralisée autour de l'activité des lymphocytes T activés. En effet, les lymphocytes T activés sont présents et augmentés dans les voies respiratoires des patients asthmatiques (9). La première partie de mon projet étudie la relation entre les LT et l'immunité cellulaire. L'interleukine(IL)-2, un facteur de croissance des cellules T, administrée en pré-traitement à des rats Brown Norway sensibilisés, à pour effet de stimuler l'immunité cellulaire et augmenter les cellules inflammatoires du lavage broncho-alvéolaire. Par conséquence, il a été démontré que la réponse des voies respiratoires à l'antigène est augmentée (10), mais que la production du LT

chez ces animaux demeure comparable aux groupes contrôles (11). La première partie de ma thèse étudie l'hypothèse que lorsque l'immunité cellulaire est amplifiée, cela crée une augmentation de la sensibilité des voies respiratoires aux LT, qui peut être mesurée en testant la réactivité des voies respiratoires à la LTD_4. En plus, mon hypothèse est que le blocage du récepteur de la cys-LT_1 avec le Montelukast (MK-0476) altérera l'effet de l'IL-2 sur la réponse tardive des voies respiratoires et affectera possiblement la production de cytokines dans les poumons.

La deuxième partie de ma thèse vérifie si l'administration de l'IL-5 peut causer des changements caractéristiques de l'asthme, chez les animaux considérés comme normaux. Pour ces expériences, j'ai étudié des rats qui ne développent pas de réponse aigu ou tardive dans les poumons, suite à la sensibilisation et à la provocation antigénique. Mon hypothèse est que l'administration intra-trachéale d'IL-5 peut affecter la réponse cholinergique des voies respiratoires, la réponse aigu ou tardive des voies respiratoires, la résistance pulmonaire, ainsi que l'inflammation des voies respiratoires, chez ces rats. En même temps, il serait important de voir si ces effets causés par l'IL-5 peuvent être bloqués par l'anticorps monoclonal anti-IL-5(TRFK5).

Mes résultats montrent qu'il existe un lien entre l'immunité cellulaire et la voie des LT. L'augmentation de l'immunité cellulaire avec l'IL-2 augmente la sensibilité des voies respiratoires aux LT. De plus, le Montelukast inhibe la réponse tardive causée par l'IL-2 et réduit d'une part la production d'ARN messagers particulièrement celles de l'IL-4 et l'IL-5 libérés par les cellules Th2 et d'autre part augmente la production de l'IFN-γ libéré par les cellules Th1. Par contre, l'administration d'IL-5 chez les rats contrôles ne permet pas de reproduire plusieurs des caractéristiques physiologiques et inflammatoires de l'asthme (i.e. réponse aigu et tardive des voies respiratoires et éosinophilie des poumons respectivement), mais cette cytokine peut déclencher une hyper-réactivité des voies respiratoires à la méthacholine, ainsi qu'augmenter la résistance pulmonaire de base, 20 heures après la stimulation antigénique. De plus, l'expression des ARN messagers des cytokines de type Th2 est augmentée dans les poumons des animaux traités à l'IL-5. En conclusion, les résultats présentés dans cette thèse démontrent bien le lien entre l'immunité cellulaire et la voie de synthese des LT. De plus ces résultats montrent qu'un seul médiateur n'est pas suffisant pour induire tous les changements physiologiques présents dans l'asthme. (Mots Clés : Asthma, Leukotriènes-cysteinyleês, interleukine-5)

Introduction

1.1.1 Epidemiology

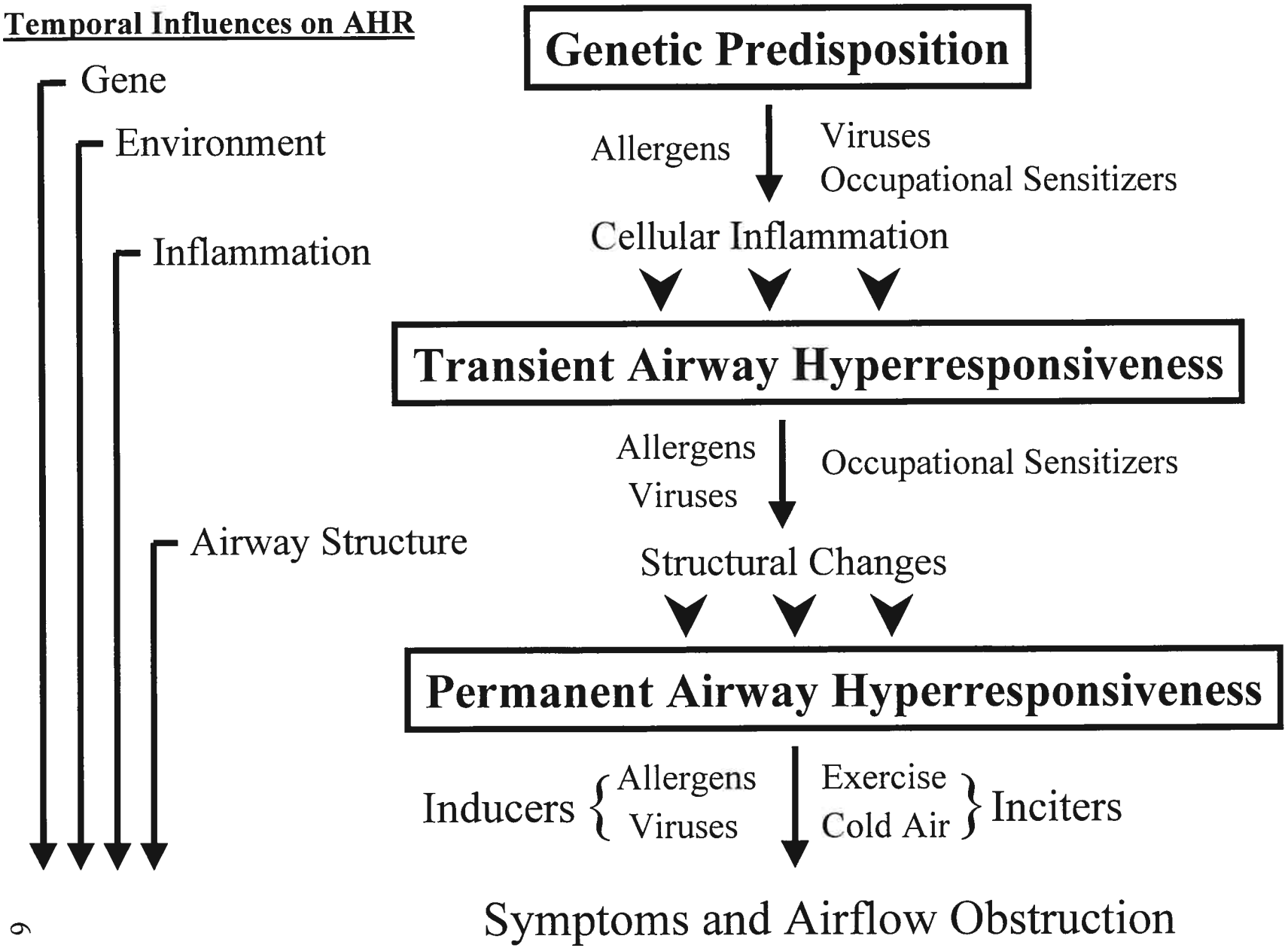
Allergic disease in general and asthma in particular have become an increasing problem for public health, especially in developed countries. Epidemiologic evidence suggests that the prevalence of asthma has increased significantly, especially among children (12,13). Asthma affects 5 to 10% of the population (14) and in the United States alone, asthma affects 14 to 15 million persons. It is the most common cause for hospitalisation of children and it continues to be fatal. In the United States, an estimated 5000 persons die of asthma per year (15).

Some scientists have questioned the validity of reports that suggest that the prevalence of asthma is increasing considerably in the population as a whole. Their main argument is that the reported increases are possibly due to a greater awareness among physicians of the importance of asthma as a cause of chronic respiratory symptoms, especially among children (16). This theory is understandable because it is still true today that many children with asthma receive other diagnoses (wheezy bronchitis, spastic bronchitis, etc.). However recent surveys that have included objective measures of risk factors for asthma, such as allergic sensitization and bronchial hyperresponsiveness (17) confirm that only a small proportion of the observed increases in the prevalence of asthma are due to a shift in diagnostic labelling. Most surveys of children in Western countries have reported that the prevalence of asthma is higher in boys with some male/female ratios exceeding 2 to 1 (18).

1.1.2 Risk Factors / Asthma Mechanisms

In recent decades, a number of studies have suggested that allergen exposure is the primary cause of asthma, and that the global increases in asthma prevalence could be the result of increases in exposure to aeroallergens (19). The hypothesized causal mechanism is that allergen exposure produces sensitization and continued exposure leads to clinical asthma through the development of airway responsiveness and inflammation **(Figure 1)**. However, there is a distinction between factors that can precipitate attacks (secondary causation of asthma) and those that increase the risk of developing asthma (primary causation of asthma). It is well established that allergen exposure is a secondary cause

Figure 1: Hypothesized development of transient and persistent hyperresponsiveness



of asthma in that it can trigger asthma attacks in sensitized asthmatic subjects and that prolonged exposure can lead to persistence of symptoms (19). Other risk factors which precipitate attacks include viral infections, cigarette smoke, atmospheric pollution, and stress (20). A factor that prolongs or exacerbates asthma symptoms may thereby increase asthma prevalence even if it has no effect at all on the incidence of asthma. The evidence linking specific allergen exposure to asthma is weak and, if the association is causal, the population attributable risk appears to be small. Some researchers have theorised that the prevalence of asthma is related more to the total burden of aeroallergens than to exposure to a particular allergen. An alternative explanation is that specific allergen exposure may not be a major primary cause of sensitisation or of asthma itself, but may determine the specificity of the sensitisation in susceptible individuals.

Several studies have shown the adverse effects of ambient air pollution on respiratory health (20,21,22,23). A major causative agent could be outdoor air pollution, derived from cars and other vehicles. Studies have demonstrated that urbanisation and high levels of vehicle emissions is correlated with increasing frequency of pollen-induced respiratory allergy. Pollen grains or plant-derived paucimicronic components carry allergens that can produce allergic symptoms. There is evidence that air pollutants may promote airway sensitisation by increasing the allergenicity of airborne allergens. By affecting plant growth, air pollutants can affect both the amount of pollen produced and the amount of allergenic proteins contained in pollen (24,25). Aeroallergens released by pollen grains can be transferred to other, small, non-biological particles of air pollution such as diesel exhaust particulate (DEP) acting as biological aerocontaminants of the inhaled air which can penetrate deep into the airways inducing allergic symptoms in sensitized subjects (26). DEP also exerts an adjuvant immunological effect on Immunoglubulin(Ig) E synthesis in atopic subjects thereby influencing sensitization to airborne allergens (27). Airway mucosal damage and impaired mucociliary clearance induced by air pollution may facilitate the access of inhaled allergens to the cells of the immune system. Despite evidence of a correlation between the increasing frequency of respiratory allergy and the increasing trend in air pollution, the link and interaction is still speculative.

Researchers have postulated that the recent increases in asthma prevalence could be due to an increase in the level of exposure to certain indoor aeroallergens (17). They reason that changes in the

structure of modern homes have rendered them more impermeable to the outdoor environment, making the inhabitants of these homes significantly more exposed to indoor allergens. In addition, it is suggested that the use of certain materials for home construction and in furniture stimulates the growth of indoor allergen sources such as house-dust mites. Also the modern tendency to keep cats and dogs inside homes can also increase the risk of sensitization to allergens produced by these pets.

It is important to note than an individual's response to allergen exposure depends on the source and components, as well as climatic factors which can favour the accumulation of allergens at ground level. Therefore, the effects of allergens on lung function depends on the environmental concentration of the allergen, the duration of exposure and the total ventilation of exposed persons.

1.1.3. Hygiene Hypothesis

There has been a significant increase in the prevalence of allergic diseases over the past 2 to 3 decades. There are also clear differences in the prevalence of allergy and asthma between rural and urban areas within one country. For example, in Ethiopia, asthma is more prevalent in urban areas than in rural villages (28), and asthma is more common in residents of urban Germany than in farmers living in rural Bavaria (29). To explain these observations, environmental factors associated with more industrialised and urban living have been studied intensively, but there is little consistent evidence to suggest that obvious risk factors, such as increased exposure to indoor allergens (such as endotoxin and lipopolysaccharides (LPS)), pollution, or changes in diet and breastfeeding, could account for the rise in atopic diseases. Indeed, it may be that endotoxin prevents the development of asthma (30). Another category of environmental factors, childhood infections, shows an overwhelming and consistent negative association with atopy and allergic diseases. Allergic sensitization is overrepresented among first-born, but is less frequent in children from large families (31), and those attending day care (32), suggesting that a frequent exchange of infections may have a protective effect (31). It has been proposed that the lack of intense infections in industrialised countries due to improved hygiene, vaccination, and use of antibiotics may alter the human immune system such that it responds inappropriately to innocuous substances. This so-called "hygiene hypothesis" (31) has a particular immunological profile, specifically a unique balance between type 1 (Th1, associated with bacterial and viral infections and autoimmune diseases) and type 2

(Th2, associated with helminth infections and allergic diseases) immune responses (33). The theory is that limited exposure to bacterial and viral pathogens during early childhood results in an insufficient stimulation of Th1 cells, which in turn cannot counterbalance the expansion of Th2 cells and results in a predisposition to allergy.

Th2 responses are characterised by increased IgE to allergens, mastocytosis, and eosinophilia. Helminths are prevalent in developing countries and lead to strong Th2 responses. Nevertheless, helminth-infected populations show little signs of allergic disorders. This difference may be explained by the differences in exposure to pathogens. A high prevalence of chronic infections in developing countries results in persistent immune challenge, with cycles of infection and inflammation, which is followed by the triggering of anti-inflammatory molecules to restrict immunopathology. This dynamic interaction educates the immune system to establish a robust regulatory network, possibly the key to controlling allergic diseases. Such a network would be weakly developed in industrialised countries with low pathogen load, allowing inappropriate immunopathologic reactions to develop more readily.

The immunological explanation for the hygiene hypothesis has been influential in directing strategies to prevent allergic diseases. Induction of allergen-specific Th1 response by Bacille Calmette-Guerin (BCG) or DNA vaccination is being advocated on the basis of the promising results obtained in experimental animals (34).

1.1.4 Genetics

The environmental modifications that are causing the increases in asthma are more likely to exert their influences in individuals who have certain genetic backgrounds. Genetic susceptibility is defined as the presence of polymorphisms in genes that determine that certain individuals are at increased risk of developing those diseases. The genetic variations that predispose those to asthma were present in the population long before the current epidemic started. However, in the absence of certain environmental influences, these variations did not lead to the development of clinical illnesses in the past. Complex diseases such as asthma and allergies are almost always the result of interactions between different sets of genes and environmental influences that, acting together with these genetic variations make these complex diseases more likely.

It is very difficult to measure simultaneously all the environmental influences that can interact to determine asthma risk, and particularly to measure them at the time in which they are active. Therefore several genome-wide screens for asthma and its associated or related traits have been carried out (35,36,37,38,39,40,41). Most of these studies lack sufficient statistical power, but some have been relatively consistent in reporting genetic linkage in the same chromosomal region. Therefore, these genetic loci may contain major genes influencing atopy and asthma (42). The latter include genes within the cytokine gene cluster on chromosome 5, 11, 12, and 16 as likely candidates that may contribute to asthma and allergy development. In addition, these data support the involvement of genes involved in antigen presentation (MHC) and T cell responses (43). Studies in identical twins have convincingly demonstrated that at least 50% of the susceptibility to asthma is determined by inherited predisposition (44).

Some of these aforementioned candidate genes could yield new therapeutic approaches once they are fully validated. It remains to be established whether there are functional polymorphisms in these candidate genes that determine the severity of the disease phenotypes. These polymorphisms could be the target of several therapeutic scenarios, including a reduction in gene expression or an elimination or alteration in the protein of interest.

1.2. Asthma Characteristics

Asthma is often associated with atopy, a disorder characterized by sustained, inappropriate IgE responses to common environmental antigens (allergens) encountered at mucosal surfaces (45). Cross-linking of surface Fc receptors to allergen-specific IgE is assumed to play a role in the pathogenesis of atopic asthma (46). However, a minority of asthmatic individuals are not demonstrably atopic according to conventional criteria, which has led to the suggestion that asthma may be divided clinically, and perhaps, mechanistically, into atopic (extrinsic) and nonatopic (intrinsic) variants (47).

Intrinsic asthma is considered to be a distinct pathogenetic variant of asthma since, unlike extrinsic asthma, patients with the disease are skin test-negative to common aero-allergens, and have total serum IgE concentrations within the normal healthy range. As well, the cellular pattern for eosinophils, neutrophils, T lymphocytes, and cytokines differs between atopic and non-atopic patients

with asthma. In atopic asthmatics, the airway inflammation is characterized by high numbers of eosinophils, mast cells, and T lymphocytes, whereas non-atopic asthmatics mainly display high number of neutrophils and mast cells (48). IL-4 and IL-5 positive cells are found in higher numbers in atopic than in non-atopic patients with asthma. There are also distinct structural alterations in the airway mucosa of patients with atopic asthma that are not found in non-atopic asthmatics.

Intrinsic asthma occurs in an older age group of people without any history of allergy to environmental factors (49). In contrast, extrinsic asthma usually develops in childhood, occurs often seasonally and remains present throughout life, with periods of remission (50,51). Intrinsic asthma has a preponderance to affect females and it is associated with nasal polyps and aspirin sensitivity (52,53). Whereas some authors suggest that around 10% of asthmatics are intrinsic, a Swiss study found that a third of total asthmatics are non-allergic (intrinsic)(53,54).

Ever since the first description of intrinsic asthma, there has been debate around the relationship of this variant of the disease to atopy (53). One suggestion is that intrinsic asthma represents a form of autoimmunity, or autoallergy, triggered by an infection since respiratory influenza-like illnesses often precedes the onset. Some researchers suggest that intrinsic asthmatics are allergic to an as yet undetected allergen and could therefore benefit from allergen avoidance, as previously demonstrated in atopic asthmatics (55).

1.2.1 Early Airway Response /Late Airway Response

Allergen challenge of sensitized atopic individuals results in an early response (ER), which in the skin is seen as the immediate wheal and flare reaction, and after inhalation as a decrease in airway caliber occurring within minutes (56). The early airway response (ER) occurs within minutes of inhalation of antigen, lasts up to an hour, and is followed by a prompt return to baseline lung resistance (57) **(Figure 2A)**. The ER is mediated by IgE antibodies, which are present on mast cells and basophils. These antibodies cross-link when in contact with allergen leading to degranulation of the cells and increased microvascular permeability (58). Bronchoconstriction of the airways during the ER is induced by the release of histamine, leukotrienes, eicosanoids, and possibly other bronchoconstrictive agents (59).

The late airway response (LR) occurs 4 to 12 hours after antigen exposure and may persist long after antigen exposure has ceased (60) **(Figure 2B)**. In general, most atopic asthmatics develop both responses **(Figure 2C)**, although occasionally, only an early or a late response is encountered (61). Airways of individuals experiencing the LR show increased edema and an infiltration with different inflammatory cells, in particular eosinophils and T lymphocytes (62,63).

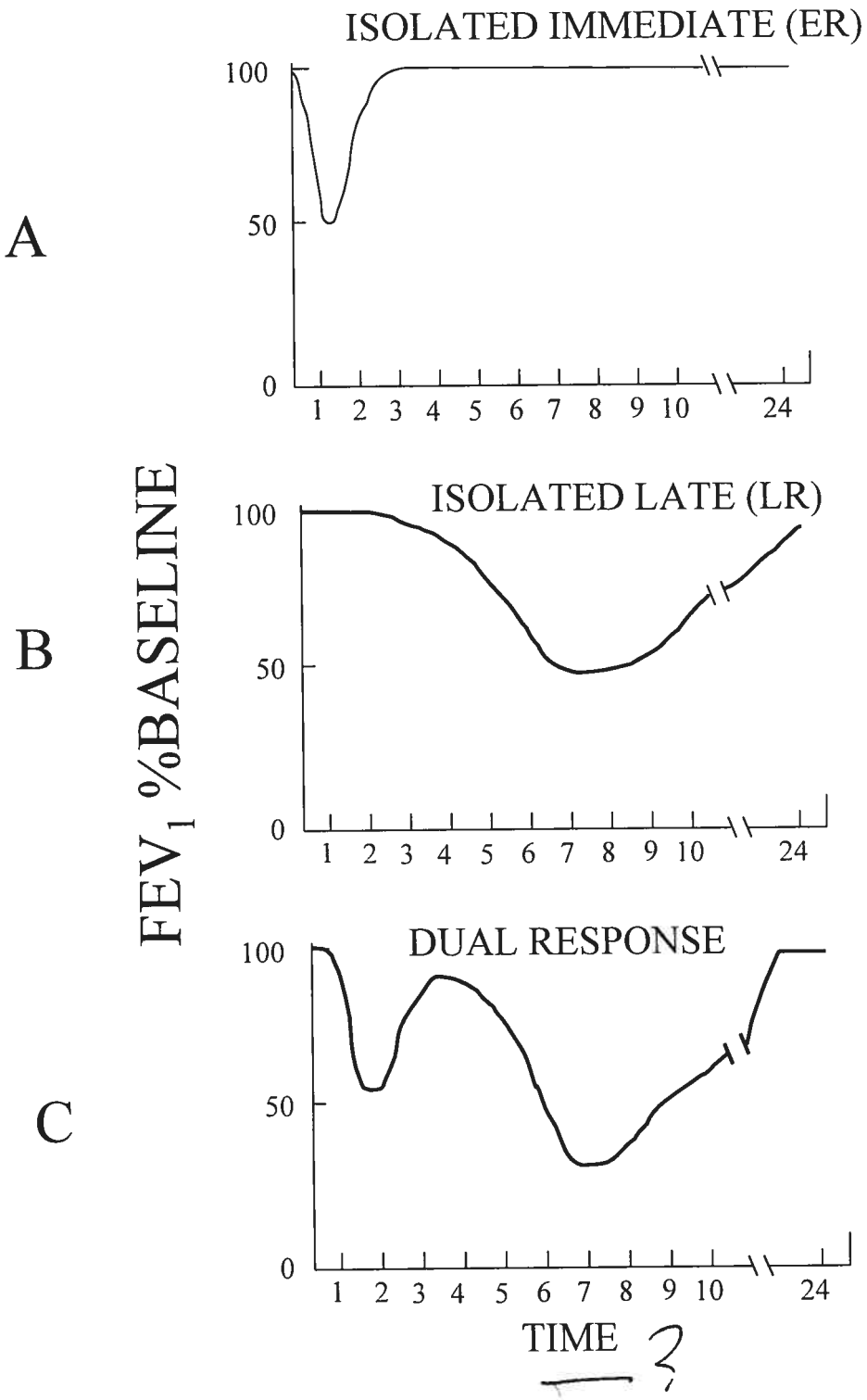
1.2.2 Airway Hyperresponsivness

Airway hyperresponsiveness (AHR) is characterized by an increase in the sensitivity and reactivity of the airways to agonists, such as allergens, and the development of a lower threshold to spasmogens that will hinder airflow (64). AHR occurs before the symptoms of the LR arise and can remain after the symptoms have subsided (65,66). The type of physiological response is directly related to the degree and method by which various antigens are presented to the immune system in the airways of atopic asthmatics (67). Exposure to an antigen to which one is sensitized to may only cause an acute, transient increase in airway hyperresponsiveness, however continuous exposure to an allergen may cause an individual to reach a chronic state of airway inflammation and hyperresponsiveness (61). Inflammation is not always necessary for AHR to occur. Bronchoconstrictive agents such as histamine, cholinergic agents and some β-agonist blockers cause bronchospasm through smooth muscle contraction without airway inflammation (61).

AHR occurs through neuronal mechanisms and inflammatory mediator release which affects the sensitivity of the airways to stimuli and accordingly the amount of smooth muscle contraction. Consequently, increased AHR leads to a decrease in airway diameter (68). The relationship between inflammation and hyperresponsiveness in the airways is especially important during the LR. There is an increased presence of inflammatory cells and mediators in the airways during the LR (69).

Type 2 T helper lymphocytes (Th2) orchestrate the inflammation and are crucial for the development of AHR. Cells and molecules involved in T cell activation (dendritic cells, T cell receptor, major histocompatability complex molecule, and costimulatory molecules) are also vital. There are at least three pathways that lead to AHR. One is dependant on immunoglobulin E and mast cells, one on

Figure 2: Common patterns of response after pulmonary antigen challenge



eosinophils and interleukin-5 (IL-5), and one on IL-13. Eosinophils are probably the most important effector cells of AHR.

The nature of the relationship between AHR and atopy (specific IgE to aeroallergens) is unknown and one of the most important questions in asthma. It is clear from epidemiological studies that these two abnormalities are linked. However, not all allergic (atopic) people have AHR and not all individials with AHR are allergic. Furthermore, there is some suggestion that the risk factors for the two abnormalities are different.

1.2.2.1. Airway Hyperresponsiveness in Humans

Researchers have long used stimuli, such as histamine and methacholine, to measure the level of AHR in patients with asthma and in animal models. By instilling or nebulizing incremental doses of these mediators to the airway, they are able to assess and to quantify the threshold of tolerance toward non-specific irritant stimuli (70,71). AHR is often defined as a 20% fall in the FEV_1 in response to a provoking agent, such as histamine, methacholine, or hypertonic saline, and sometimes to exercise or cold air hyperventilation. However a measure of AHR is not always considered to be a sign of asthma or even of airway inflammation (72). The amount of AHR to cholinergic agonists correlates only with the level of certain asthmatic symptoms such as wheezing and nocturnal cough and can give a limited prognosis of the severity of an asthmatic attack (72). Airway hyperresponsiveness is not restricted to non-specific stimuli. AHR can occur in normal subjects following viral respiratory infection and can be present in atopic non-asthmatic individuals, in patients with chronic obstructive pulmonary disease or cystic fibrosis (73,74,75).

1.2.2.2. Airway Hyperresponsiveness in Animal Models

Airway responsiveness in the intact animal depends on tracheal smooth muscle airway contractility, chest wall compliance, bronchiolar mucus plugging, airway fibrosis, and other factors (76). AHR is a function of both airway hyper *reactivity* (an increased response to a given dose of bronchoconstrictor) and airway hyper *sensitivity* (the ability to respond to a smaller dose of bronchoconstrictor) (77).

There are three ways to measure AHR (78). The *ex vivo* technique examines the contractility of dissected tracheal smooth muscle stimulated by electricity or methacholine. The first *in vivo* method

measures the airway pressure and lung resistance of an anaesthetised animal with an intratracheal and intra-esophageal cannula (79). The second *in vivo* method uses unanaesthetised and unrestrained animals in a plethysmography box to produce a mathematically derived parameter called the enhanced pause (Penh) that reflects airway obstruction (78).

The *ex vivo* method differs from *in vivo* measurements because it neglects the effects of edema, mucus, or chest wall recoil on airway narrowing. The first *in vivo* method requires the technique of trachea and esophageal cannulations. The second *in vivo* method permits uninterrupted measurements, enabling the evaluation of early and late phase bronchial response and the discernment of airway hyperreactivity and airway hyperresponsiveness (80). However, in some circumstances, the nasopharynx contributes significantly to the total airway resistance with this method.

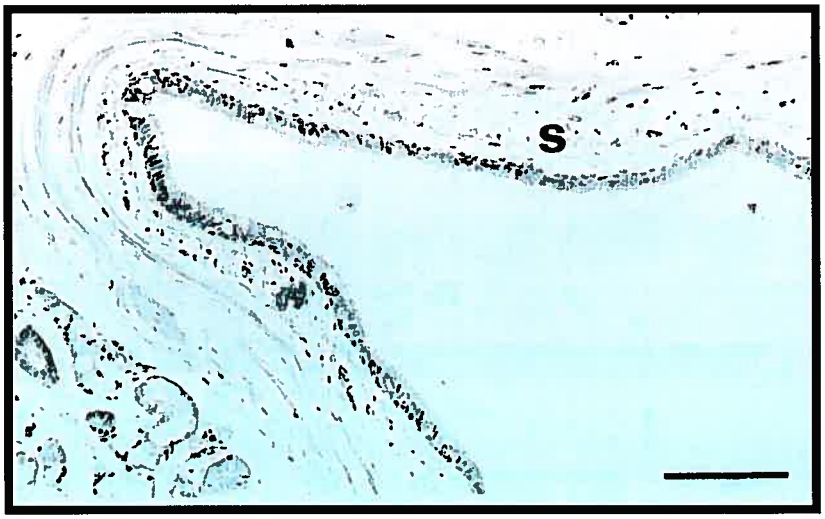
1.2.3 Airway Remodelling/Obstruction

Asthma has traditionally been thought of as an entirely reversible disorder. However, a number of studies have demonstrated that individuals with asthma experience an accelerated rate of respiratory function deterioration (81). Patients with asthma can develop a physiological state characterized by irreversible, or partially reversible, airway obstruction, and they manifest persistent AHR even after prolonged corticosteroid therapy. The pathogenic mechanisms responsible for these findings are poorly understood but they may be the result of structural changes, referred to as airway remodelling. Remodelling in chronic asthmatic airways is characterized by wall thickening, subepithelial fibrosis, mucous metaplasia, myofibroblast hyperplasia, vascular abnormalities, and myocyte hyperplasia and hypertrophy (82).

1.2.3.1 Airway wall thickening

All components of the airway wall (inner, outer and total) have been reported to be thickened in asthma. Many elements contribute to this response, including an increase in airway smooth muscle. edema, inflammatory cell infiltration **(Figure 3)**, glandular hypertrophy, and connective tissue deposition. Compared to nonasthmatic subjects, airway wall thickness is increased from 50% to 300% in cases of fatal asthma, and from 10% to 100% in cases of non-fatal asthma (83). The exact physiologic and

Figure 3: Photomicrographs of airway inflammation



(a) the airway submucosa (S) from a nonasthmatic individual with few inflammatory cells, bar = 0.1 mm

(b) the submucosa from an individual with fatal asthma with polymorphonuclear and mononuclear cell infiltration, bar = 0.05 mm

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pathologic consequences of airway wall thickening are incompletely understood. Some models have demonstrated that the thickening response reduces the amount of smooth muscle shortening required to cause airway closure (84).

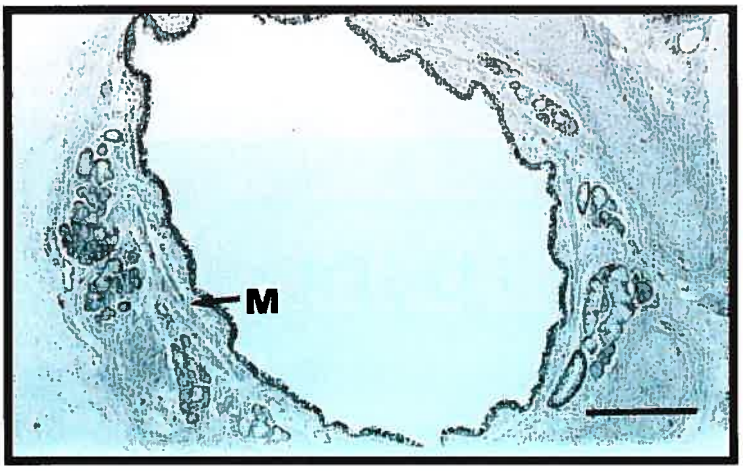
1.2.3.2 *Basement Membrane Thickening*

Collagen deposition beneath the basement membrane is described as "basement membrane thickening" (85). This has been recognized as increased collagen types III and V, as well as matrix components laminin and fibronectin along the basement membrane (composed of type IV collagen) (86). Multiple factors have been associated with subepithelial collagen thickening including increased frequency of asthma (87), longer duration of symptoms (88), airway hyperresponsiveness (89), increased T lymphocyte and fibroblast activity (90,91), epithelial damage (92), as well as mast cell and eosinophil infiltration (93). A possible mechanism is that growth factors released by cellular activity lead to increased myofibroblast numbers and activation observed below the basement membrane (94) where they appear in relative abundance. Enhanced collagen production by myofibroblasts and fibroblasts beyond the rate of collagen breakdown will lead to increased deposition. Potentially, the increased airway rigidity may contribute to mortality in asthma by reducing the maximal bronchodilator response. In addition, internal mucosal edema may cause significant internal luminal narrowing, without allowing outward radial expansion of the wall.

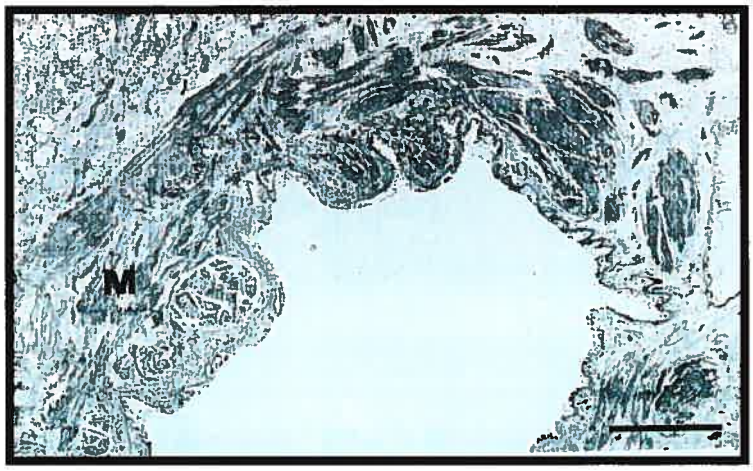
1.2.3.3 *Smooth Muscle Hypertrophy/Hyperplasia*

A 50-230% and 25-150% increase in the area of airway smooth muscle has been demonstrated in fatal and nonfatal asthma, respectively (83) **(Figure 4)**. These findings imply that the increased smooth muscle mass in some asthmatics may predispose to poorer lung function and a poorer response to a severe attack. Presumably, the factors known to be trophic for smooth muscle growth act unopposed in persistent airway inflammation. These include histamine, thrombin, thromboxane A2, and epidermal and platelet-derived growth factor (95). The modelling studies of Wiggs and colleagues have indicated that shortening of inner airway wall smooth muscle by 40% may be sufficient to lead to airway closure (96).

Figure 4: Photomicrograph of airway smooth muscle



(a) a nonasthmatic individual showing normal airway smooth muscle (M), bar = 0.5 mm



(b) an individual with fatal asthma showing a prominent layer of smooth muscle (M) surrounding the folded airway mucosa, bar = 0.05 mm

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Moreover, the added thickness may account for the loss of dynamic expansion of deadspace seen during inspiration in asthma (97).

1.2.3.4 *Mucus Metaplasia*

Mucus hypersecretion, epithelial mucus metaplasia, and airway obstruction due to bronchial mucus plugging are well-documented features of chronic asthma and *status asthmaticus* (98). Morphometric and immersion fixation studies demonstrate that the area of mucus glands is increased in fatal and nonfatal asthma. Liquid and mucus can also fill in the interstices and folds of the airway surface. This could add to the forces tending to narrow the airway by amplifying the effect of muscle shortening and increasing the surface tension at the air-liquid interface.

1.2.3.5 *Airway Vascularity*

The bronchial mucosa of endobronchial biopsies from mild asthmatics has been shown to contain increased numbers of vessels per unit area (738 per mm^2) (99). The increase in vascularity below the basement membrane and adjacent to the musculature is potentially capable of causing further luminal narrowing. Using anti-collagen type IV antibodies on biopsies from mild asthmatics, researchers have found significantly more vessels overall and more vessels larger than 300 μm^2 in asthmatic airways, suggesting bronchial vasodilation in addition to angiogenesis. Canine models have demonstrated *in vivo* correlation between airway thickening and airflow obstruction (100). This engorgement might also contribute to the loss of airway distensibility seen in asthma (97).

1.3 Airway Inflammation

1.3.1 *IgE*

The secretion of IgE by B lymphocytes defines the allergic state, and the association between allergy and asthma is well established (101). The percentage of asthmatic subjects defined as allergic depends on whether subjects have a positive skin test to an aeroallergen in addition to elevated levels of serum IgE. IgE binds to high or low affinity receptors (FcεRI and CD23, respectively) on the surface of a variety of effector cells, the most important of which are mast cells and basophils. The cognate antigen

crosslinks IgE bound to high affinity receptors on mast cells and on basophils causing the cells to release a variety of preformed and newly generated mediators that promote airway hyperresponsiveness, mucus hypersecretion, and increases in vascular permeability. The early asthmatic response is clearly dependant on IgE-mediated activation of mast cells through high affinity receptors (FceRI). Antigen specific IgE responses are regulated by HLA class II and T-cell receptors and involve T-B cognate interaction while nonantigen-specific IgE response involve noncognate interaction of mast cells, basophils, and T and B cells (102). IL-4 is the most important cytokine mediating IgE synthesis and together with IL-13 plays a central role in the IgE-dependent allergic reaction.

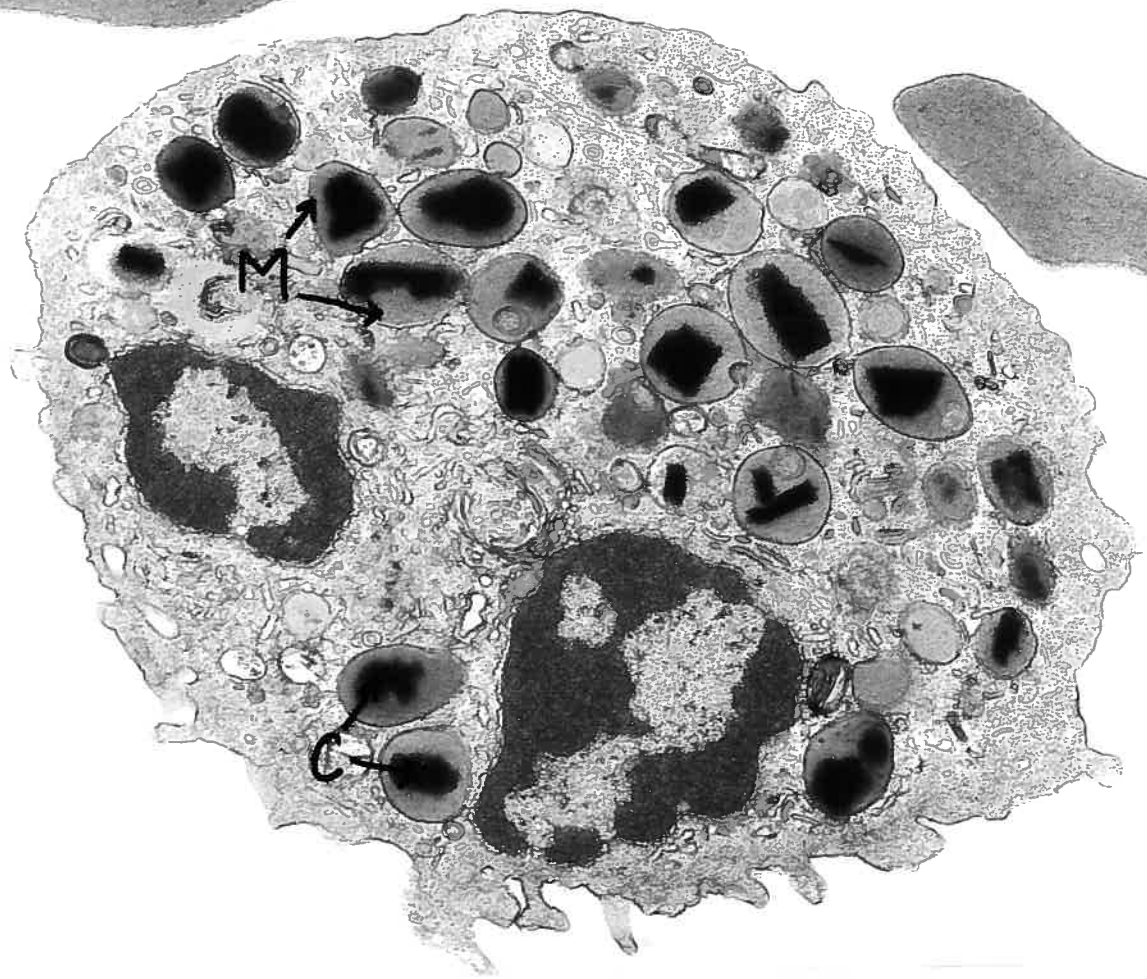
The role of IgE in mediating an allergic airway reaction is confirmed by the finding that treatment of allergic asthmatic patients with monoclonal anti-IgE antibodies attenuated airway eosinophilia, increased the dose of allergen needed to provoke an early reaction, and reduced the mean maximum fall in FEV_1 during the early and late responses to allergen challenge (103). Therefore, IgE has a direct role in mediating not only the ER, but also the LR.

While IgE-dependant inflammation plays a major part in allergic asthma, there are many inconsistencies. One study has demonstrated that total IgE is a poor diagnostic indicator of respiratory allergic disease (104) and in the African population serum levels of IgE have been reported to be higher in nonasthmatics than in asthmatics (105). Moreover, IgE knockout mice when challenged with allergen can elicit an inflammatory response in the airway as well as airway hyperresponsiveness (106). Even the syndrome of active anaphylaxis, with mast cell activation and mediator release can be displayed by both ovalbumin (OVA) sensitized $IgE^{-/-}$ and FceRI-deficient mice after intravenous challenge with OVA (107,108). These studies cast a question mark on the precise role of IgE in allergic disease.

1.3.2 *Eosinophils*

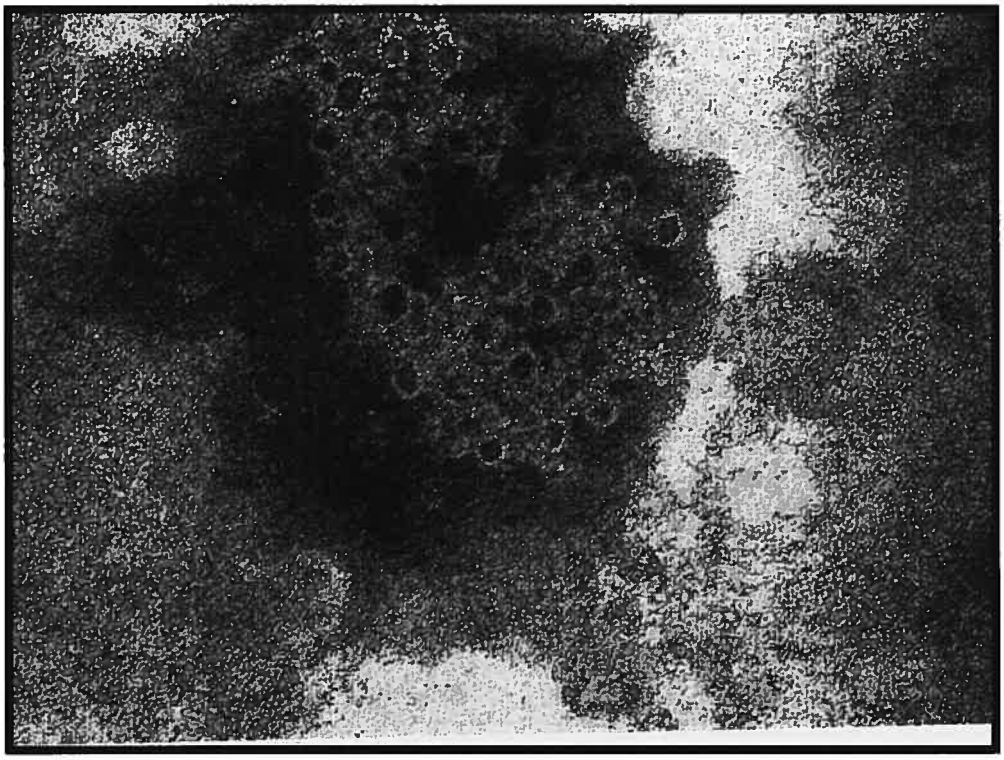
The current view of the eosinophil is that it is a proinflammatory cell with substantial tissue destructive potency. The biological activities exerted by the eosinophil are related to the products released from its granules, including the eosinophil cationic protein (ECP) and the major basic protein (MBP) **(Figure 5)** . These two potent cytotoxic proteins have the capacity to kill both mammalian and non-mammalian cells, such as parasites, by making pores in cell membranes, which leads to osmotic lysis

Figure 5: Eosinophil granules



Electron photomicrograph of a human peripheral blood eosinophil with the crystalloid-containing specific granules showing electron-dense cores (C) of various shapes embedded in less dense matrix (M).
Photo courtesy of Dr. Hirohito Kita, Mayo Clinic, Rochester, MN

Figure 6: Eosinophil Cationic Protein (ECP)



Electron microscopic demonstration of the effect of eosinophil cationic protein on cellular membranes which can produce pores with an approximate diameter of 5nm.
Photo courtesy of Dr. Per Venge, Uppsala University, Uppsala, Sweeden

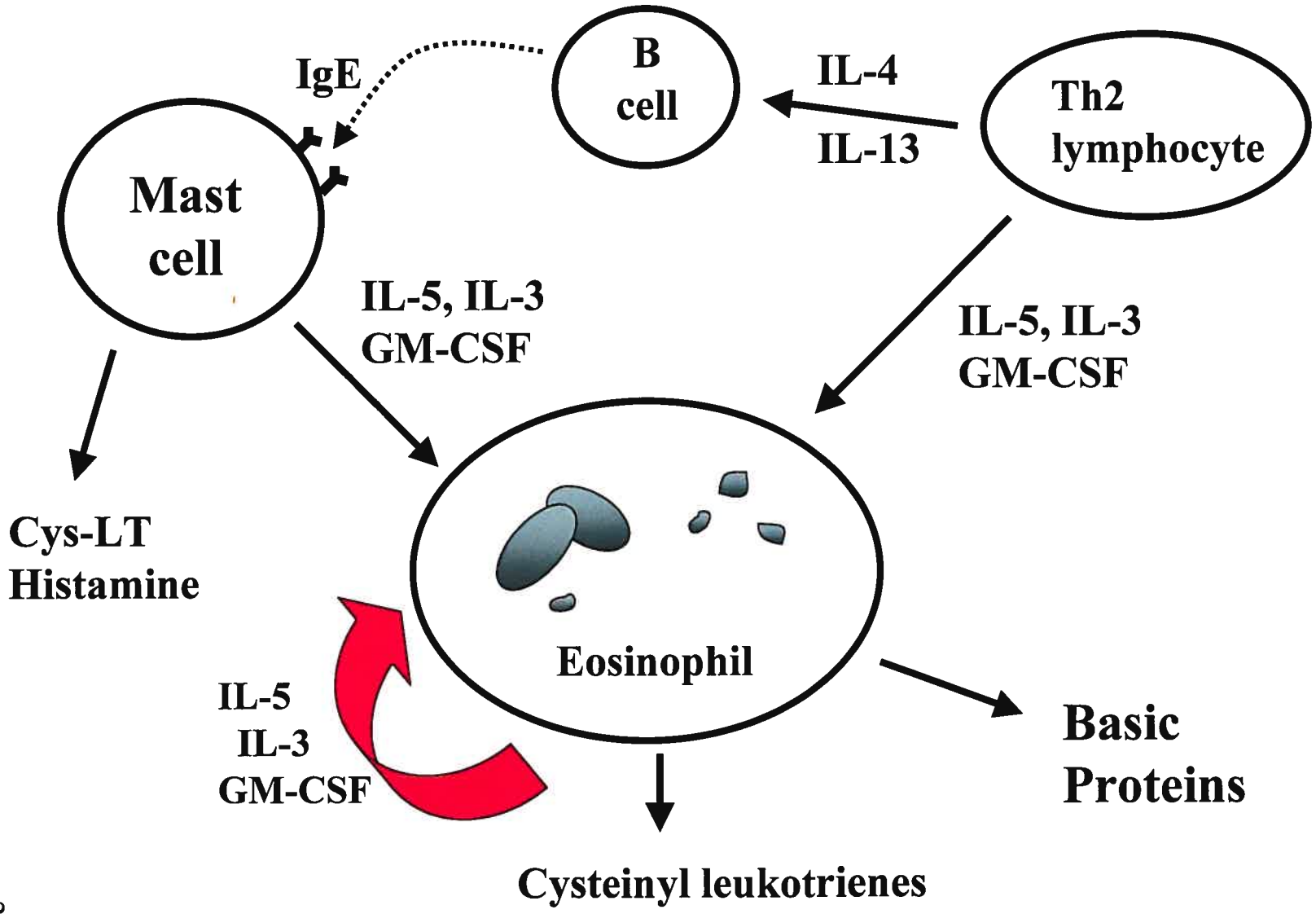
(Figure 6). The accumulation and activation of eosinophils from the bone marrow to the lungs is governed by the upregulation of adhesion molecules on lung endothelial cells and the production of various cytokines and chemotactic molecules by mast cells and T cells. Of these cytokines, IL-5 seems to play a central role, because it regulates most aspects of eosinophil behaviour, such as growth, apoptosis, adhesion, and secretion (109). Activation of the endothelium by cytokines such as IL-4 favours their migration to the lungs by upregulating the expression of vascular cell adhesion molecule 1 (VCAM-1) on endothelial cells (110).

Eosinophils differentiate within the bone marrow under the influence of GM-CSF, IL-3, and IL-5 **(Figure 7)**. In response to allergic stimuli in the lung, the eosinophil pool in the bone marrow expands and the number of eosinophils residing in the blood and at the site of allergen provocation increase markedly. Circulating eosinophils are recruited into tissues following adhesion to endothelial cells expressing specific adhesion molecules, including VCAM-1, which recognizes vascular leukocyte antigen-4 (VLA-4) found on eosinophils, and ICAM-1 that binds CD11/CD18 molecules on a range of leukocytes types. Eosinophils migrate in response to chemoattractants, including lipid mediators, complement components, chemokines, and cytokines. Eosinophils express receptors for complement components C3a and C5a, for chemokines including IL-8, RANTES (CCR-1) and eotaxin (CCR-3), for cytokines including IL-1, IL-2, IL-3, IL-4, IL-5, IL-9, IL-16, GM-CSF, IFN-γ, and TNF-α, and for immunoglobulins (Ig) A, G, and E (high and low affinity) (111). Eosinophils are thus well equipped to respond to a range of inflammatory stimuli.

Eosinophils are also an important source of inflammatory mediators. Among the lipid derivatives, eosinophils have the capacity to produce an amount of leukotrienes comparable with that of mast cells and basophils and higher than that of monocytes **(Figure 7)**. Eosinophils also generate 15-HETE, lipoxins, platelet-activating factor (PAF), and small amounts of thromboxane$_2$ (TXA_2) and prostaglandin$_2$ (PGE_2). The eosinophil specific granule contains MBP in its core, and ECP, EPO, and EDN in its matrix. These granule-derived proteins have cytotoxic activity for helminths and are implicated in bronchial epithelial damage (112).

Early studies on post-mortem lungs obtained from patients who died of asthma showed significant eosinophilia. Influx of eosinophilia into the bronchoalveolar lavage (BAL) fluid was demonstrated during the late response after allergen challenge of atopic subjects, at a time when

Figure 7: Mast cells and T cell cytokines regulate the expression of cytokines and basic proteins and the activity of the leukotriene synthetic pathway in eosinophils



bronchial responsiveness is enhanced. In numerous clinical asthma studies, eosinophilia has been demonstrated in bronchial biopsies, induced sputum, BAL fluid, and blood of allergic and nonallergic patients (113). Levels of MBP and ECP are elevated in lung tissue and BAL fluid obtained from asthmatics, suggesting eosinophil activation (112). Eosinophil numbers in biopsies and BAL fluid correlate with asthma symptom scores, lung function (FEV_1) and nonspecific AHR.

Although there is evidence of the eosinophil as a primary effector cell leading to bronchoconstriction, epithelial damage and AHR in clinical asthma, its contribution in individual subjects may be highly variable (113). In several studies, a distinct proportion of patients with clinically significant asthma had negligible counts of eosinophils in bronchial biopsies or BAL fluid. Correlations between eosinophilia and AHR may be statistically significant, but individual patients may differ from this simple relationship, with activated T cells sometimes showing stronger correlations with measures of disease severity.

In contrast to wild-type litter mates, allergic IL-5 -/- mice do not generate eosinophilia in the blood and bone marrow compartments in response to allergen provocation of the lung, and this greatly reduces the level of eosinophils recruited to the airways (114). However, mature eosinophils still reside in the blood (albeit reduced numbers) and bone marrow compartments, indicating that baseline differentiation, maturation, and subsequent extramedullary migration persist in the absence of IL-5 (114). Results suggest that the primary role for IL-5 is in the promotion of peripheral eosinophilia in response to allergic stimulation.

1.3.3 *Neutrophils*

The neutrophil has only recently been the target of considerable interest regarding asthma pathogenesis. In recent years, the expansion of invasive studies to more severe forms of asthma, the advent of sputum analysis, concerns regarding the eosinophil as the most important effector cell and an appreciation of the properties intrinsic to the neutrophil, have led to an increased interest in this cell type. The inflammatory products generated by the neutrophil range from cytokines, chemokines, and lipid mediators to reactive oxygen species, various proteases and growth factors (115). The most important of

these are IL-8, LTB_4, and matrix metalloproteinase (MMP)-9. IL-8, a potent chemoattractant for neutrophils, has been reported to be increased in asthmatic patients.

Neutrophils may predominate in the airways of patients with nocturnal asthma, sudden-onset fatal asthma, acute exacerbations or severe asthma poorly controlled by high dose glucocorticoids (116). Sputum analysis of asthmatics demonstrated that neutrophils were the prominent cell in subjects with severe asthma, as well as mild asthmatics. Although the sputum reflects pathology in the larger airways, in both transbronchial biopsies and surgical evaluation of neutrophils in the small airways, there appears to be an increase in neutrophils, with specific localization to the small airway inner wall (117).

It is not likely that the neutrophil plays any role in the acute bronchospasm associated with asthma, given the profile of the mediators generated by these cells. Neutrophils are more likely to be involved in chronic inflammation, wound repair, and remodelling processes of asthma. The neutrophil is well-known to be part of the wound repair processes in the skin, eyes, and blood vessels (118). Studies indicate that the neutrophil may contribute to the fibrotic processes associated with asthma, particularly the basement membrane thickening, through its production of TGF-β and MMP-9. TGF-β has been reported to be increased in bronchoalveolar fluid and in the cells of patients with asthma, with studies showing that at least 50% of these $TGF\text{-}\beta^+$ cells are neutrophils (117). MMP-9 has also been reported to be increased in asthma and in patients with *status asthmaticus*, but whether their source is the neutrophil or not is difficult to prove. (119).

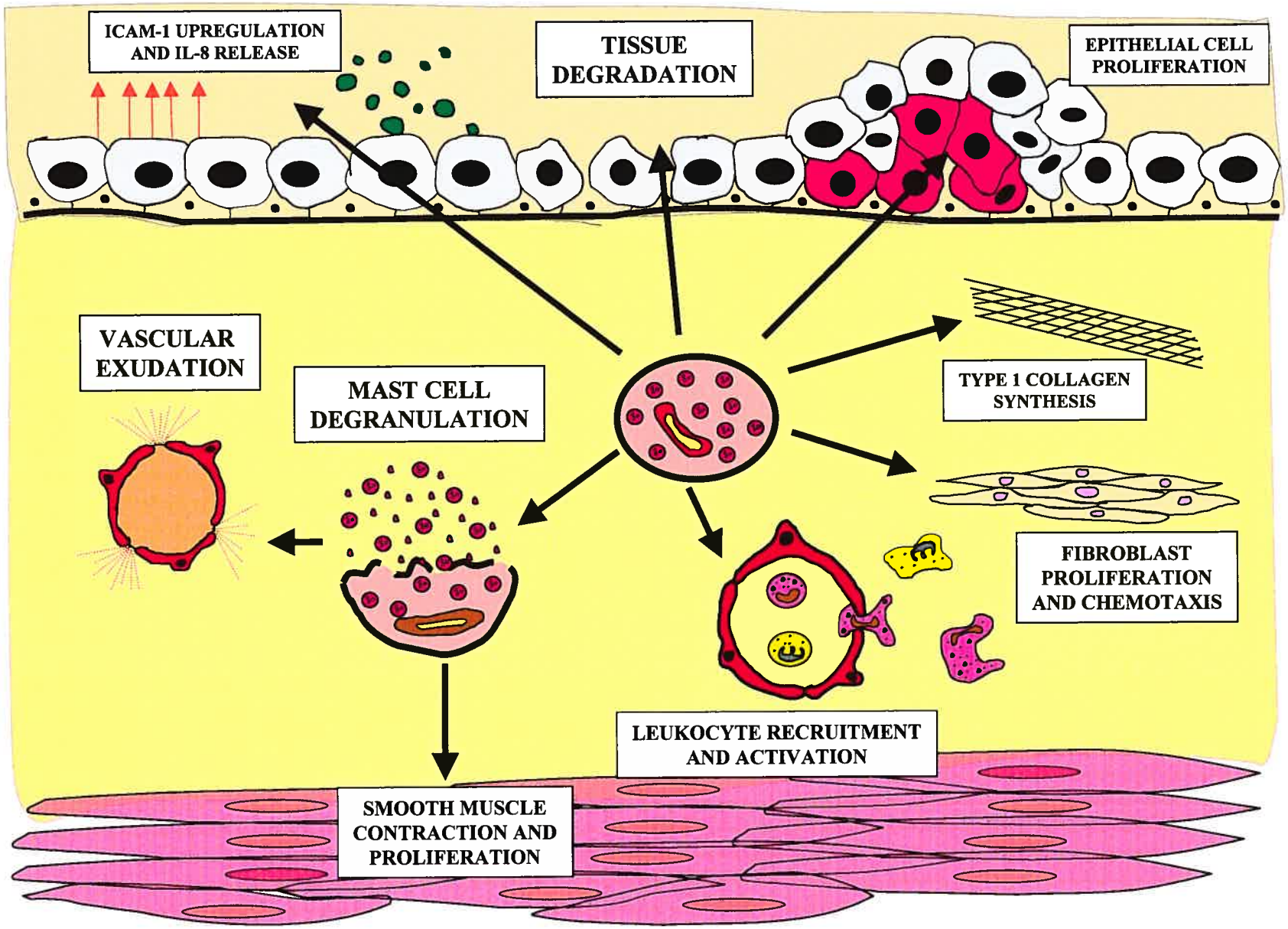
Neutrophils have also been suggested to play a role in mucus production and secretion, both prominent features in severe asthmatics. Several animal studies support a role for neutrophils and neutrophil elastase in both the upregulation of MUC-5 mRNA and protein, and in the degranulation of goblet cells (120,121). In animal models, removing the neutrophil appears to decrease mucus production (122). As well, data suggest that the neutrophil might be an important mediator of airway caliber in asthma but might not be an important mediator of bronchial hypersensitivity.

1.3.4 Mast Cells

Mast cells are key cellular participants in allergic disease **(Figure 8)**. Their potential role in asthma was recognized early on with the identification of these cells as major sources of the spasmogenic mediator histamine. The activation of mast cells is known to release a range of potent mediators of inflammation, including proteoglycans (heparin, chondroitin sulphate), proteases (tryptase, chymase), cytokines (IL-4, GM-CSF) and lipid mediators such as prostaglandins(PG) D_2 and LTC_4 which leads to bronchoconstriction. These products may be stored in the prominent secretory granules of these cells and are released following cell activation. Mast cells can be stimulated to degranulate by cross-linking allergen-specific IgE bound to high-affinity IgE receptors on the cell membrane. Mast cells express approximately 300 000 high affinity IgE receptors/cell, but cross-linking only 100 receptors will result in detectable responses (123). Mast cells may also be activated by diverse stimuli acting through other receptors (specific allergens, adenosine, neuropeptides, opiates). Histamine, the best studied of mast cell products, accounts for 5-10% of mast cell granule content, and is stored in association with proteoglycans. Histamine receptor stimulation results in smooth muscle contraction, increased vascular permeability, and prostaglandin generation.

In the airways, mast cells are abundant in the mucosa. They may also be present in the submucosa, particularly in the vicinity of mucus glands, and small numbers are free in the lumen, where they are well placed to respond to inhaled allergens. Mast cell derived mediators have been found in lavage fluid from patients with asthma, supporting the role of these cells in the immediate or early allergic reaction in asthma (123). When allergen challenge preceded BAL, increases were documented for histamine, LTD_4, PGE_2, and tryptase (123). The role of mast cells in the late allergic response has been more difficult to resolve. However, they are thought to play a key role in the development of the chronic inflammatory phase through their production of cytokines and chemotactic factors that lead to the recruitment of other cell types such as eosinophils (107).

Figure 8: Cellular and tissue targets of human mast cell tryptase



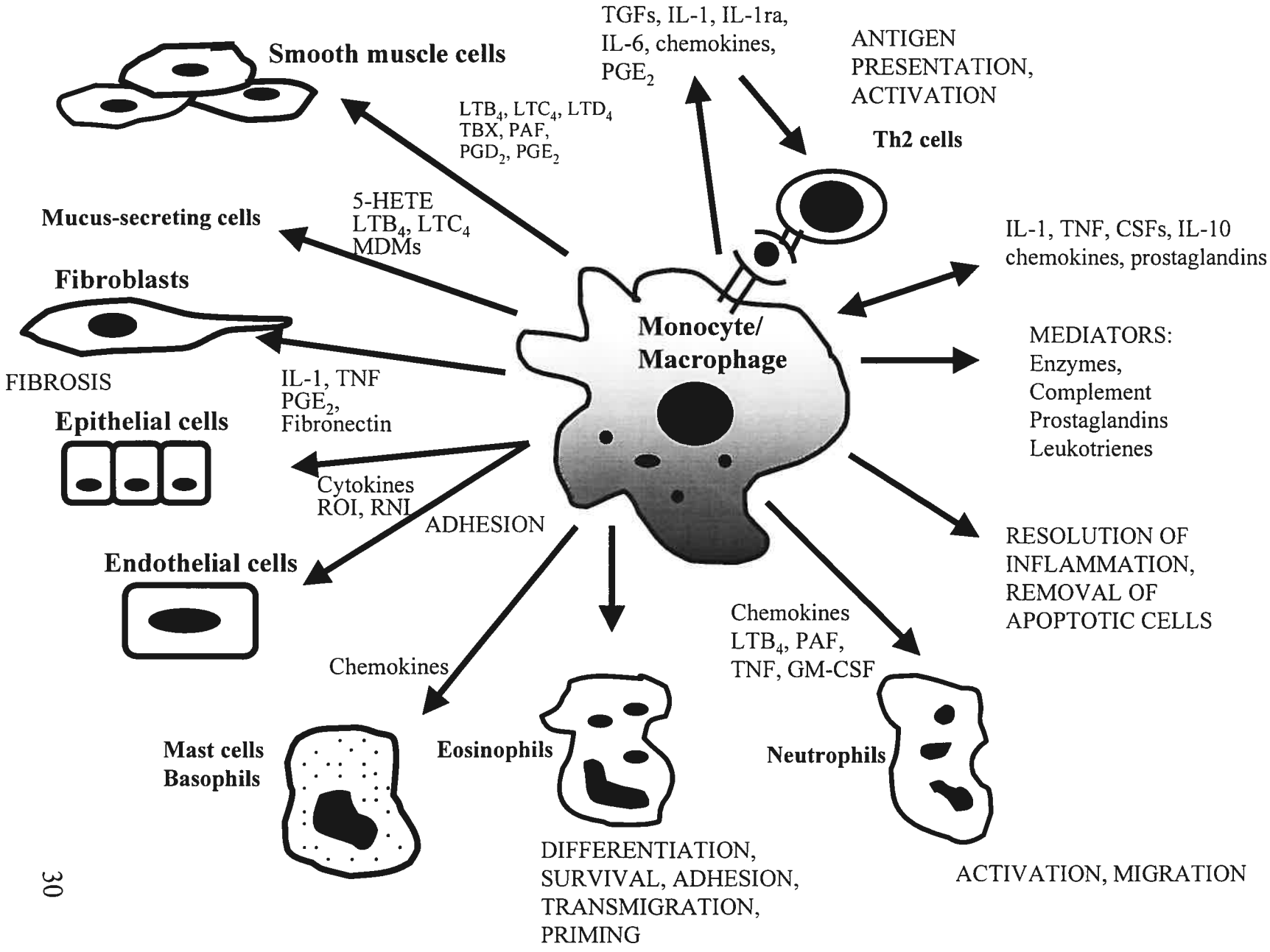
1.3.5 Macrophages

Monocytes and macrophages are cells of the mononuclear phagocyte lineage and are found in essentially every human tissue and body cavity. They derive from immature bone marrow precursors that are released into the blood, where they are termed monocytes, and are then recruited to tissues where they undergo tissue-specific terminal differentiation to macrophages. Macrophages exhibit a wide range of functions, ranging from the capacity to phagocytose and kill microorganisms, to presentation of antigen to T cells and the release of large quantities of numerous soluble mediators **(Figure 9)**.

Macrophages can be activated via the cross-linking of surface IgE bound to the high and low affinity receptors for IgE. Activation through the low affinity IgE receptor, FceRII, leads to release of soluble mediators, including proinflammatory cytokines and leukotriene B_4 and C_4 (124,125). Recent studies have shown greatly increased efficacy by monocytes of antigen uptake and presentation to T cells via the high affinity IgE receptor, FceRI (126).

The pulmonary immune system represents a highly specialized and unique environment. Despite containing the greatest proportion of memory T cells of any compartment of the body and having continual exposure to foreign antigen in the air we breathe, little or no immune activation is observed in the lungs of normal healthy individuals. Such activation would be likely to result in damage to the fragile, permeable epithelial lining of the airways that permits gaseous exchange to occur, a primary and essential function of the lung. Macrophages play a central role in preventing such activation from occurring. Alveolar macrophages, located on the distal side of the epithelial lining of the lung, have a highly phagocytic and microbicidal nature. They are responsible for eliminating inhaled particulate antigens, such as microbes, allergens, and toxic substances, by physical means, namely ingestion followed by degraduation and elimination. In doing so, inadvertent and unnecessary immune activation is prevented.

Figure 9: Soluble mediators synthesized by mononuclear phagocytes and their potential role in regulating events in asthmatic inflammation



1.3.6 Dendritic Cells

Over the last 25 years, it has become clear that dendritic cells (DC) are the major antigen-presenting cells inducing the primary immune response *in vivo* (127). DCs capture antigens in the peripheral tissues and carry it into the T cell area of draining lymph nodes, where naive T lymphocytes are continuously re-circulating in search of specific antigens. Although many cell types such as macrophages and eosinophils have been shown to transport antigens into the draining nodes, the directed migration into the T cell area is a specialised function of DCs (128). The capacity to uptake antigens is a feature of immature DCs residing in peripheral tissues, and is largely lost during the migration of DCs into the draining lymph nodes. This way, immature DCs effectively make a "snapshot" of the antigens present in a peripheral inflammatory site. Interactions in the draining lymph nodes are important for clonal expansion, differentiation, and avoidance of anergy in T cells.

Lung DCs have an immature phenotype, specialized for uptake and recognition of inhaled antigen, but not yet capable of stimulating naive T cells, because they lack co-stimulatory molecules (129,130,131,132). When antigen is encountered in an inflammatory context, there is a dramatic change in the behaviour of the DCs, a process called maturation. Upon recognition of foreign antigens, DCs have to migrate from the periphery to the draining lymph nodes against the chemotactic gradient that attracts immature DCs. Therefore, upon recognition of antigen, DCs lose responsiveness to lung-expressed chemokines, e.g. by downregulation of the CCR6 receptor, but at the same time increase the expression of the CCR7 molecule, which directs the DCs towards the lymph nodes (133). The migration of airway DCs in response to an immunogenic stimulus is rapid and within 12h, lung derived DCs can be traced in the T cell area of the mediastinal lymph nodes of the lung (128,134,135).

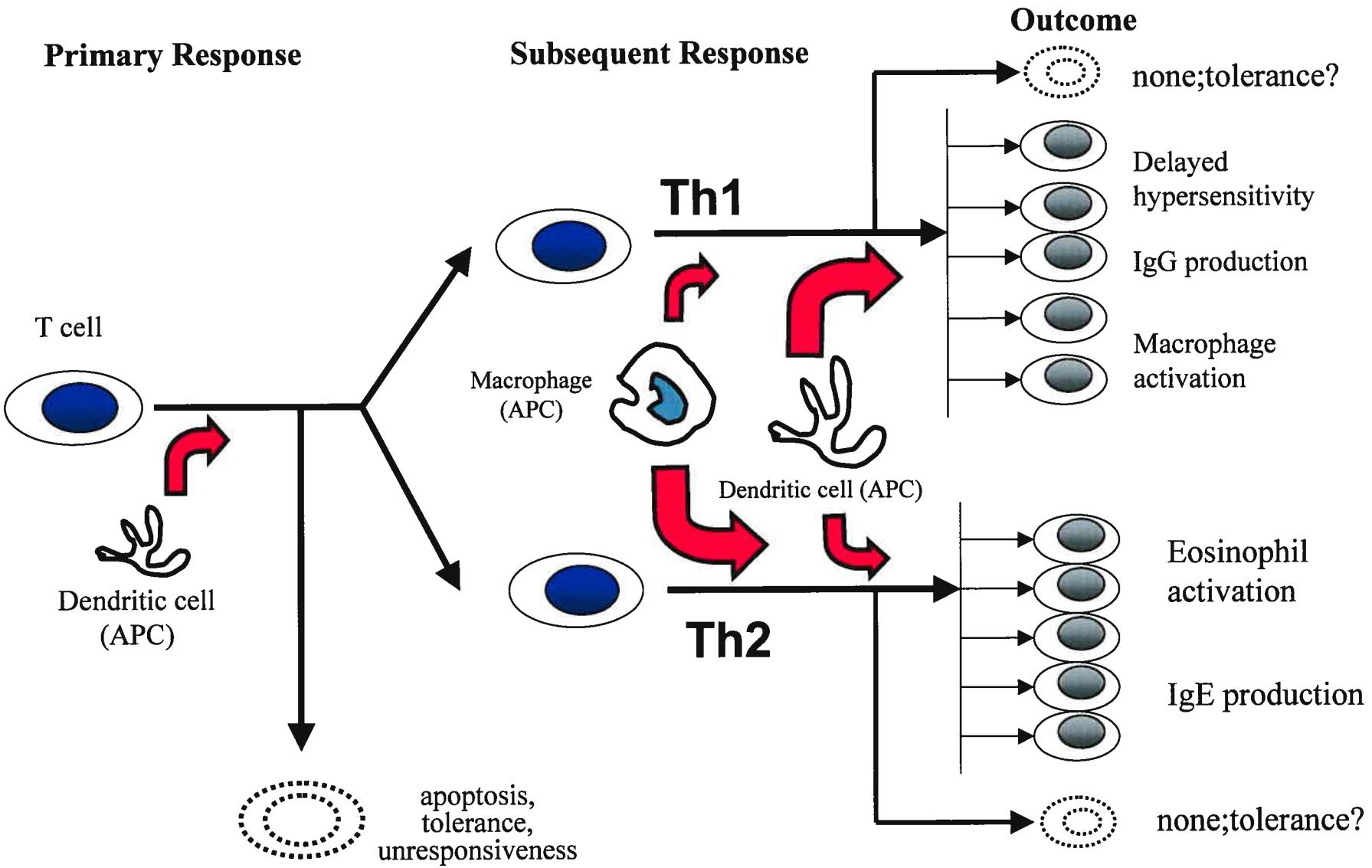
The increased presence of DCs in the airways of atopic asthmatics and allergen exposed animals suggests that DCs have a critical contribution to the disease pathogenesis (136,137,138). They have been associated with Th2-dependant sensitization leading to eosinophillic airway inflammation (139). Reducing the number of DCs either experimentally or by inhaled corticosteroids is associated with a reduction in eosinophillic airway inflammation (140).

1.3.7 Lymphocytes

It is widely believed that T cells acting via the release of cytokines are central regulators of human airway inflammation and in turn, the abnormalities in lung patho-physiology in asthma such as wide spontaneous fluctuations in airway caliber, bronchial hyperreactivity and airflow obstruction are the direct consequence of inflammation of the bronchial mucosa. Th2-based T cells (T cell populations able to secrete IL-4, IL-5, IL-6, IL-9, and IL-13) are dominant effector cells in the pathogenesis of asthma. Lymphocytes are activated by antigen-presenting cells and adopt functional phenotypes under instruction from soluble and physical signals that they receive during antigen presentation. They expand by proliferation, reaching sites of inflammation under the instruction of patterns of chemokines and adhesion molecules where, as armed effector cells, they affect other leukocytes. The abbreviations Th1 (T helper cell type 1) and Th2 (T helper cell type 2) have been classically referred to $CD4^+$ $\alpha\beta$ TCR T cell subsets that are crucial to both the innate and adaptive immune systems. Other lymphocytes have been reported to produce Th1/Th2-type subsets including $\gamma\delta$ TCR T cells (141,142), and $CD8^+$ $\alpha\beta$ TCR T cells (143). Multiple Th forms have been described (e.g. Th1, Th2, Thp, Th0, and Th3) (141,144,145,146,147), and the terms naive, effector, and memory are often mentioned within the same context (141,144,148,149). Antigen-naive T cells are designated Thp for precursor of T helper cell (144). Antigen exposure to a Thp cell results in the selective maturation to either Th1 or Th2 cells. The Th phenotypes are characterized by the cytokines they produce. The first Th cell types characterized were mouse Th1 and Th2 cells. Mouse Th1 cells secrete IFN-γ, while Th2 cells secrete IL-4 (150). In humans, Th1 cells have been identified to secrete IFN-γ, while Th2 cells secrete IL-4 and IL-5 (151). Subsequent studies have established that Th1 cells produce IFN-γ, TNF-β, and IL-2, while Th2 cells produce IL-4, IL-5, IL-6, and IL-13 (144,147). Another Th cell type with a unique cytokine secretion pattern is the Th3 cell (146,152,153) which appears to be a $CD4^+$ immune regulatory T cell that secretes TGF-β (146). Th0 cells have been described as producing both IL-4 and IFN-γ (141,144), but their actual existence is controversial.

Th1 and Th2 cells have been associated with specific immune responses due to the cytokines they secrete **(Figure 10)**. For pathogens that require internalization, the presence of Th1 cytokines (IFN-γ and TNF-β) is considered necessary. Conversely, for large extracellular parasites such as helminths, Th2-type cytokines (IL-4 and IL-5) have been considered most protective (145,154,155,156). In the case of

Figure 10: Antigen-presenting cells (APC) and the nature of immune responses



Th1-type cytokines, IFN-γ has a multitude of functions. It promotes phagocytosis and upregulates microbial killing. In particular, it induces IgG_{2a} (in mice) which is known to opsonize bacteria. On phagocytes, IFN-γ promotes the expression of $Fc\varepsilon R_I$ receptors, which are used for phagocytosis. IFN-γ provides all the tools necessary to eliminate most external microbes (155,156,157,158). For the classic Th2 cytokines, IL-4 promotes production of neutralizing antibodies (IgG_1) and the mast cell/eosinophil degranulating antibody IgE (157,158). IL-4 also promotes upregulation of IgE receptors on mast cells, eosinophils and macrophages, and it induces membrane expression of MHC class II molecules and the IL-4 receptor on macrophages (157). IL-4 and IFN-γ often exist in an antagonistic relationship. IFN-γ blocks IgE and IgG_1 production, while IL-4 blocks IgG_{2a} secretion (158).

The majority of allergen-specific T cell clones derived from the peripheral blood of atopic individuals produce increased amounts of IL-4 and IL-5 and lower levels of IFN-γ (159). Collectively the current biology of T cells in asthma suggest that they have the most important role in regulating cellular inflammation.

1.3.8 Leukotrienes

The cysteinyl leukotrienes (cys-LT) LTC_4, LTD_4, and LTE_4, originally described as the slow-reacting substance of anaphylaxis (SRS-A), are proinflammatory mediators that play an integral role in the pathophysiology of asthma. Cys-LTs function as potent bronchoconstrictors and asthmatic patients demonstrate increased production of cys-LT during naturally occurring asthma and acute asthma attacks as well as after allergen and exercise challenge (160).

Leukotrienes are eicosanoids that are produced *de novo* from the cell membrane phospholipid arachidonic acid by the activity of 5-lipoxygenase in conjunction with 5-lipoxygenase-activating protein. This reaction generates the unstable leukotriene A4, which is, in turn, converted either to the chemotaxin LTB_4, or to cysteinyl leukotrienes LTC_4, LTD_4, and LTE_4 (161). In humans, the cysLTs are at least 100-1000 times more potent bronchoconstrictors than histamine (162). They also increase airway blood flow and vascular permeability, allowing the exudation of plasma macromolecules and contributing to the airway edema that characterizes asthma (163,164,165). In addition, the cys-LTs induce mucus secretion (166) and reduce respiratory ciliary motility, hampering mucociliary clearance (167).

LTD_4 and LTE_4 are potent and specific chemoattractants for eosinophils which are recognized as the predominant cell type in asthma-related inflammation (168). A recent study has forged an important link between cys-LTs and the variable eosinophil response to allergen challenge (169). Of 18 asthmatic patients, 9 produced a significant (>200-fold) increase in cys-LT levels in the BAL 24 hr after inhaled allergen challenge, while the other 9 showed no significant increase (<20-fold). Only the high LT producers showed a significant rise in BAL eosinophils after allergen challenge, accompanied by increases in LTB_4, IL-5, IL-6, and total protein. The "high LT producer" phenotype may thus be synonymous with a subgroup of asthmatics with a high level of airway eosinophilia. The cellular sources of excess cys-LT production in the airway are unclear. Following inhaled allergen challenge, release of LTC_4 into BAL fluid is associated with simultaneous release of histamine, PGD_2, and tryptase, suggesting a mast cell source for all four mediators (170). However, in persistent asthma, the eosinophil itself may be an important source of LTC_4. In allergic and nonallergic asthmatics with mild, moderate and severe disease, eosinophils represent the majority of bronchial mucosal cells expressing LTC_4 synthase, the terminal enzymes in the LTC_4 synthetic pathway, with only small numbers of mast cells and macrophages expressing the enzyme. In mild allergic asthma patients with seasonal symptoms, the numbers of cells expressing LTC_4 synthase rose 4 to 5 fold in the pollen season and the overwhelming majority of these cells are eosinophils (171). Taken together, the data suggest that the release of cys-LTs from eosinophils themselves may sustain a self-perpetuating cycle of eosinophil recruitment and further cys-LT synthesis in a significant subpopulation of asthmatics. A high capacity for cys-LT synthesis appears to be the predisposing factor, possibly triggered by cys-LTs released from allergen-activated mast cells. Within the airway, the capacity of recruited eosinophils to generate cys-LT may be upregulated by Th2 type cytokines (172) and also by contact with human bronchial epithelial cells (173). Eosinophil-derived cys-LTs may be most important in chronic impairment of lung function, with mast cell-derived cys-LTs more important in the acute response to allergen and other stimuli.

1.3.9 Cytokines

Inflammation of the airways underlies the pathology of respiratory disorders such as asthma and chronic obstructive pulmonary disease. No one mediator of inflammation is responsible for all the clinical

and pathological changes of asthma **(Figure 11)**. The primary inflammatory profile of asthma consists of an accumulation of CD4+ T helper type 2 (Th2) lymphocytes and eosinophils in the airway mucosa. Th2 cells orchestrate the asthmatic inflammation through the secretion of a series of cytokines, particularly IL-4, IL-13, and IL-5.

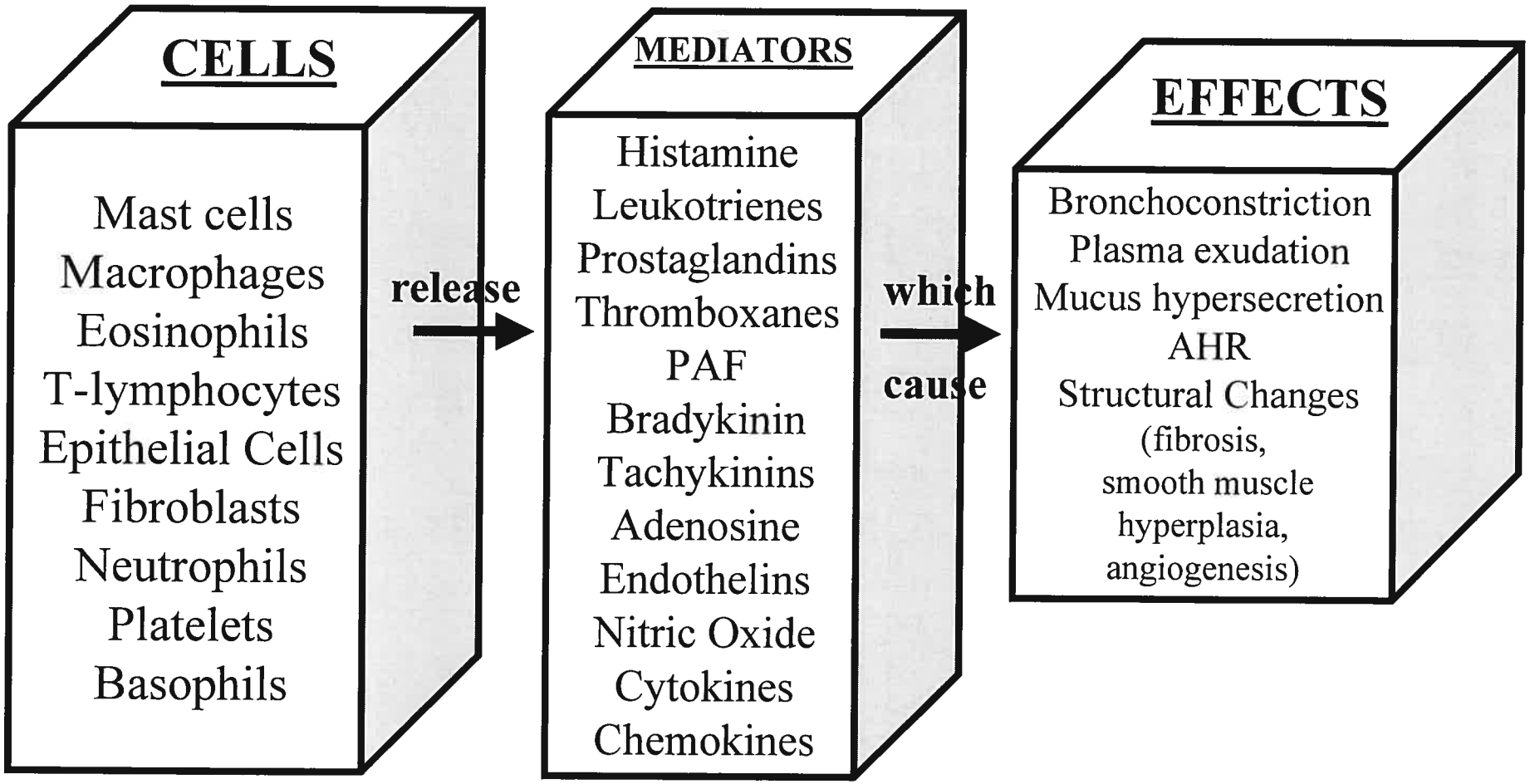
1.3.9.1 Interleukin (IL)-4

IL-4 is a 20 kDa monomer secreted by Th2 cells, mast cells, basophils, and eosinophils. It was originally identified as a B cell growth factor, which drives the optimal stimulation of B cells by antigen (174). In addition, IL-4 stimulates B cell expression of major histo-compatability complex (MHC) class II molecules, b7-1 (CD86), CD40, surface IgM, and the low affinity IgE receptor, resulting in enhanced antigen presenting capacity of B cells (144). IL-4 also induces the immunoglobulin isotype switch from IgM to IgE (175,176,177,178). Using *in vitro* priming models, it was shown that IL-4 is required for the generation of IL-4 producing T cells (144). Another important activity of IL-4 in allergic inflammation is inducing the expression of VCAM-1 on endothelial cells. This will produce enhanced adhesiveness of the endothelium for T cells, eosinophils, basophils, and monocytes, which is characteristic of allergic reactions (179).

The role of IL-4 in IgE production can not be extended to inducing AHR, which is a major characteristic of asthma. Overexpression of IL-4 in lungs leads to a lymphocytic and eosinophillic inflammation, but without AHR (180). Moreover, mice deficient in B cell antibody production can exhibit the phenotype of the experimental asthma model (181,182). Thus, an essential effector role for IL-4 in experimental asthma has been difficult to show. Although IL-4 appears to play an important role in Th2 cell development and recruitment to the airways (183,184,185), it is now generally accepted that IL-4 does not have a similar role in the development of AHR, at least in mouse models.

Nevertheless, it has been shown that IL-4 receptor (IL-4R) blocking antibodies inhibit allergen-induced AHR (186), which is in contrast with the lack of effect of anti-IL-4 antibodies on AHR in the same model. Similar observations were obtained with IL-4Rα deficient mice (187), which turned out to be more resistant to the development of asthma than IL-4 deficient mice, suggesting that another IL-4Rα interacting cytokine plays an important role in this model. The obvious candidate is IL-13, a cytokine

Figure 11: Cells and Mediators involved in Asthma Phenotype



closely related to IL-4, which binds to IL-4Rα and is also produced by Th2 cells from patients with asthma (188). Overexpression of IL-13 in the lungs induces inflammation, mucus hypersecretion and subepithelial fibrosis. In addition, intratracheal IL-13 administration can lead to airway hyperresponsivness, eosinophilia, and increased IgE production in a mouse model (189,190). The candidate mechanisms for IL-13 effects include direct alterations in epithelial cells or smooth muscle function because IL-13 receptors have been detected recently on these cell types (191). Direct evidence for the role of IL-13 is confirmed by the observation that blocking its activity by intratracheal administration of soluble IL-13 receptor (IL-13R) reduced AHR and mucus production in a mouse model of asthma (189,190,192).

1.3.9.2 Interleukin (IL)-5

Several lines of evidence suggest that IL-5 is important in asthma. IL-5 is an important growth factor for eosinophils (193) and is increased in the lungs of allergic and nonallergic asthmatics (194). IL-5 expression in the lungs and sputum of patients with allergic asthma increases 24 hours after antigen challenge (195) and a clear correlation exists between IL-5 expression and the presence of eosinophils in the airways of patients with asthma (196). Intra-tracheal administration of IL-5 to human asthmatics or to sensitized animals with the characteristics of atopic asthmatics increases the airway response to antigen (197,198).

IL-5 acts on B cells and eosinophils in the mouse and seems to be restricted to eosinophils in humans (199). IL-5 is crucial in regulating the eosinophillic response both *in vitro* (200) and *in vivo*, as seen during helminth infections (201). Transgenic mice in which IL-5 is constitutively expressed in all T cells show a profound and life long eosinophilia, with large numbers of eosinophils in the blood, spleen, and bone marrow (202). When the IL-5 gene is inactivated in sensitized mice challenged with an antigen aerosol, lung eosinophilia is absent and very little inflammation and lung damage is observed (114). Similarly, anti-IL-5 antibodies decreases the eosinophil infiltration induced by OVA inhalation in the trachea of sensitized mice (203). Whereas blocking IL-5 reduces eosinophil responses to allergen, this strategy falls short of inhibiting AHR, both in mouse experimental models (204) and in preliminary human observations (205). Thus, airway eosinophilia is not a requirement for allergen induced airway responsiveness (206,207). Although blocking IL-5 might fail to prevent the acute phase of asthma, this

approach could be an efficient way to interfere with long term airway remodelling, a process in which eosinophils are thought to be crucial.

1.3.9.3 Interferon-γ

IFN-γ is a glycoprotein produced by T cells ($CD4^+$, $CD8^+$, γδ) and NK cells (208). It binds the IFN-γ receptor (IFN-γR) as an antiparallel dimer. Although the IFN-γRα subunit binds IFN-γ with high affinity, it requires an additional IFN-γRβ subunit to transduce intracellular signals (209,210). The resultant cascade involving tyrosine phosphorylation of signal transducers and activators of transcription (STATs) activates up to at least 20 identifiable IFN-γ responsive genes. To date, 12 of these are unique to IFN-γ. The transcription of these genes determines the biological and biochemical effects of IFN-γ (211).

The ability of IFN-γ to activate macrophages is central to its role in cell-mediated immunity. IFN-γ enhances macrophage microbicidal actions through the regulation of genes coding for the enzymes of nitric oxide (NO) synthase and the NADPH oxidase system (212). In addition to activating macrophages, IFN-γ has been shown to recruit and activate inflammatory and Th1 cells. IFN-γ can directly induce Th1 cell proliferation and augment antigen presentation and T cell activating capacity of macrophages. It achieves this function by increasing the cell surface expression of major histocompatability class II (MHC II) and B7 on macrophages (213). IFN-γ facilitates the recruitment of lymphocytes and macrophages to inflammatory sites by inducing ICAM expression on endothelial cells and promotes furthur accumulation by the induction of chemotactic factors such as IFN-γ inducible protein 10 (IP10) and LTB_4 (214,215).

IFN-γ promotes the differentiation of $CD8^+$ and $CD4^+$ T cells into active cytotoxic cells (216,217). In addition, IFN-γ enhances cytotoxic T-cell recognition and destruction of infected cells by increasing the expression of surface MHC I. This cytotoxic response releases viable organisms either protected in the cytosolic compartments of cells or persisting in senescent phagocytes, exposing them to a different display of effector inflammatory cells.

A number of cytokines antagonize the effects of IFN-γ. Since inflammation due to Th1 immune responses is associated with tissue damage, during the course of protective immunity against microbes such as mycobacteria, the presence of a system to counterbalance Th1 responses is essential to prevent

progressive tissue damage. This is particularly important because once a Th1 response begins, they tend to become self-perpetuating. The Th2 response is important in the regulation of the Th1 process. IL-4, the signature Th2 cytokine, inhibits inflammatory functions of macrophages and Th1 cells (218). IL-4 inhibits macrophage secretion of the monokines IL-1 and TNF-α and suppresses cytokine production by Th1 cells (218). It also stimulates macrophages to produce IL-1 receptor antagonist (IL-1ra), a naturally occurring inhibitor of IL-1 activity. Both IL-4 and IL-13 activate intracellular STAT proteins (STAT6) (219). These pathways appear to facilitate IL-4 inhibition of the transcriptional activation of IFN-γ inducible proteins. Furthermore, IL-4 inhibits the ability of IFN-γ to augment microbicidal activities including superoxide production. Th1 responses often appear early while Th2 cells predominate later in an immune response that suggests that the *in vitro* observation of Th2 responses may occur *in vivo* to limit the consequences of Th1-mediated protective immunity.

The mechanism(s) by which IFN-γ protects against allergen-induced AHR are unknown. It is possible that the inhibitory effect of IFN-γ on AHR may be mediated by its action in inhibiting eosinophil recruitment. However, there is no significant increase in airway and BAL eosinophilia with anti-IFN-γ antibody treatment, despite a significant enhancement of AHR (220). The protective function of IFN-γ might be conducted by Th1 T lymphocytes or $CD8^+$ T cells suppressing Th2 cells, eosinophils and neutrophils, and ultimately reducing the Th2-orchestrated airway inflammation and AHR.

1.3.9.4 Interleukin-2

IL-2 is pivotal for the generation and regulation of the immune response. In mice, IL-2 is regarded as a defining Th1 cytokine, while in humans, this distinction is less clear (221). IL-2, a glycoprotein with a single molecular size of 15.5 kD, is a pleiotrophic cytokine produced principally by activated T-helper cells (222). It primarily functions in an autocrine fashion to clonally expand antigen-specific T cells and generate memory phenotypes. The IL-2-secreting T helper cells expand early after antigenic stimulation and the subsequent differentiation into Th1 or Th2 cells occurs under the influence of other cytokines (223). Unlike IFN-γ production by $CD4^+$ cells, which requires IL-2 during both priming and expression phases, IL-4 production requires IL-2 only during priming phase (223). IL-2 also acts as a paracrine factor

influencing NK cell, B cell, and phagocyte function (224,225,226). IL-2 augments microbicidal activity, cytokine production, and cell surface expression of cytokine receptors (226).

IL-2 stimulates T cells to undergo cell cycle progression via interactions with its specific membrane receptors (227). Resting T cells do not produce IL-2, nor are they capable of responding to IL-2 when it is added exogenously (228). It has been shown that signals sent out from the T cell antigen-receptor complex co-ordinate the transcriptional activation of both the IL-2 gene and the genes encoding IL-2 receptors (229). Activation of the T cell receptor renders the cells "competent" to receive the cell cycle "progression" signals that are provided by IL-2. IL-2 promotes a gradual and sustained increase in cell size and prepare the cell metabolically for DNA replication. IL-2 is considered the authentic G_1 progression factor for T cells (230).

The essential nature of IL-2 and IL-2 receptors for the generation of a normal immune response is readily demonstrated by a deficiency in the system. For example, immunodeficient athymic mice lack the capacity to produce IL-2 but can respond to IL-2 when it is supplied exogenously (231). Patients suffering from acquired immunodeficiency syndrome have similar immune responses as these athymic mice, namely these patients have a selective loss of helper T cells resulting in the inability to produce IL-2 and other lymphokines in response to antigen challenge.

Stimulation of the immune response via the IL-2 receptor is under clinical investigation as a potential new form of immunotherapy for cancer (232). The exact mechanism responsible for the beneficial effects is unclear, but it could be explained either by the IL-2-directed clonal expansion of tumour-reactive cells or by stimulation of natural killer cells. Others have used IL-2 as an adjuvant for stimulating the immune response to vaccines (233).

1.3.10 Chemokines

Chemokines are small secreted proteins, whose main function is to regulate cell trafficking. They are classified into four subclasses: CC, CXC, C, and CX3C chemokines, based on the location of the first two cysteine residues in their sequence. To date, 23 human CC chemokines, 14 human CXC, and one each of the C and CX3C chemokine subclasses have been described (234). The biological effects of chemokines are mediated by cell surface receptors. Nine CC chemokine receptors, five CXC chemokine

receptors, one CX3C chemokine receptor, and one C chemokine receptor have been characterized. Interestingly, there is a certain degree of promiscuity in the chemokine superfamily, with many ligands binding different receptors and vice versa (234).

The identification of eotaxin as the first chemokine with the preferential ability to recruit eosinophils has drawn much attention to this molecule (235). Eotaxin was first discovered in the BAL fluid of guinea pigs after allergen challenge (236). Unlike other eosinophil chemotactic factors, eotaxin binds to a single receptor, CCR3, which is highly expressed on eosinophils (237,238). In human asthma, eotaxin is produced at high concentrations and localises in the airway epithelium (238). Several other chemokines including RANTES, MCP-3, and MCP-4 can also recruit eosinophils, probably through the CCR3 receptor (238).

In the lung, eotaxin is produced by many cell types. The primary source of eotaxin following allergen exposure is thought to be epithelial cells (239), but eotaxin can be produced by a large number of other cell types including lung fibroblasts, airway smooth muscle, endothelial cells, alveolar macrophages, eosinophils themselves, and lymphocytes (234).

Eotaxin acts in the early stages following allergen exposure, in conjunction with IL-5 to co-ordinate the differentiation of myeloid progenitor cells in the bone marrow and induce the export of these cells into the peripheral blood and subsequently recruited to local sites of inflammation (240). Eosinophil degranulation can be elicited by eotaxin, which has been found to be able to induce respiratory burst and actin polymerisation (240).

The eotaxin-CCR3 axis is also responsible for the recruitment of a subset of Th2 lymphocytes to the allergic site. This has been demonstrated by the ability of a CCR3 antibody to isolate IL-4 and IL-5 producing cells from the peripheral blood of atopic patients and that antibody inhibition of eotaxin in a mouse adoptive transfer model can block the recruitment of Th2 cells into the lung and inhibit eosinophilia and allergen-induced bronchoconstriction (241,242,243). It has also been shown that human blood basophils express high levels of the CCR3 receptor and *in vitro* will release inflammatory mediators such as histamine and leukotrienes following stimulation with eotaxin.

One of the several possible explanations for the redundancy of chemokines, particularly for the CCR3 receptor, is that they may be expressed in a temporal fashion. Studies on the kinetics of cell

recruitment and chemokine expression showed that eotaxin plays a major role in the recruitment of eosinophils during the early phase reaction at 6h, but that eotaxin-2 and MCP-4 are involved in the late phase accumulation of eosinophils at 24h post-challenge (244). Moreover, recent data from mouse studies have produced evidence that Th2 lymphocyte recruitment into the lung is at least partially dependent on the involvement of eotaxin up to a 4 day time point, but that by day 7, monocyte-derived chemokine (MDC) becomes predominant in regulating Th2 cell infiltration in an adoptive transfer model of lung inflammation (243). Thus, the recruitment of inflammatory cells over the entire period of the course of a disease state like allergic asthma will likely be dependent on the spatial and temporal regulation of chemokine expression.

1.4 **Asthma Therapy**

1.4.1 Drugs

Management of asthma begins with educating the patient about the disease. It also includes environmental control interventions and the management of complicating factors such as sinusitis and gastroesophageal reflux. Recent advances in understanding asthma pathogenesis, including the roles that cytokines, leukotrienes, adhesion molecules, and transcription factors play in the recruitment and infiltration of inflammatory cells have led to promising therapies, potential or already in use. These include new generation inhaled steroids, leukotriene blockers, and antibodies against IgE. New inhalation devices are using dry-powder formulations and vehicles such as hydrofluoroalkanes to deliver the active drug. Experimental therapies include cytokine antagonists, adhesion-molecule blockers, and transcription factor inhibitors. Oligonucleotide therapy, DNA vaccination, and new phosphodiesterase and tryptase inhibitors are also being tested. Much of the research has focused on airway remodelling, and so the outlook for not only asthma management but even reversibility or a cure is becoming optimistic.

The current options for asthma drugs can be placed in 4 categories: bronchodilators, anti-inflammatory drugs (nonsteroidal and glucocorticosteroids), leukotriene modifiers, and phosphodiesterase inhibitors.

1.4.1.1 Bronchodilators

Short-acting bronchodilators such as β_2-adrenergic agents salbutamol and penoterol and pirbuterol are useful as rescue medication and for acute symptom relief but they tend to have increased systemic side effects. A long-acting bronchodilator, salmeterol, combines the active site of salbutamol with a highly lipophillic side chain that appears to attach to an exosite of the β_2-receptor on bronchial smooth muscle, holding the salmeterol molecule in place for an extended period of time. Salmeterol is a highly selective β_2-agonist. Combined with moderate or even low doses of inhaled corticosteroids, it improves lung function in patients with asthma and increases the number of symptom-free days.

1.4.1.2 Anti-inflammatory drugs

In persistent asthma, anti-inflammatory medication is crucial. The cromones – cromolyn sodium and nedocromil, are acceptable for mild disease. However, they are not as effective as inhaled glucocorticoids for controlling inflammation or preventing airway remodelling. For many years, the treatment of asthma has depended heavily on the use of glucocorticoids. These agents were introduced into clinical practice in the 1940's and proved extremely effective in controlling asthma in most patients with the condition (245). However, long-term use of oral steroids leads to significant and unacceptable side effects, so oral corticosteroids (OCS) therapy is restricted to those whose disease cannot be treated effectively by any other means. The introduction of inhaled corticosteroids (ICSs) in 1970 was a major advance, allowing many patients with relatively mild asthma to receive regular inhaled steroid therapy (246).

Until recently, metered-dose inhalers (MDI's) were the only devices available to deliver inhaled asthma drugs. These hand-held, pressurized, multiple-dose systems administer aerosolized particles measuring one to seven microns. They have disadvantages, including difficulty of use, need for spacer devices, and use of chlorofluorocarbons (CFCs), considered as the primary cause of ozone depletion, as vehicles. The first CFC-free inhaler became available in 1995. Since then other types of inhalers have been introduced including dry powder inhalers, and inhalers using hydrofluoroalkanes as vehicles (permitting a reduced particle size, which in turn improves distal penetration). Inhaled glucocorticoids include beclomethasone, triamcinolone, flunisolide, budenoside, and fluticasone. A preparation of

beclomethasone with ozone-friendly hydrofluoroalkane propellant is now available. A combination of fluticasone and salmeterol is available as well as a combination of budenoside with another long-acting β_2-agonist formoterol. At low doses, inhaled glucocorticoids are considered highly effective and safe. At higher doses, bone growth and metabolism may be affected and bone density reduced.

1.4.1.3 Leukotriene Modifiers

These agents are fairly new and controversies persist on precisely when to use them. Zafirlukast and Montelukast are Cys-LT_1 receptor antagonists. Zileuton is the only available inhibitor of leukotriene synthesis, acting by inhibiting 5-lipoxygenase. Zafirlukast is approved for use in asthmatic children at least seven years old and Montelukast for children at least two years old. As a group, leukotriene modifiers alleviate asthma symptoms, improve objective measures of airway function, and decrease the need for concomitant β_2 rescue medication and inhaled corticosteroids. Individually, Montelukast has been shown to be effective in reversing exercise-induced bronchospasm. Leukotriene modifiers can be beneficial given in concert with other agents (more in 1.4.2.1).

1.4.1.4 Phosphodiesterase Inhibitors

Theophylline, a bronchodilator was employed for many decades as asthma therapy and may have potential as an adjunctive agent or for severe asthma in patients not optimized on high dose inhaled glucocorticoids. The drug is known to inhibit several of the intracellular enzyme phosphodiesterases, which protect signal pathways essential for β-adrenergic cellular responses, but studies have also demonstrated anti-inflammatory effects unrelated to phosphodiesterase inhibition.

1.4.1.5 Steroid-sparing strategies

Much effort has gone into the search for safer inhaled steroids since it has been shown that inhaled beclomethasone is absorbed and has detectable systemic effects in terms of reductions in short-term linear growth rates (247), but at present it remains uncertain whether these effects have any long-term adverse consequences on final height or on any other aspect of health (248).

Troleandomycin: A macrolide antibiotic that was formerly used quite widely in asthma exacerbations. It also inhibits the hepatic metabolism of methyl prednisolone (249), and it seems likely that the beneficial effects results from this mechanism.

Cyclosporin A (CyA): A fungal metabolite that has successfully been used as an immunosuppressant agent in the prevention of rejection after organ transplantation. The principal action of CyA is to inhibit T-cell activation and suppress the production of cytokines associated with activation. CyA blocks the late asthmatic reaction and inhibits eosinophil-associated cytokines after allergen challenge (250). However, given the potential severity of side effects, there is insufficient evidence of benefit to recommend its use.

Gold: Gold can inhibit some of the processes implicated in asthma, including IgE dependant histamine release and neutrophil chemotaxis. Although gold can reduce doses of oral corticosteroids, there is evidence of side effects which precludes its use as a promising therapy.

1.4.2 Therapeutic guidelines

Expert panels have classified asthma as intermittent or persistent. Persistent disease is further classified as mild, moderate, or severe. Intermittent asthma can be managed with inhaled β_2-agonists. Exercise-induced intermittent asthma may be relieved by warm-up exercises and use of β_2-agonists, cromolyn sodium, or nedocromil taken prior to exertion. Recent reports suggest that for exercise-induced asthma a formoterol inhaler or Montelukast may provide more sustained relief than short-acting β_2-agonists (251).

Mild persistent asthma requires long-term control medication, usually meaning the initiation of anti-inflammatory agents such as low-dose inhaled corticosteroids. Other alternatives include leukotriene modifiers, cromolyn or nedocromil. Acute asthma symptoms can be managed with intermittent use of inhaled β_2-agonists or occasional bursts of oral glucocorticoids. For moderate persistent asthma, management includes long-term control medications such as moderate-dose inhaled corticosteroids or a combination of low-dose inhaled steroids with a long-acting bronchodilator such as salmeterol or formoterol. Severe persistent asthma usually requires oral glucocorticosteroids. It is important to use the lowest possible dose, to administer the drug on alternate days, and to monitor closely for adverse effects

including osteoporosis and cataracts. Leukotriene modifiers may have some steroid-sparing potential and under adequate management and with adequate compliance, many patients may be weaned from oral glucocorticoids.

1.4.2.1 Efficacy of Inhaled corticosteroids vs antileukotriene drugs

The cornerstone of asthma treatment is inhaled corticosteroids. Their efficacy is a result of their potent and broad anti-inflammatory properties. Antileukotriene drugs provide an alternative and novel approach to the treatment (252). The novelty of these new compounds is that they are targeting cysteinyl leukotrienes, a major player in the pathophysiology of asthma. The antileukotriene drugs are more effective than placebo, but they are not as effective as inhaled corticosteroids in improving lung function, reducing β_2-agonist use, and decreasing symptom-free days. In contrast, they may have similar beneficial effects on reducing asthma exacerbations and decreasing peripheral blood eosinophil counts. For patients with mild and moderate disease there are a number of circumstances that support using an antileukotriene drug first, such as in cases of aspirin tolerance, predominantly exercise-induced symptoms and problems with using an inhaler or the adverse effects of inhaled corticosteroids such as dysphoria and thrush. In addition, it has been shown that inhaled corticosteroids are not effective in all patients with asthma (253). Corticosteroids produce minimal suppression of leukotriene production, and in some cases enhance production (254,255).

Currently, four antileukotriene drugs are approved for use in one or more countries. They are the leukotriene synthesis inhibitor zileuton and the cys LT_1 receptor antagonists zafirlukast, montelukast, and pranlukast. Although these drugs differ in several pharmacokinetic and pharmacodynamic properties, and in other properties as well (e.g. adverse effects, drug interactions), they are all effective in treating patients with asthma (256,257).

The first published study that directly compared an antileukotriene drug and an inhaled corticosteroid was reported by Malmstrom and colleagues in 1999 (253). This was a randomized, double-blind, placebo-controlled, parallel group design, 12-week trial comparing the oral leukotriene receptor antagonist Montelukast (10mg once daily at bedtime) with the inhaled corticosteroid beclomethasone (200µg twice daily). Both treatments produced clinically important beneficial effects, reduced peripheral

blood eosinophils and did not cause rebound loss of asthma control when therapy was stopped. The key differences were that beclomethasone produced a larger beneficial effect, while montelukast had a more rapid onset of action. Overall, using the patient populations described in the studies (mostly patients with mild to moderate asthma) inhaled corticosteroids are more effective in the clinical trial setting. They produce a greater improvement in lung function, as measured by FEV_1, and a similar or greater reduction in daytime and night-time symptoms and β_2-agonist use. However, these results do not take into account differences in adherence to prescribed therapies in the "real world" setting. Problems related to improper use of inhaled corticosteroids, and the unwillingness of some individuals to take inhaled medications, result in beneficial effects that are typically less than what one would expect based on the controlled clinical trials. These issues appear to be less important with the orally administered anti-leukotriene drugs.

On the basis of the available data, inhaled corticosteroids have the advantage over the antileukotriene drugs as monotherapy for the treatment of mild to moderate asthma. However, in patients with moderate to severe disease, treatment requires the use of more than one medication. The overwhelming consensus is that inhaled corticosteroids should be one of the medications. Which drug to add next is more controversial. One potential advantage of the anti-leukotriene drugs over the others is their ability to reduce airway inflammation in addition to their beneficial effect on pulmonary physiology.

1.4.3 Alternative Therapies

1.4.3.1 Anti-IgE

Since its role in the pathogenesis of allergic disease is central, IgE is an attractive target for therapy. The first specific, selective anti-IgE therapy developed for study of humans is a unique humanized monoclonal anti-IgE antibody called rhuMAb-E25 (or omalizumab). Omalizumab inhibited both early and late phase reactions (258,103). Asthma exacerbations were significantly less frequent in the omalizumab group than in the placebo group. Interestingly, even subjects receiving placebo experienced some improvement, meaning that the difference from active treatment is difficult to demonstrate (259).

1.4.3.2 Anti-histamine

Most new antihistamines display additional antiallergic effects and can modulate some inflammatory phenomena (i.e. expression of adhesion molecules on epithelial and endothelial cells, recruitment of eosinophils and mononuclear cells at the site of disease) (260). Therapeutically, its use is improbable because antiallergic effects are usually obtained *in vitro* at concentrations much higher (10-1000 times) than *in vivo*. Therefore, antihistamines cannot be presently considered as an alternative to any of the standard antiasthma drugs.

1.4.3.3 Cytokines

Strategic approaches for cytokine inhibition include the blocking of transcription factors that lead to their expression, blockade after release, cytokine receptor antagonism, and the inhibition of signalling pathways that are activated after cytokine-receptor binding. Results with a humanized anti-IL-5 antibody have been disappointing (205). Although successful in markedly reducing circulating eosinophils and in preventing eosinophil accumulation in the airways, it is unable to reduce airway reactivity to methacholine challenge in patients with asthma. A soluble IL-4 receptor antagonist has shown clinical benefits for patients with moderate asthma, who require daily inhaled corticosteroids (186). Agents that target IL-13 and TNF-α remain to be evaluated in asthmatic inflammation. The use of cytokines with anti-inflammatory effects may also have therapeutic value. The evaluation of such agents in human beings, including IL-10, IL-12, and IFN-γ, is at a preliminary stage, but so far the results have not been encouraging.

1.4.3.4 Immunotherapy

Vaccination with allergen extracts are being considered as strategies for asthma. Vaccination with mycobacteria has antiallergic properties. In Japan, early vaccination with BCG is associated with a substantial reduction in the risk of developing allergy (261) although similar associations were not observed in studies performed in Sweden (262).

Two new approaches using DNA vaccines are undergoing serious consideration. The first involves the use of CpG oligodeoxynucleotides (ODNs). Preadministration of CpG ODNs prevented both airway eosinophilia and bronchial hyperresponsiveness in a mouse model of asthma (263).

An alternative approach is to use allergen-specific naked DNA sequences as vaccines. Preliminary data suggest that giving naked DNA leads to production of allergens from within the airways' epithelial cells. Because of the different handling pathways for endogenous and exogenous allergens (262), it seems that the endogenously produced allergen elicits a Th1-type response which in theory can overcome the existing Th2 pattern response in human asthmatics and eliminate the allergy.

1.5 Animal Models

Animal models, including guinea pigs, monkeys, rats and mice, have been used to study the pathogenesis of asthma. Rat and mouse models of allergic lung disease have been utilized to dissect the complex pathophysiological mechanism underlying the asthma phenotype. A wealth of research activity has shown that rats and mice can be induced to display a range of the pathophysiological features that are hallmarks of the human disease. These animal models have been shown to develop inflammatory infiltrates in the lungs, both in peribronchiolar tissues (as shown in lung sections) and in the airway lumen (collected in BAL). Although eosinophils are generally the most prolific cell type within these infiltrates, lymphocytes are also present in significant numbers. Lung sections show that there is an increase in mucus secretion from the bronchoepithelial surface. Analysis of serum reveals that rats and mice show an increase in both total and allergen-specific IgE, as well as increased IgG_{2a} titers. This Th2-type profile is reflected in the cytokines generated within the lungs namely IL-4 and IL-5 being present in significantly greater quantities than IFN-γ. Many investigations have also documented changes in lung function following allergen provocation, using a variety of techniques. The variety of protocols that have been used to induce pulmonary eosinophilia, AHR, and mucus hypersecretion is tremendous. The use of animal models has enabled researchers to highlight specific pathways and to study the function of these pathways *in vivo*.

1.5.1 Brown Norway Rat Model of Allergic Asthma

Brown Norway (BN) rats are a well established model of allergic asthma. This model reflects many features of human allergic asthma, including both early and late phase reactions (264), elevated antigen-specific IgE (265), airway inflammation (266), and increased airway responsiveness to several

stimuli (267). Allergen exposure of sensitized animals induces airway accumulation of inflammatory cells, such as eosinophils, lymphocytes, neutrophils in BAL fluid, eosinophils, $CD2^+$, $CD4^+$, $CD8^+$ T cells in the airway submucosa and expression of mRNA for Th2 cytokines. The BN rat is known to produce IgE in large quantities after sensitization (268,269). Injecting BN rats with gold salts causes an important increase in serum IgE concentration and this technique is used to identify the genetic determinants controlling the IgE response (270). Mas *et al.* showed that rat chromosome 9, together with a region on rat chromosome 10, plays a major role in the control of the IgE response (271). Sensitization of the BN rat can be performed using a variety of antigens; the most popular of which are ovalbumin and house dust mite injected subcutaneously. The sensitization procedure does not change the cellular profile of BAL fluid, but even after a single exposure to aerosolised antigen following sensitization, airway inflammation develops. After repeated exposure to antigen, BAL fluid contains an increased percent of neutrophils, eosinophils, and lymphocytes. This pattern is similar to the cellular distribution of BAL fluid recovered from asthmatic subjects after a local allergen challenge (272). Neutrophil infiltration in humans is commonly found in cases of severe asthma or *status asthmaticus*, while in the BN rat model, neutrophil influx to the BAL induced by allergen is usually prominent early on (273,274,275,276).

Increased numbers of $CD4^+$ and activated IL-2 receptor-positive T cells can be observed in the BAL and lung parenchyma, demonstrating that activated T helper cells are involved in the allergic response in the lung of the BN rat asthma model (277,278). This observation is similar to the findings in human asthma (277,278). Stimulation of similar amounts of $CD4^+$ or $CD8^+$ T cells revealed that both T cell populations of BN rats produce significantly less Th1 and more Th2 cytokines than their Lewis rat strain counterpart (279). The former group also showed that the BN rat $CD8^+$ T cell compartment produces only one-quarter the amount of IFN-γ produced by the $CD4^+$ T cell compartment, suggesting that the defective IFN-γ production by the BN rat $CD8^+$ T cell compartment may account for the susceptibility of this rat strain to develop Th2-type immune responses. In another study comparing rat strains, Sirois *et al.* showed that alveolar macrophages (AM) from BN and Sprague-Dawley (SD) rats are functionally different (280). LPS and OX8-stimulated AM from BN rats produce more Th2-type cytokines (IL-10, IL-13) than AM from SD rats, suggesting that these cells may play an important role in creating a cytokine milieu that may favour the development of allergic reactions.

The link between airway inflammation and airway responsiveness in BN rats has not been clearly established. It could be that the inflammation alters the mucoepithelial barrier, thus selectively increasing the airway responsiveness to inhaled broncho-constrictor agents. BN rats develop a transient episode of AHR within 24hr of an ovalbumin challenge which is associated with an increased number of eosinophils and activated T lymphocytes bearing the CD25 marker in the BAL (273,281). Moreover, the cell influx and AHR are abolished by corticosteroids (276). No clear relationship between eosinophilia and AHR has been demonstrated in studies using the BN rat. For example, inhibition of allergen-induced eosinophilia with the immunosuppressant cyclosporin A is not accompanied by a reduction in AHR (270), while inhibition of AHR has been observed in the absence of any reduction in airway eosinophilia following anti-ICAM-1antibody treatment (282). Intravenously administered ovalbumin in the antigen-sensitized BN rat causes bronchospasm (283) and microvascular leakage into the airways (284). Nagase *et al* have demonstrated a role for 5-HT and leukotriene D_4 in the increase in tissue resistance seen in the airways after antigen administration in sensitized BN rats (283). BN rats antigen-induced bronchospasm appears to be mediated equally by the activation of 5-HT and Cys-LT_1 receptors with little or no involvement of histamine or its receptors.

Recently, inconsistencies in eliciting a LR after ovalbumin challenge in the highly inbred BN rat animal model prompted Turner *et al* (284) to study the airway responsiveness to allergen in BN rats from 2 different continents. This study concluded that the same substrain of BN rat, namely SSN, showed different responses to allergen. Specifically, BN (SSN) rats from the United Kingdom (UK) produced greater allergen-induced LR and associated cellular influx into the BAL than USA BN SSN rats. Moreover, Th2 cytokines were expressed more in UK BN rats than in USA BN rats while the latter expressed more Th1 cytokine in the lung tissue. We chose to use the USA BN (SSN strain) rats in our studies despite these differences and re-establish a strong asthma phenotype, including eosinophilia, AHR, and a LR by giving these animals IL-2 injections (11).

1.6. Active Immunization Models

1.6.1. Sensitization and Challenge

This protocol relies on the delivery of an antigen to replicate a sensitization and challenge phase, in order to mimic the allergic response to exogenous or innocuous stimuli. This protocol involves preimmunization with the allergen before a challenge phase in which the allergen is introduced to the target organ, in the case of the lungs, either intranasally, as an aerosol, or via the trachea (286). This basic protocol induces a pulmonary eosinophilia generally in conjunction with an increase in circulating IgE levels. There are countless variations to this protocol including subtle differences in the route of administration and the use of a variety of antigens, from complex microorganisms to simple proteins and chemicals (287). Soluble protein antigens are widely used to elicit allergic pulmonary inflammation and range from simple proteins such as ovalbumin (OA) to complex, environmentally relevant antigens, such as cockroach or house dust mite proteins (287). The most used protein is chicken egg OA, the use of which models late-phase events such as eosinophilia, and in some protocols, AHR *in vivo*. OA is an important human allergen and has the advantage of reliably inducing in rats and mice antigen-specific IgE responses that are largely dependant on IL-4. Zhang and colleagues (1997) (288) found that a combination of systemic and local exposure to OA resulted in a maximal and reproducible induction of responses. These responses included AHR *in vivo*, production of allergen-specific IgE, peri-airway and bronchoalveolar infiltration of eosinophils and increased expression of Th2 cytokines in the local lymphoid tissues.

1.6.2 Adjuvant

The use of adjuvant during the priming phase is to boost the immune response to the allergen in use. Specifically, the use of aluminium compounds (alum) is associated with the induction of Th2 responses (289, 290). OA/alum was found to induce IL-4 and IL-5 production in the absence of IL-4 signalling in IL-4R and Stat6 deficient mice (291).

1.6.3 Dose

Constant and Bottomly (1997) concluded that there is no clear-cut conclusion regarding dose and a corresponding development of specific immunity but pointed out that the type of antigen used is critical (292). They remarked that studies in which low dose Th1-type responses were elicited used parasite antigens, whereas low doses of soluble proteins tended to elicit a Th2-type response.

Antigen dose is an important issue in the context of immunotherapy. Immunotherapy with suboptimal doses of OA has been found to down-regulate AHR and BAL eosinophilia with a concomitant decreased production of Th2 cytokines (293). However, the same study found that immunotherapy with an immunodominant epitope of OA aggravates AHR and increases BAL eosinophilia (293).

Avant-Propos

Much of the research in asthma has focused on the multiple proinflammatory mechanisms involved in this complex inflammatory disease (3). The therapeutic approach to this disorder has focused on symptomatic control through the alleviation of bronchospasm and the elimination of inflammation. The direct relation of airway inflammation to airway remodelling and disturbances of lung function, development of irreversible changes in lung function, and increased bronchial responsiveness is complex and difficult to assess. This thesis focuses on two important components of airway inflammation: cysteinyl-leukotrienes and interleukin-5, and their roles in the asthma phenotype.

The most widely studied biological effects of the cysteinyl leukotrienes are smooth muscle contraction and vascular leakage. There is much less convincing evidence that the cysteinyl leukotrienes cause any effects on cell-mediated inflammation. We decided to use the Brown Norway rat model to investigate how cysteinyl leukotrienes influences airway inflammation in asthma. This animal model has been used extensively in the study of asthma because of its ability to mount a high IgE response, a common indicator of asthma, following antigenic stimuli. However, these animals are unable to mount a physiological response to antigen. A recent study by Renzi *et al* showed that by giving these animals IL-2, they were able to have an increased airway response to antigen (10). Therefore, to study how leukotrienes affect the physiological and inflammatory response in BN rats, we first promoted the animals' cell-mediated immunity with IL-2 and then gave the animals Montelukast, a cysteinyl leukotriene receptor antagonist to inhibit leukotriene activity. We measured the physiological and inflammatory response with the following tests: airway responsiveness to leukotrienes, the late airway response following antigen challenge and airway inflammation, namely cell influx and cytokine production. These tests allowed us to characterize the physiologic effects of leukotrienes in a pseudo asthmatic airway and the changes in the cellular and cytokine profile in the lung.

Having measured cytokine expression in the lung, we decided to furthur study specific changes that cytokines, specifically IL-5, can cause in the airways. In order to study these changes, we again used the Brown Norway rat model, as our experimental system because it represents an animal model not predisposed to mount a physiologic or a complete immunological response (ie. Eosinophils, Th2 cytokines) following antigenic stimuli. Therefore by challenging these animals with IL-5, we would observe

functional and cellular changes caused directly by IL-5. The ultimate question being answered is can IL-5 alone convert normal rats into rats with an allergic phenotype? Once the animals were challenged with IL-5, we measured airway responsiveness to methacholine, the early and late airway response to antigen, lung resistance, and airway inflammation.

These experiments increase in recognition and understanding of asthma as an immune-mediated disease. Because airway inflammation, such as leukotrienes and IL-5, has profound effects on the immune system, they influence both the pathogenesis of asthma and its ongoing status. This study concludes that there is indeed a direct relationship between cysteinyl leukotrienes and the airway response and cell–mediated immunity, however one such mediator, IL-5, is unable to cause a complete asthmatic phenotype.

Materials and Methods

2.1. Cysteinyl Leukotrienes, Cellular Immunity and the Airway Response to antigen of BN rats

2.1.1 Animals and sensitization

Sixty-two highly inbred male BN SSN rats, 7-8 weeks old and weighing 200 to 240g were obtained from Harlan Sprague-Dawley Inc. (Walkerville, MD). Rats were maintained in conventional animal facilities at the Research Centre of Notre-Dame Hospital, Montreal. The Animal Care Committee approved all the experiments that were performed in this study.

Rats were sensitized on day 1 by subcutaneous injection of 1 ml of saline containing 1 mg of OA and 200 mg of aluminium hydroxide (Sigma Chemicals, St. Louis, MI).

2.1.2 Measurement of lung resistance

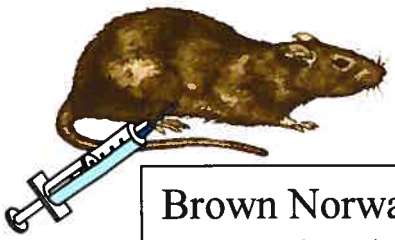
Rats were anaesthetised with either somnotol (50 mg/kg i.p.) [LTD_4 responsiveness] or urethane (1g/kg i.p.) [ER/LR]. A heating pad was used to maintain body temperature constant during the experiment and rectal temperature was monitored continuously with an electronic thermometer. Lung resistance (R_L) was measured during spontaneous tidal breathing with the animals in the supine position as previously described (11). Flow was measured by placing the tip of the tracheal tube inside a small Plexiglas box (265 ml in volume). A Fleisch no.O pneumotachograph coupled to a piezoresistive differential pressure transducer (Micro-Switch 163PCOID36, Honeywell, Scarborough, Ont. Canada) was attached to the other end of the box to measure airflow. Transpulmonary pressure (Ptp) was measured using a water-filled catheter placed in the lower third of the esophagus connected to one port of a differential pressure transducer (Transpac II, Abbott, Illinois); the other port was connected to the Plexiglas box. The esophageal catheter consists of a polyethylene tube (PE-240, 6 cm. long).

Figure 12: Animal airway response measurement protocol

Sensitization

- Ovalbumin (sc) 1 mg/ml
- $Al(OH)_2$ (sc)adjuvant

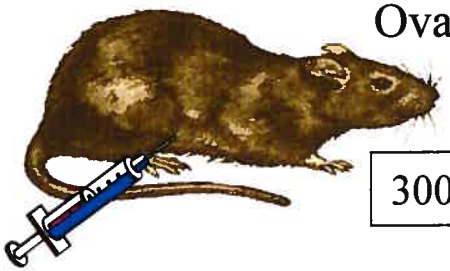
2 weeks



Brown Norway (BN) rat
7-8 weeks old
200-250g

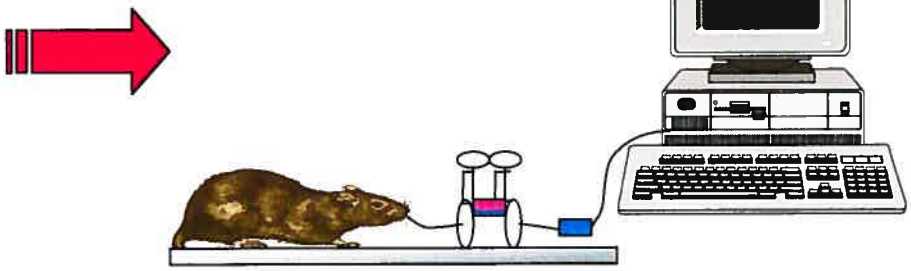
Antigen Challenge

Ovalbumin (1 mg/ml; Aerosol)



300-350g

Somnotol (anaesthetic)



Responsiveness studies

Methacholine (aerosol) or
Leukotriene (intratracheal)

- Doubling doses after baseline saline challenge (aerosol) until airway resistance is doubled.
- Recover BAL
- Recover lungs

The pressure and flow signals were amplified, passed through eight-pole Bessel filters (model 902LPF, Frequency Devices, Haverhill, MA) with their cut off frequencies set at 100 Hz. The data was recorded and stored on a computer. The airway response was evaluated from R_L, which was determined by fitting the equation of motion of the lung by multiple linear regression using commercial software (RHT-Infodat Inc, Montreal, Quebec, Canada). Endotracheal tube resistance was 0.11 cm H_2O/ml/s at a flow of 25 ml/s. Tube resistance was subtracted from all values of R_L.

2.1.3 Experimental Protocol

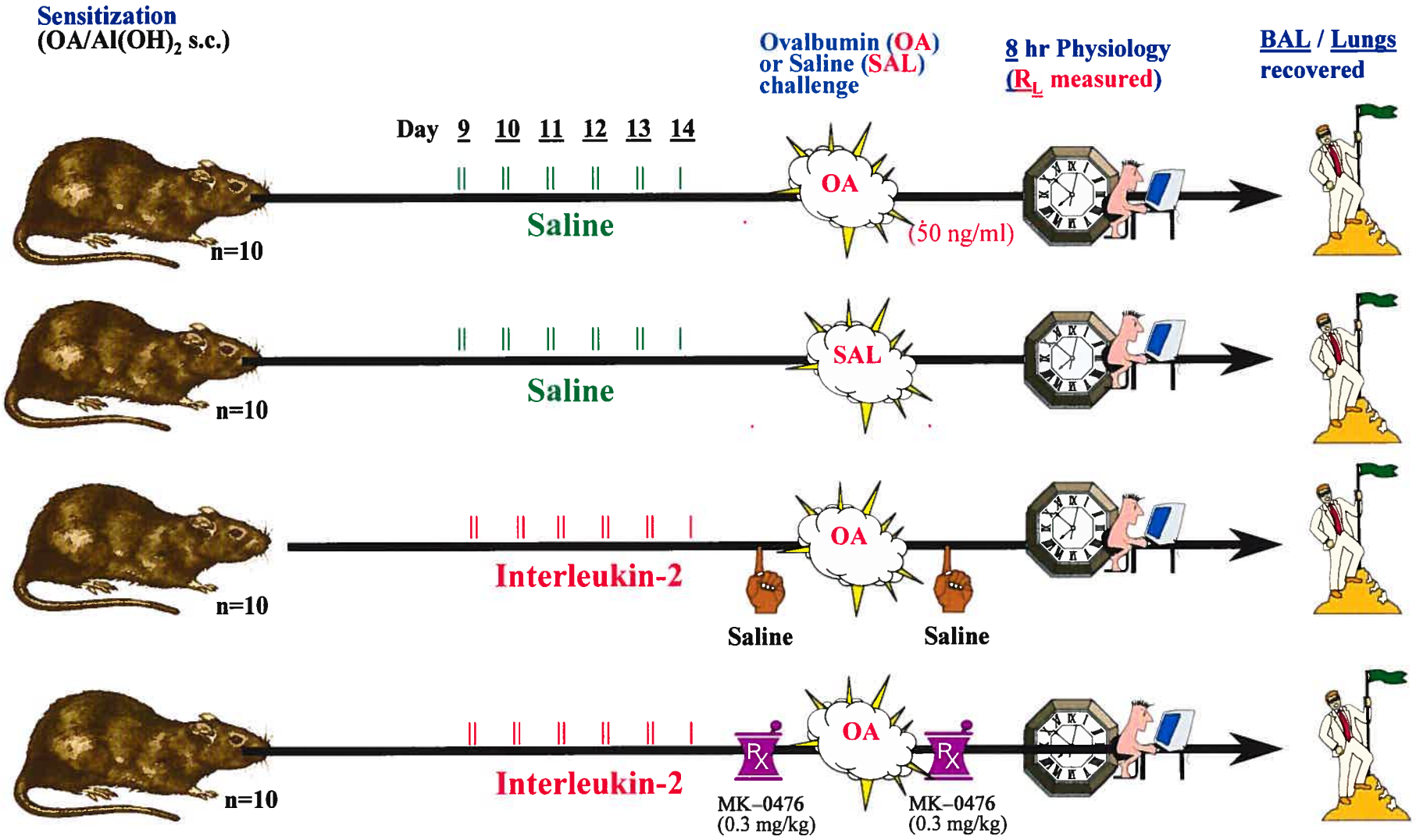
2.1.3.1 Experiments assessing the airway responsiveness to LTD_4

Our study investigated the effects of lung agonists, such as LTD_4, in an animal model at a state of heightened cell-mediated immunity; a state commonly found in asthmatics. Therefore, we chose to inject IL-2, a known T-cell growth factor, into rats to boost their cellular immunity. Twenty-two rats were given either 0.2 ml of saline or 20,000 units of human recombinant IL-2 diluted in 0.2 ml of saline subcutaneously twice a day for 4.5 days from the 9^{th} to the 14^{th} day after sensitization. On day 14, rats were challenged with incremental doses of LTD_4 (0.05 ng/ml to 1000ng/ml) (Cayman Chemical Company, Ann Arbor, Michigan) intratracheally in 50µl until a doubling in lung resistance occurred **(Figure 12)**. LTD_4 was given to rats intratracheally at doses established in previous studies (294).

2.1.3.2 Experiments assessing the effects of montelukast on IL-2 induced increased airway responsiveness after OA challenge

Four groups of rats were studied **(Figure 13)**. The first 2 control groups consisted of twenty sensitized rats that were given 0.2 ml of saline subcutaneously twice a day for 4.5 days, from day 9 to day 14 after sensitization, and were challenged with an aerosol of saline or OA on day 14. The second 2 experimental groups consisted of twenty sensitized rats that received 20,000 units of human recombinant IL-2 subcutaneously twice a day for 4.5 days from the 9^{th} to the 14^{th} day after sensitization. On day 14, prior to and 2 hours following OA challenge, rats received an i.v. injection of 0.36 ml of saline or

Figure 13: Experimental Animal Groups



montelukast (MK-0476, 0.5mg/kg, Merck Frosst, Montreal, Quebec). MK-0476 was not tested alone for two reasons. One, the objective of this experimental protocol was limited to studying the IL-2-mediated lung response in BN rats. BN rats not treated with IL-2 do not mount an airway response, therefore giving MK-0476 to these rats could not be justified. Moreover, MK-0476, an experimental drug at the time, was graciously donated by Merck Frosst and in a quantity sufficient to test only the principal questions of our study. All rats were challenged with an aerosol of saline or OA (1mg/ml) for 5 minutes. Lung resistance (R_L) was measured at baseline, 5, 10, 15, 20 and 30 minutes after challenge and subsequently every 15 minutes for a period of 8 hours.

2.1.4 Bronchoalveolar Lavage (BAL)

Eight hours after OA challenge, the lungs were lavaged through the tracheal tube by five instillations and immediate retrieval of 5ml of saline at room temperature. The cells were separated from the supernatant by centrifugation, washed and the total cell count was determined with a hemacytometer as previously described (10). The differential count was assessed on a cytospin slide that was prepared with a Cytospin model III (Shandon, Pittsburgh, PA) and stained with Wright-Giemsa (Biochemical Sciences, Swedesboro, NJ). At least two hundred cells were counted under oil-immersion microscopy.

2.1.5 Lung Retrieval and Preparation

After performing BAL, the chest wall was opened and animals were exsanguinated by cardiac puncture. The pulmonary vasculature was washed by slowly injecting balanced salt solution (10ml) into the right ventricle until the lungs were white. The right lung was fixed in fresh 4% paraformaldehyde for *in situ* hybridization, while the right lung was snap frozen in liquid nitrogen for semi-quantitative polymerase chain reaction (SQ-PCR).

2.1.6 RNA preparation, reverse transcription and PCR

TRIzol reagent (Gibco BRL, Montreal, Quebec, Canada) was used as a monophasic solution to homogenize tissue and to isolate total RNA from frozen biopsies according to the manufacturer's instructions. Reverse transcription was performed on 5 µg of total RNA with Moloney murine leukemia

virus (M-MLV) reverse trancriptase (Gibco BRL, Montreal, Quebec, Canada) in the presence of RNasine (Pharmacia, Montreal, Quebec, Canada). PCR was performed by using an automatic thermal cycler (MJ Research Inc., Ottawa, ON, Canada). Cycle conditions were 94°C for 1 min, 60°C for 2 min, 72°C for 3 min. cDNA (2µl) was amplified in a 25-µl reaction volume containing 0.5 µM (each) dNTPs, 0.5 µM primers, 1U Taq Polymerase (Gibco BRL, Montreal, Quebec, Canada).

2.1.7 Semi-Quantitative Reverse Transcription Polymerase Chain Reaction

For semi-quantitative experiments, the PCR was set up as described above, except that the reaction mixture contained 5 µCi/ml of [α^{32}P]dATP as a tracer. Specific primers were used to amplify selected cytokine messages (295). Preliminary experiments determined the optimal number of cycles for each primer, which were as follows: cyclophillin (a housekeeping gene): 19 cycles, and IL-4, IL-5, and IFN-γ: 30 cycles. The above cycle numbers were selected as midpoints of their respective linear ranges for amplification of cDNA (2µl), and there was a linear correlation between input cDNA and the yield of PCR products. Quantities of cDNA were standardized to yield equivalent amounts of PCR products for cyclophillin and compared with each other. In order to determine the relative mRNA expression of each cytokine present in different samples, 20 µl of the amplified product was electrophoresed through a polyacrylamide gel, containing 5% urea, in Tris-acetate/EDTA(TAE) buffer. The gels were dried and exposed overnight at -80°C using autoradiography film (Kodak, Rochester, MY). The radioactive signal-specific bands were quantified by an Instant Imager System 2000 (Alpha Innotech Corporation, CA). The relative amount of radioactive signal (RS) of specific bands for each cytokine and cyclophillin were calculated as follows: relative amount of cytokine mRNA = (RS for cytokine in sample)/(RS for cyclophillin in sample).

2.1.8 In situ hybridization

In order to confirm the differences in Th1/Th2 cytokine expression obtained by SQ-PCR, we performed *in situ* hybridization for IL-4 and IFN-γ as previously described (295). Cryostat blocks were made from stored lungs and eight micron sections were cut consisting of an intact airway and surrounding submucosa and placed on poly-L-lysine coated slides. Sections were incubated at 37°C for 12 hours and

either processed immediately or stored at -80°C until used. The cDNA for IL-4 and IFN-γ was subcloned into the Bluescript RNA vector (pBluescript SK, Stratagene, La Jolla, CA) and sense and antisense probes were generated by T7 and SP6 RNA polymerases. Labelling of RNA probes with digoxigenin (DIG)-11-UTP was performed according to the manufacturer's recommendations (Boehringer Mannheim, Mannheim, Germany). The labelled probes were digested by alkaline hydrolysis to an average length of 100 to 200 bases before precipitation and hybridization was performed at 42°C for 12h. The slides were washed in decreasing concentrations of SSC (4 X SSC to 0.1 X SSC) and RNase A (20µg/ml) to remove unhybridized probe. As a negative control, preparations were hybridized with DIG-UTP-labelled sense probes under the same conditions. Specimens were then stained for 1 minute with Hoechst 33258 dye (bisbenzimide; Sigma Chemical Co., St.Louis, MO.) at a dilution of 1 µg/ml in PBS for visualisation under a Zeiss Axiophot fluorescence microscope (Carl Zeiss (Oberkochen), Ltd., Welwyn Garden City, UK). Positive cells were counted in a random coded order at x200 magnification. In the airway submucosa, positive cells were counted along the entire length of the epithelial basement membrane in a minimum of 6 sections. Results are expressed as the mean number of positive cells per millimeter square of airway basement membrane for each RNA probe.

2.1.9 Data Analysis (Section 2.1 to 2.1.8)

The concentration of LTD_4 required to double R_L ($EC_{200}R_L$) was obtained by linear interpolation between the two concentrations bounding the point at which R_L reached 200% of the control value. Comparisons of airway responsiveness to LTD_4 between groups were performed with log transformed data and analyzed using unpaired, non-parametric Mann-Whitney tests. The LR was calculated as the area under the curve of R_L above the baseline value over the 3-8 hour period following challenge (9). Data were analyzed using a Kruskal-Wallis nonparametric ANOVA test for groups of rats followed by Dunn's multiple comparisons test between two independent groups of rats. Differences were considered to be statistically significant when p values were less than 0.05.

2.2 Interleukin-5 and the Airway Response of BN rats

2.2.1 Animals and sensitization

Ninety-three highly inbred male BN SSN rats, 7-8 weeks old and weighing 200 to 240 g were obtained from Harlan Sprague-Dawley Inc. (Walkerville, MD). Rats were maintained in conventional animal facilities at the Meakins-Christie Labs of McGill University or the CHUM Research Centre. The Animal Care Committees of each institution approved all the experiments that were performed in this study.

We actively sensitized the rats by subcutaneous injection of 1ml of saline containing 1 mg of ovalbumin (OA) and 200 mg of aluminium hydroxide (Sigma Chemicals, St.Louis, MI).

2.2.2 Eosinophil colony proliferation from peripheral blood mononuclear cell progenitors (PBMNC)

These experiments were performed to assess whether rhIL-5 induced eosinophil proliferation from progenitors in rats as previously described in humans (296). We isolated PBMNC from ten BN rats by centrifugation over Ficoll Hypaque. The cells were plated at a concentration of 1×10^6 cells per ml in 35 x 10 mm tissue culture dishes (Falcon) in supplemented Iscove's modified Dulbecco's medium containing 20% heat inactivated fetal bovine serum, 1% penicillin, 1% streptomycin, 5×10^{-5} mol/L 2-mercaptoethanol, 0.9% methylcellulose with or without rhIL-5 (0.25, 1, 2 or 5 ng/ml). After 14 days of culture at 37°C in a humidified atmosphere containing 5% CO_2 in air, colonies (defined as any aggregation of more than 40 cells) were counted by inverted microscopy and eosinophil-type colonies (CFU-Eo) were counted as a function of their morphologic appearance. CFU-Eo appeared as tight, compact aggregations of round, refractile cells and showed varying combinations of eosinophilic, basophilic, and mixed eosinophilic-basophilic cells on May-Grunwald-Giemsa staining.

2.2.3 IgE determination

Specific IgE levels to OA were determined by ELISA as previously described (297). Assays were performed on 96-well microtiter plates (Immulon II, Fisher, Pittsburgh, PA). Plates were coated overnight

with mouse monoclonal antibody to rat IgE (Zymed Labs Inc., San Francisco, CA) diluted 1:500 in carbonate/bicarbonate buffer. Plates were blocked with PBS-0.5% Casein. A 1:10 dilution of serum in PBS-0.5% Casein was prepared prior to addition of 100µl to the plates. Biotin-labelled OA (0.02 mg/ml) was added to the wells. The above steps were done at 2h intervals at 37°C. Between steps the wells were washed three times with PBS-0.5% casein. Horseradish peroxidase conjugated avidin (Sigma Chemicals, Montreal, Quebec) in PBS-0.5% casein (1:500 dilution) was added for 30 minutes. Plates were developed at room temperature for 15 min. after addition of TMB substrate (Sigma Chemicals, Montreal, Quebec) diluted (1:10) in substrate buffer, and were read with an ELISA plate reader (SLT Lab Instruments, Fisher Scientific Co., Pittsburgh, PA) at 450nm.

2.2.4 Endotoxin determination and challenge

The concentration of endotoxin in the administered rhIL-5 was quantified using the Limulus Amebocyte Lysate (LAL) test (Associates of Cape Cod, Falmouth, MA). The same concentration of endotoxin (Lipopolysaccharide (LPS), Sigma, St.Louis, MO), diluted in 0.9% saline was given intra-tracheally to animals and airway responsiveness to Mch and inflammatory cells in the BAL were measured.

2.2.5 Lung lavage (BAL)

These experiments were performed 14 days after sensitization. Forty-three rats were anaesthetised with somnotol (50 mg/kg), intra-tracheally intubated and were awakened after intra-tracheal injection of BSA, rhIL-5 (1 to 10 µg) or 10 µg rhIL-5 and 50 ng TRFK5 (BD Biosciences, Canada). Twenty hours later the animals were again anaesthetised with somnotol, intubated and BAL was performed after methacholine (Mch) challenge. The BAL retrieval is described earlier in section 2.1.4.

2.2.5.1 Staining of BAL cells for major basic protein (MBP)

The cells obtained from BAL were fixed in acetone-methanol, washed in Tris-Borate solution (TBS), incubated with blocking solution (Dako Diagnostics, Mississauga, Ontario) (10 minutes) and incubated with 60μl of primary monoclonal MBP IgG antibody (diluted 1:30 in Tris Borate Solution (TBS)) at 4^{o}C in a humid chamber overnight (11). The next day, slides were again washed with TBS, and

incubated with secondary anti-primary antibody for 45 minutes at room temperature. The slides were washed again in TBS, and incubated with Streptavidin-Alkaline phosphatase antibody (Dako Diagnostics, Mississauga, Ontario) for 45 minutes at room temperature. Signals were revealed using a Fast Red stain (Sigma Chemicals, St.Louis, MO) and viewed under a Nikon Eclipse E600 microscope (Nikon, USA) at 40X magnification.

2.2.6 Measurement of lung mechanics

Lung mechanics measurements followed protocols detailed in section 2.1.2.

2.2.7 Airway responsiveness to methacholine

Fourteen days after sensitization, rats were given either rhIL-5 (1, 3 and 10μg), 10μg of rhIL-5 and 50 ng TRFK5 or 60 Endotoxin Units (EU) of LPS **(Figure 14)**. The latter two groups underwent airway responsiveness to methacholine tests only 20 hours later. The dose of rhIL-5 was based upon previous studies involving guinea pigs (298) and humans (299). 10 ug of rhIL-5 given nebulized to asthmatics was shown to be sufficient to increase airway responsiveness and infiltration of eosinophils into the airways. 30 minutes, 20 hours or 72 hours later rats were anaesthetised with somnotol (50 mg/kg), intubated and baseline R_L was measured. Rats receiving 10μg of IL-5 was also challenged with a nebulized aerosol of OA (50 mg/ml in sterile water) for 30 seconds 20 hours after IL-5 administration. All rats were given an aerosol of phosphate-buffered saline (PBS) followed by progressively doubling concentrations of Mch (from 0.0625 to 32 mg/ml in sterile saline) and R_L was measured after each aerosol until it increased to at least 200% of the PBS baseline value. Aerosols were generated from 3ml of solution using a Hudson nebulizer with an airflow of 10 L/min; each administration lasted for 30 seconds.

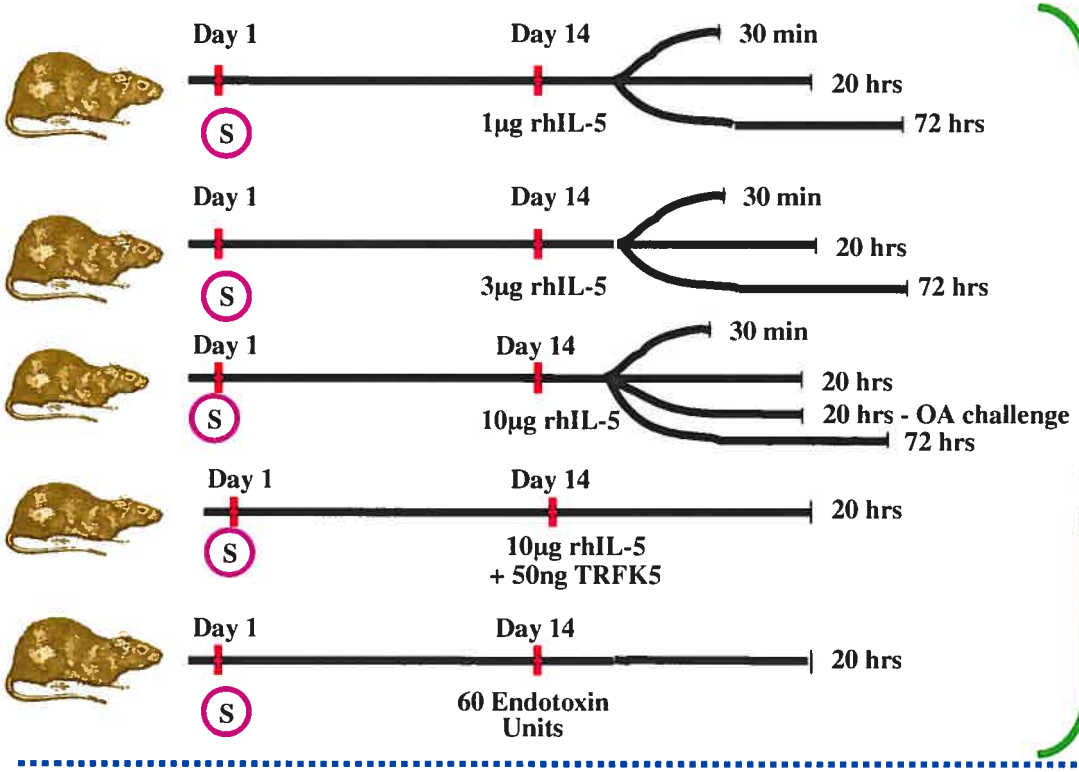
2.2.8 Measurement of airway responses to OA

Fourteen days after sensitization, rats were anaesthetised with somnotol (50 mg/kg), intra-tracheally intubated and received either IL-5 (3μg) or the same weight of BSA intra-tracheally prior to being awaken **(Figure 14)**. This specific dose of IL-5 was given to animals based on a previous

Figure 14 : IL-5 Experimental Animal Groups

Airway Responsiveness to Methacholine

S = Sensibilization (1 mg OA + 200 mg $Al(OH)_2$ (s.c.))



PBS Aerosol for R_L Baseline

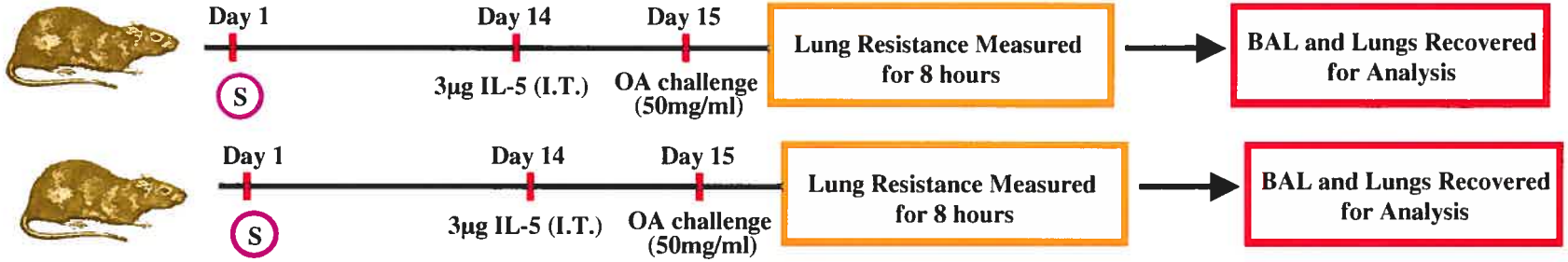
↓

Followed by Progressive Methacholine challenges (0.0625 to 32 mg/ml)

↓

Lung Resistance is measured after each Mch challenge until 200% increase in R_L from PBS challenged baseline

Airway Response to Ovalbumin



experiment by Lilly *et al* (298) that showed that instilling 5μg of IL-5 to guinea pigs caused a peak in airway eosinophilia. We chose 3μg as a comparable dose to a smaller mass animal model. The following day, rats were anaesthetised with urethane (1.1 g/kg), intubated and baseline R_L was measured. The rats were then challenged with aerosolized OA (50 mg/ml in sterile water) by using a Hudson nebulizer (Model 1400; Hudson, Temecula, CA) at airflow of 10 L/min for 5 minutes. R_L was measured before and at 5, 10, and 15 min after the OA challenge, and at 15 min intervals for a total period of 8h.

2.2.9 Lung mincing and digestion

These experiments were performed 8 hours after OA challenge as previously described (296) in 16 rats either pre-treated with 3 μg of IL-5 or 3 μg of BSA. The chest wall was opened, and the animal was exsanguinated after puncture of the left ventricle by section of the abdominal aorta. Blood was kept in a heparinized tube for analysis of lymphocyte subsets by flow cytometry. The pulmonary vasculature was washed by slowly injecting balanced salt solution (10ml) into the right ventricle until the lungs were white. The lungs were dissected from the chest and mediastinal structures, weighed, and separated into the large airways (trachea and large bronchi until approximately the fifth generation) and small airways and parenchyma (S/P). Tissue digestion and cell retrieval through a #60 sieve (Sigma) was performed and slides were prepared by centrifuging 500,000 cells in enriched RPMI 1640 for 5 min at 400 rpm in a cytocentrifuge. The cellular differential was assessed on a Wright-Giemsa stained slide by counting 200 cells under oil-immersion microscopy.

2.2.10 Isolation and staining of blood lymphocyte subsets

Peripheral blood mononuclear cells were isolated from fresh heparinized peripheral blood by standard Ficoll-Hypaque methods and prepared for flow cytometry as previously described (300). The isolated cells were washed with Hanks balanced salt solution and stained directly by incubating with the monoclonal antibodies W3/25 (equivalent of the CD4 helper lymphocytes) or OX8 (CD8 suppressor/cytotoxic lymphocytes) for 30 min. Cells were studied immediately or fixed in 1% paraformaldehyde/0.85% saline. The fixed cells were stored at 4°C in the dark until analysis. Flow

cytometry was performed with an argon laser, 488nm FACScan analyzer (Becton Dickinson FACS Division, Sunnyvale, CA) focusing on the lymphocyte cluster. Controls consisted of cells stained with polyclonal antibodies obtained from mouse ascites fluid.

2.2.11 Measurement of cytokine mRNA expression

These experiments were performed 20 hours after OA challenge as previously described (298) in 16 rats either pre-treated with 3 µg IL-5 or BSA. All the animals were killed by exsanguination. The lungs were dissected from the chest after perfusion of the pulmonary vessels, fixed in 4% paraformaldehyde and then transferred an hour later into a 15% sucrose in PBS solution at 4°C. Airways from the lung were cut transversely into 1cm pieces, and blocked with liquid nitrogen. Cryostat blocks were cut at a depth of 8 µm/ section, placed on poly-L-lysine coated slides and stored at -80°C.

In situ hybridization was performed as previously described (301) and as detailed in section 2.1.8.

2.2.12 Statistical analysis (Section 2.2 to 2.2.11)

The concentration of Mch required to double R_L ($EC_{200}R_L$) was obtained by linear interpolation between the two concentrations bounding the point at which R_L reached 200% of the control value. Comparisons of airway responsiveness to Mch between groups were performed with log-transformed data. To compare the prevalence of ER among treatment groups, we defined a significant ER as an increase in R_L to at least 150% of the baseline value within 1 hour after OA challenge. The late response was calculated as the area under the R_L vs. time curve from 180-480 min post challenge. The differences between the tested and control group means were analyzed using Student's t test or the Mann-Whitney U-test, as appropriate, to compare magnitude, time to peak, ER and LR, inflammatory cells and cytokines. Results are presented as mean $\pm$ SEM, except for values of $EC_{200}R_L$, which are reported as geometric means. To determine the statistical significance of the airway responsiveness to Mch challenge, we employed unpaired student's t test. Significance was accepted when the probability (p) value was ≤ 0.05.

2.2.13 Chemicals

Ovalbumin, BSA, APAAP, urethane, Mch, paraformaldehyde, collagenase, and Wright-Giemsa stain were purchased from Sigma Chemicals (St.Louis, MI); *Bordetella pertussis* vaccine was purchased from the Armand Frappier Institute (Montreal, Quebec, Canada); Fetal calf serum, penicillin, streptomycin, L-glutamine, nonessential amino acids, RPMI 1640 medium, Iscove's modified Dulbecco's medium, HBSS and tryptan blue were obtained from GIBCO Laboratories (Grand Island, NY). Ficoll-Hypaque was obtained from Pharmacia (Montreal, Quebec, Canada). RhIL-5 was graciously donated by Merck Frosst (Montreal, Quebec, Canada). Somnotol was used to put rats to sleep and was obtained from BDH Pharmaceuticals (Montreal, Quebec, Canada). Harris Haematoxylin was purchased from Zymed Chemicals (California, USA). W3/25 and OX8 were obtained from DAKO, Cedarlane (Carpinteria, CA).

Results

Pre-treatment with IL-2 increases airway responsiveness to LTD_4

To determine the relationship between LTs and upregulation of cell-mediated immunity, rats were pre-treated with either saline or IL-2 for 4.5 days and after general anaesthesia and endotracheal intubation were challenged on the 14th day after sensitization with exponentially increasing doses of LTD_4 to measure airway responsiveness. Rats pre-treated with IL-2 showed increased airway responsiveness to LTD_4 (fig. 15). The mean dose of LTD_4 that caused a doubling in resistance was 88.2 $\pm$ 38.4 ng/ml (n=10, 1 rat died during the procedure) for IL-2 treated rats. Rats pre-treated with saline required a higher dose of LTD_4 in order to double lung resistance (665.7 $\pm$ 47.6 ng/ml, n=11, $p < 0.05$).

Montelukast, a cys-LT1 receptor antagonist, inhibits the IL-2-mediated increase in LR after OA challenge

Montelukast (MK-0476) was given i.v. in order to block the effects of LTs on the cys-LT_1 receptor. In animals given IL-2 and then challenged with OA, montelukast blocked the LR significantly (n=10, 1 rat died during the procedure) when compared to IL-2 pre-treated animals that received saline i.v. instead of montelukast and were then OA challenged (n=10, 1 rat died during the procedure, LR: IL-2 + MK = 4.54 $\pm$ 0.61 vs. IL-2 + Saline = 26.49 $\pm$ 5.96, $p<0.05$) (fig. 16).

Effect of IL-2 and montelukast on the differential cell count in the BAL

The administration of IL-2 significantly increased the total number of cells recovered in lung lavage 8 hours after antigen challenge when compared to rats that received saline and were not OA challenged (IL-2 + OVA: 6.47 $\pm$ 1.78 x 10^6 cells/ml vs. SAL + SAL: 2.93 $\pm$ 0.77 x 10^6 cells/ml; $p<0.05$). The number of eosinophils in the BAL were significantly increased in animals pre-treated with IL-2 and OA challenged when compared to saline pre-treated and either saline or OA challenged rats ($p<0.05$, fig. 17). The absolute numbers of eosinophils rather than the percentage of eosinophils per total cells counted in each group is reported here. Although the volume of cells was equal in each group, the total number of cells recovered was significantly different. Therefore, it is statistically incompatible to compare

percentages between groups with unequal population sizes. Rats that received IL-2 and montelukast and were OA challenged showed a trend for a decrease in the number of total cells and eosinophils found in the BAL compared to those that received just IL-2 and OA challenged but this difference was not significant ($p>0.05$).

Effect of IL-2 and montelukast on cytokine mRNA production

SQ-PCR analysis of lung cytokine mRNA obtained 8 hours after OA challenge showed that IL-2 pre-treatment increased the expression of Th2 cytokine (IL-4 and IL-5) mRNA 8 hours after OA challenge (fig.18a, 18b). IL-2 pre-treatment also decreased the expression of the Th1 cytokine mRNA IFN-γ after OA challenge (fig. 18c). Montelukast inhibited the effect of IL-2 on lung cytokine mRNA expression and caused a decrease in IL-4 and 5 and an increase in IFN-γ mRNA 8 hours after OA challenge.

Results obtained by SQ-PCR for Th1 (IFN-γ) and Th2 (IL-4) cytokines were confirmed by *in situ* hybridization (figure 19a and b). Analysis of lung cytokine mRNA obtained 8 hours after OA challenge by *in situ* hybridization showed that IL-2 pre-treatment increased the number of IL-4 mRNA positive cells (19a) and decreased the number of IFN-γ mRNA positive cells (fig. 19b) in the submucosa of the airways when compared to control challenged rats ($p<0.05$). Montelukast inhibited the increase in cells expressing IL-4 mRNA but also increased the number of cells expressing IFN-γ mRNA in IL-2 pre-treated rats after OA challenge.

Effect of rhIL-5 on eosinophil colony formation from progenitors

To determine if rhIL-5 exhibits significant functional activity in BN rats, BN rat PBMNC's were incubated in 0, 0.25, 1, 2, and 5 ng/ml of rhIL-5 in enriched RPMI medium for 14 days. Concentrations of IL-5 above 1 ng/ml caused a significant increase in eosinophil/basophil colonies when compared to controls ($p<0.05$) and the number of eosinophil/basophil colonies increased with the concentration of rhIL-5 (fig. 20).

Effect of ovalbumin sensitization on OA-specific IgE levels from serum

To show that BN rats were properly sensitized to OA, serum from two actively sensitized rats was collected 0, 1, 2, and 3 weeks following sensitization. OA-specific IgE levels were measured in duplicate and showed an increase in OA-specific IgE levels (fig. 21).

Effect of intra-tracheal administration of rhIL-5 on airway responsiveness to Mch

Bovine serum albumin, 1, 3 or 10μg of rhIL-5 were administered intra-tracheally and airway responsiveness to Mch was measured 20 hours later. The amount of Mch that caused a doubling in R_L decreased significantly as doses of IL-5 increased from 1μg to 10μg (fig. 22). The airway response to Mch was significantly increased in the rats that received 3 µg of IL-5 when compared to the group that received BSA ($p< 0.05$). The airway response to Mch was also significantly increased in the group that received 10 µg of rhIL-5 when compared to the BSA group (10µg IL-5: 1.3 $\pm$ 0.4 mg/ml vs. BSA: 6.5 $\pm$ 0.9 mg/ml; $p<0.01$) (fig. 22). 50µg of TRFK5 significantly reduced the effect of 10µg of rhIL-5 on the airway response to Mch (4.75 $\pm$ 0.48 mg/ml; $p<0.05$). Experiments testing the effect of LPS at a concentration equal to that measured in the rhIL-5 (60 EU) showed no significant difference in airway responsiveness to Mch with control animals. Airway responsiveness to Mch 30 min. and 72 hours after administration of 10µg of IL-5 was not significantly affected (Fig. 23).

Effect of dose of intra-tracheal rhIL-5 on the cellular return from lung lavage

Intra-tracheal administration of rhIL-5 increased the cellular return from BAL 20 hours later. The total cellular return was for 1μg: 6.65 $\pm$ 2.08 x 10^6 cells, for 3μg: 9.75 $\pm$ 1.84 x 10^6 cells and for 10µg: 13.1 $\pm$ 3.07 x 10^6 cells ($p<0.05$). 50 ng of TRFK5 given in combination with 10µg of rhIL-5 significantly reduced the total cellular return from the BAL (3.28 $\pm$ 0.53 x 10^6 cells; $p<0.05$). The number of neutrophils increased significantly in the rats that received 10µg of IL-5 when compared to the group that received no IL-5 (10 µg IL-5: 8.01 $\pm$ 2.21 x 10^6 cells vs. no IL-5: 2.78 $\pm$ 0.73 x 10^6 cells; $p<0.05$). The effect of IL-5 on BAL neutrophils was significantly reduced by TRFK5 (2.23 $\pm$ 0.42 x 10^6 cells; $p<0.05$). No difference was found in the total number of macrophages, lymphocytes, and basophils that were present in the BAL of each group (Macrophages: 10µg IL-5: 4.53 $\pm$ 0.92 x 10^6 cells vs. Controls: 3.95 $\pm$ 0.27 x 10^6 cells;

Lymphocytes: 10µg IL-5: 0.252 ± 0.067 x 10^6 cells vs. Controls 0.154 ± 0.09 x 10^6 cells; Basophils: 10µg IL-5: 0.0655 ± 0.0403 x 10^6 cells vs. Controls: 0.05 ± 0.03 x 10^6 cells). Also, rats given TRFK5 did not show a significant difference between controls or IL-5 alone. There was no difference in total eosinophils between all groups when evaluated by Wright Giemsa stain or with an anti-MBP stain (Eosinophils: 10µg IL-5: 0.243 ± 0.0403 x 10^6 cells vs. Controls: 0.092 ± 0.07 x 10^6 cells vs 10 µg IL-5 and 50 ng TRFK5: 0.0689 ± 0.02 x 10^6 cells; $p>0.05$).

Effect of rhIL-5 on the physiological response after ovalbumin challenge

Pre-treatment with 3µg of rhIL-5 did not affect the ER (Figure 24a) or LR (Figure 24b) but significantly increased R_L 20 hours after challenge with OA (IL-5: 0.178 ± 0.046 cm H_2O/ml/s vs. Control: 0.094 ± 0.057 cm H_2O/ml/s, $p = 0.01$, Figure 25). There was no significant difference in responsiveness to Mch between the group that was saline-challenged and given IL-5 and the OA-challenged groups that were given BSA or IL-5 ($EC_{200}RL$: IL-5 + Saline: 10.69 ± 0.55 mg/ml vs. BSA + OA: 11.8 ± 2.93 mg/ml vs. IL-5 + OA: 17.95 ± 3.48 mg/ml; $p>0.05$, Figure 26).

Effect of rhIL-5 on blood lymphocyte subsets 8 hours after ovalbumin challenge

When compared to controls, pre-treatment with rhIL-5 caused significant differences in blood lymphocyte subsets 8 hours after OA challenge. We found a higher percentage of blood $CD8^+$ cells in IL-5 pre-treated and challenged rats (mean control: 20.6 ± 5.8 vs. challenged: 30.6 ± 3.7, $p < 0.05$), while the mean percentage of $CD4^+$ cells was lower (control mean: 43.0 ± 6.4 vs. challenged: 32.8 ± 5.2, $p < 0.05$). The CD4/CD8 ratio in the blood of IL-5 pre-treated and challenged rats was significantly lower when compared to BSA pre-treated rats (IL-5: 1.18 ± 0.47 vs. BSA: 2.92 ± 0.57, $p < 0.05$, Figure 27).

Effect of IL-5 on lung cytokine mRNA expression after ovalbumin challenge

More cells were expressing mRNA for IL-4 in the airways of IL-5-pre-treated rats 8 hours after antigen challenge (IL-5 pre-treated: 1.92 ± 0.28 positive cells/0.45 mm^2 basal membrane vs. BSA Control: 0.33 ± 0.04 positive cells/0.45 mm^2 basal membrane, $p<0.05$). Similar results were found for

cells expressing IL-5 (IL-5 pre-treated: 1.19 $\pm$ 0.08 positive cells/ 0.45 mm^2 basal membrane vs. BSA Control: 0.25 $\pm$ 0.09 positive cells/0.45 mm^2 basal membrane, $p<0.05$). However, we found no difference in interferon-γ mRNA expressing cells between both groups (IL-5 pre-treated: 0.482 $\pm$ 0.042 positive cells/ 0.45 mm^2 basal membrane vs. BSA Control: 0.394 $\pm$ 0.086 positive cells/0.45 mm^2 basal membrane, $p>0.05$).

Figure 15: Effect of IL-2 on airway responsiveness to LTD_4

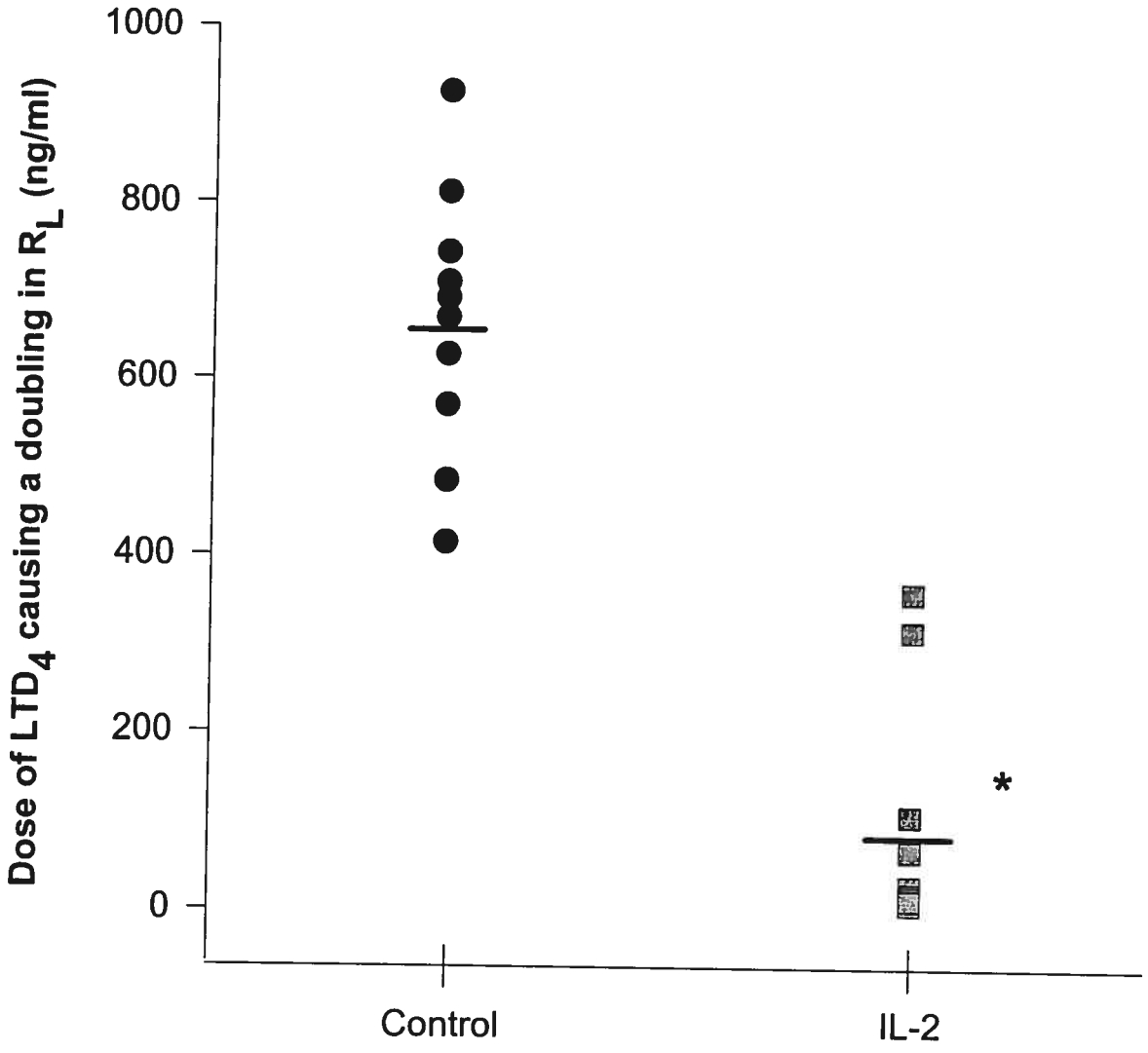


Figure 15: Effect of IL-2 on airway responsiveness to LTD_4. Rats (n=21) received either saline or IL-2 s.c. (20,000 Units/twice a day for 4.5 days pre-LTD_4 challenge) and were then challenged with increasing doses of LTD_4 (0.05, 0.5, 5.0, 50, 500, 1000 ng/ml) until baseline R_L doubled. $EC_{200}R_L$ was calculated as the amount of LTD_4 necessary to double R_L. * $p<0.05$ between IL-2-treated and saline-treated control rats.

Figure 16: Effect of Montelukast on IL-2-induced Late Airway Response

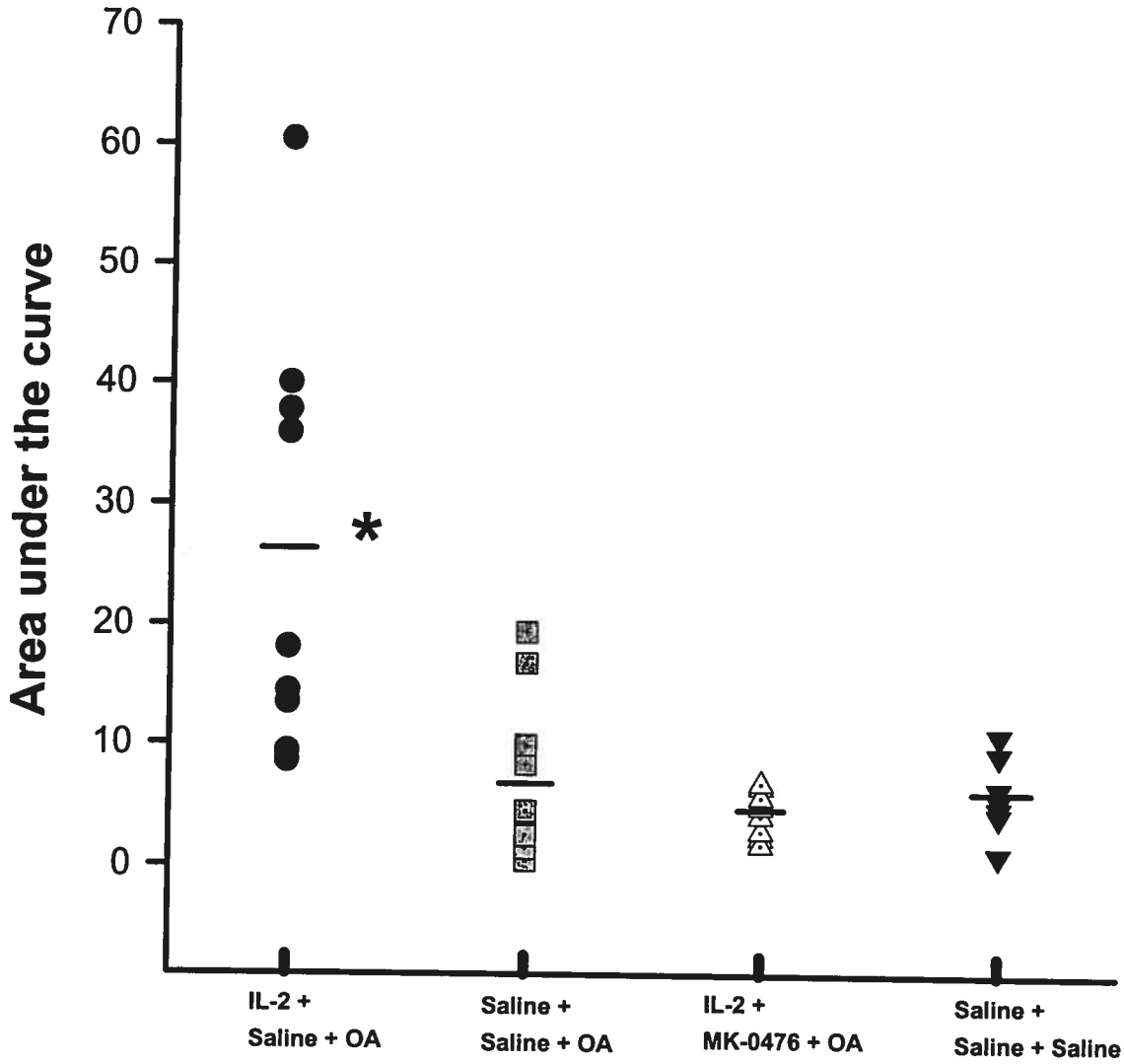


Figure 16: Effect of IL-2 alone or IL-2 and montelukast on the late airway response to ovalbumin. OA-sensitized rats (n=35) were given either IL-2 (20,000 Units/twice a day) or saline for 4.5 days starting on day 9. On the 14th day they received either montelukast (0.5mg/kg) or saline intravenously prior to and 2 hours following OA challenge. R_L was measured for 8 hours after OA challenge and the LR was calculated as the area under the curve for R_L values obtained from 4 to 8 hours after OA challenge.
* $p<0.05$ between IL-2 treated animals that received saline i.v. and IL-2 treated animals that received montelukast i.v. prior to and 2 hours following OA challenge

Figure 17: Effect of IL-2 and Montelukast on BAL Inflammatory cells

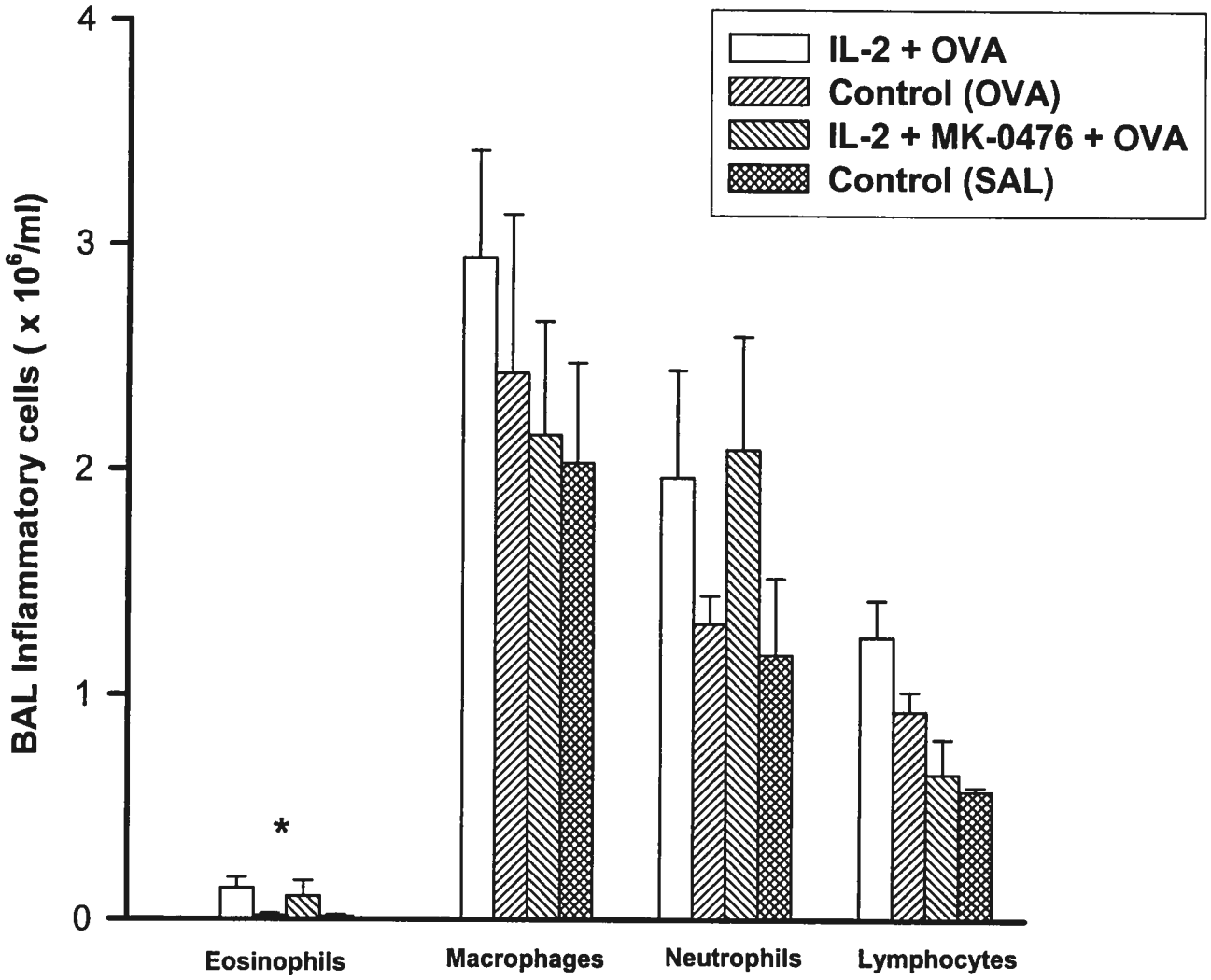


Figure 17: Effect of IL-2 pre-treatment or IL-2 and montelukast on BAL inflammatory cells after OA challenge. BAL (n=35) was recovered 8 hours after OA or saline challenge for cell differential analysis. * $p<0.05$ between OA-challenged IL-2 pre-treated rats and saline pre-treated and saline-challenged rats

Figure 18A: Effect of IL-2 and Montelukast on lung IL-4 mRNA expression

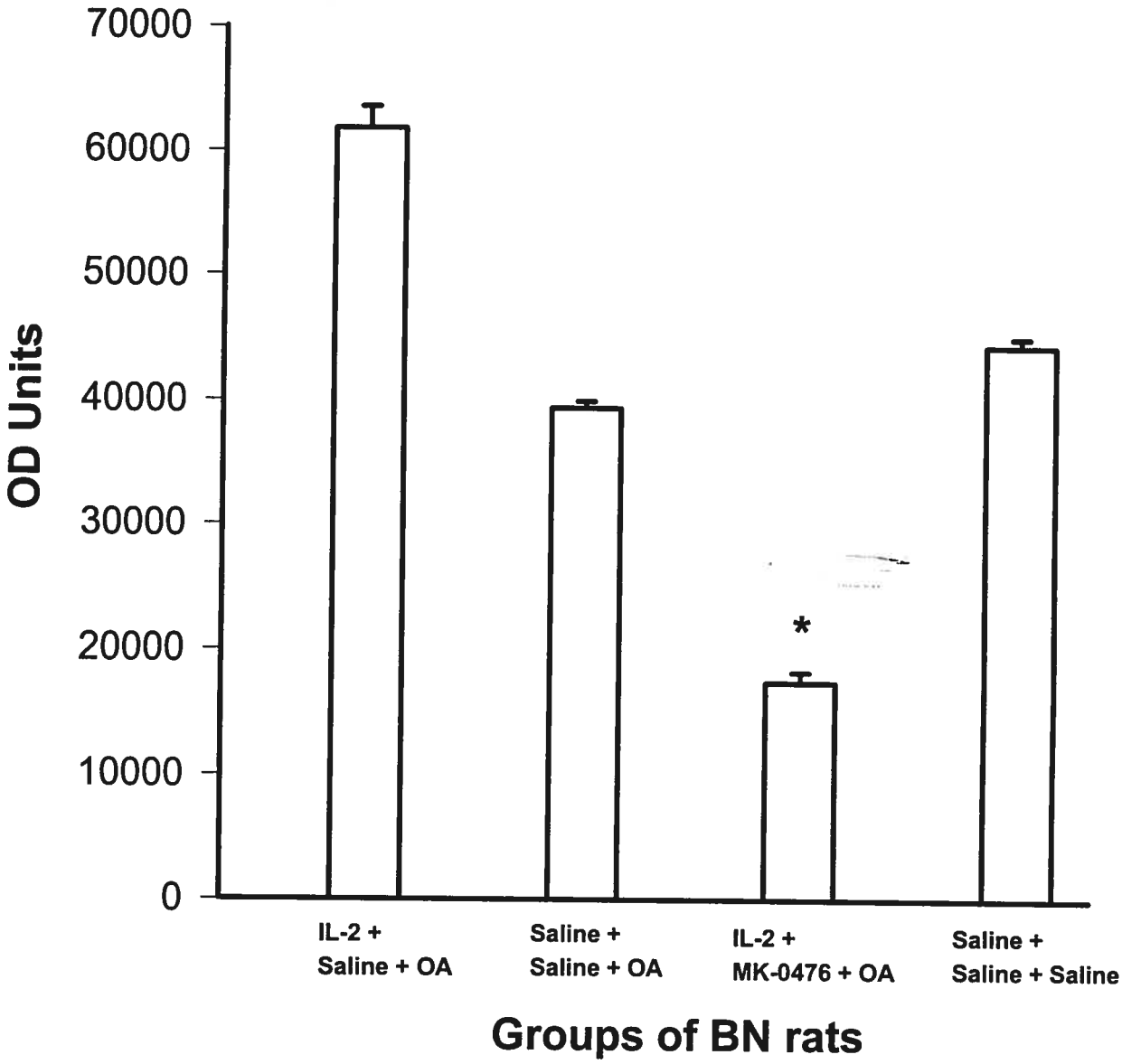


Figure 18A: Effect of IL-2 pre-treatment with or without montelukast on IL-4 total ng cytokine mRNA expression after OA challenge. Lungs (n=10) were fixed in liquid nitrogen 8 hours after challenge for SQ-PCR analysis.
*$p<0.05$ between IL-2 pre-treated rats that received either saline or montelukast i.v. prior to and 2 hours following OA challenge.

Figure 18B: Effect of IL-2 and Montelukast on lung IL-5 mRNA expression

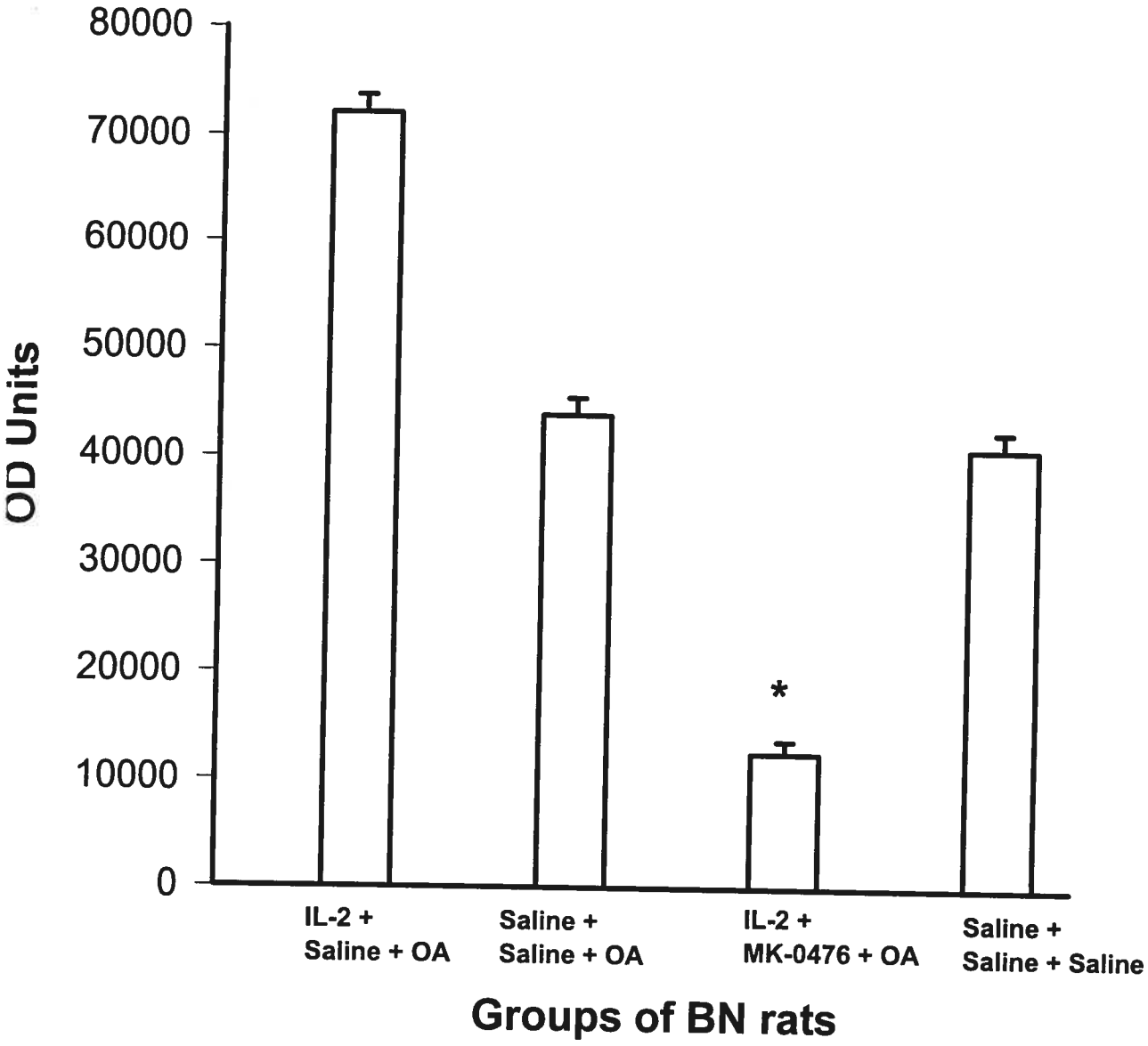


Figure 18B: Effect of IL-2 pre-treatment with or without montelukast on IL-5 total lung cytokine mRNA expression after OA challenge. Lungs (n=10) were fixed in liquid nitrogen 8 hours after challenge for SQ-PCR analysis.
*$p<0.05$ between IL-2 pre-treated rats that received either saline or montelukast i.v. prior to and 2 hours following OA challenge.

Figure 18C: Effect of IL-2 and Montelukast on lung IFN-γ mRNA expression

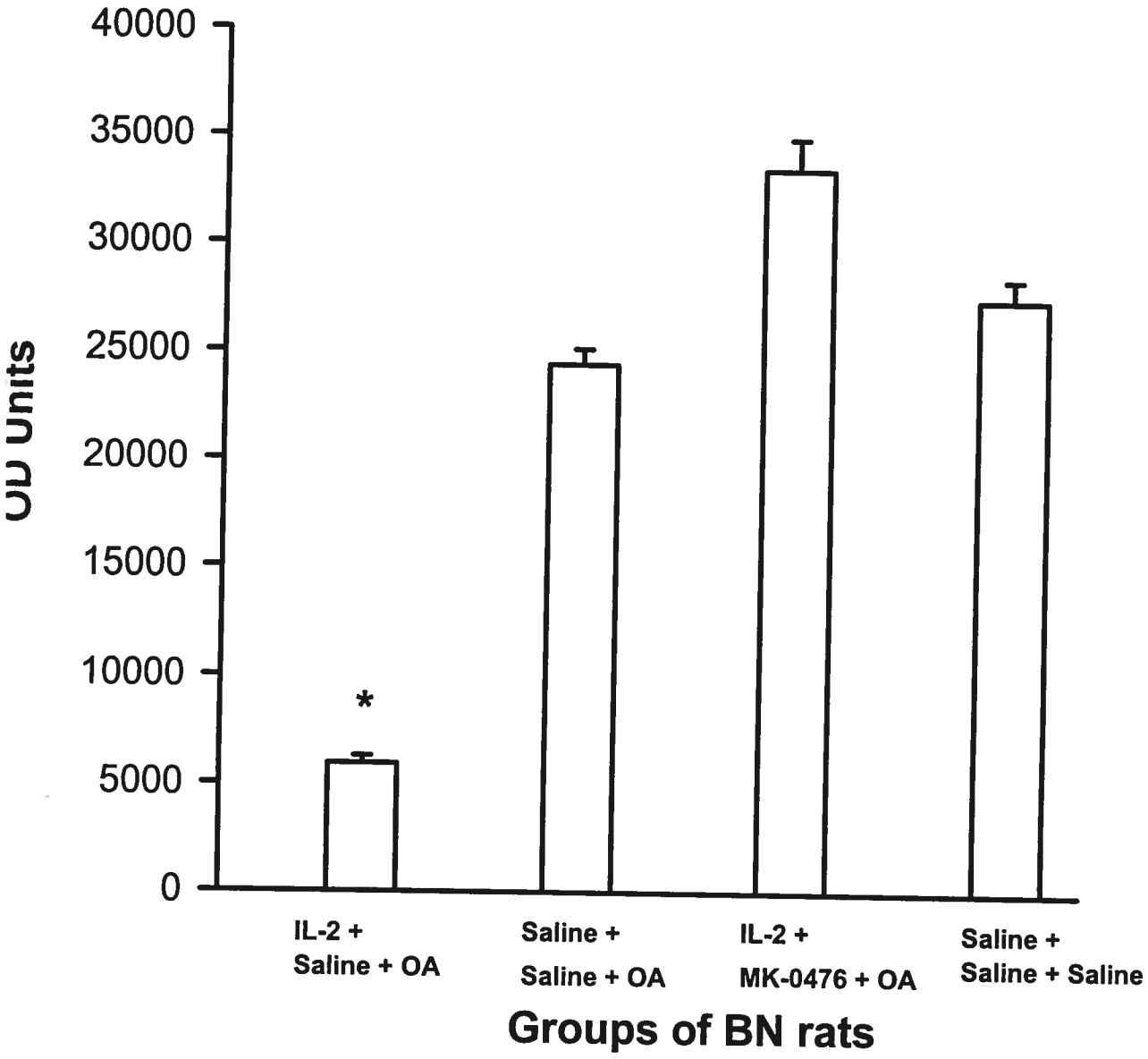


Figure 18C: Effect of IL-2 pre-treatment with or without montelukast on IFN-γ total lung cytokine mRNA expression after OA challenge. Lungs (n=10) were fixed in liquid nitrogen 8 hours after challenge for SQ-PCR analysis.
*$p<0.05$ between IL-2 pre-treated rats that received either saline or montelukast i.v. prior to and 2 hours following OA challenge.

Figure 19A: Effect of IL-2 and Montelukast on IL-4 mRNA positive cells in the airways

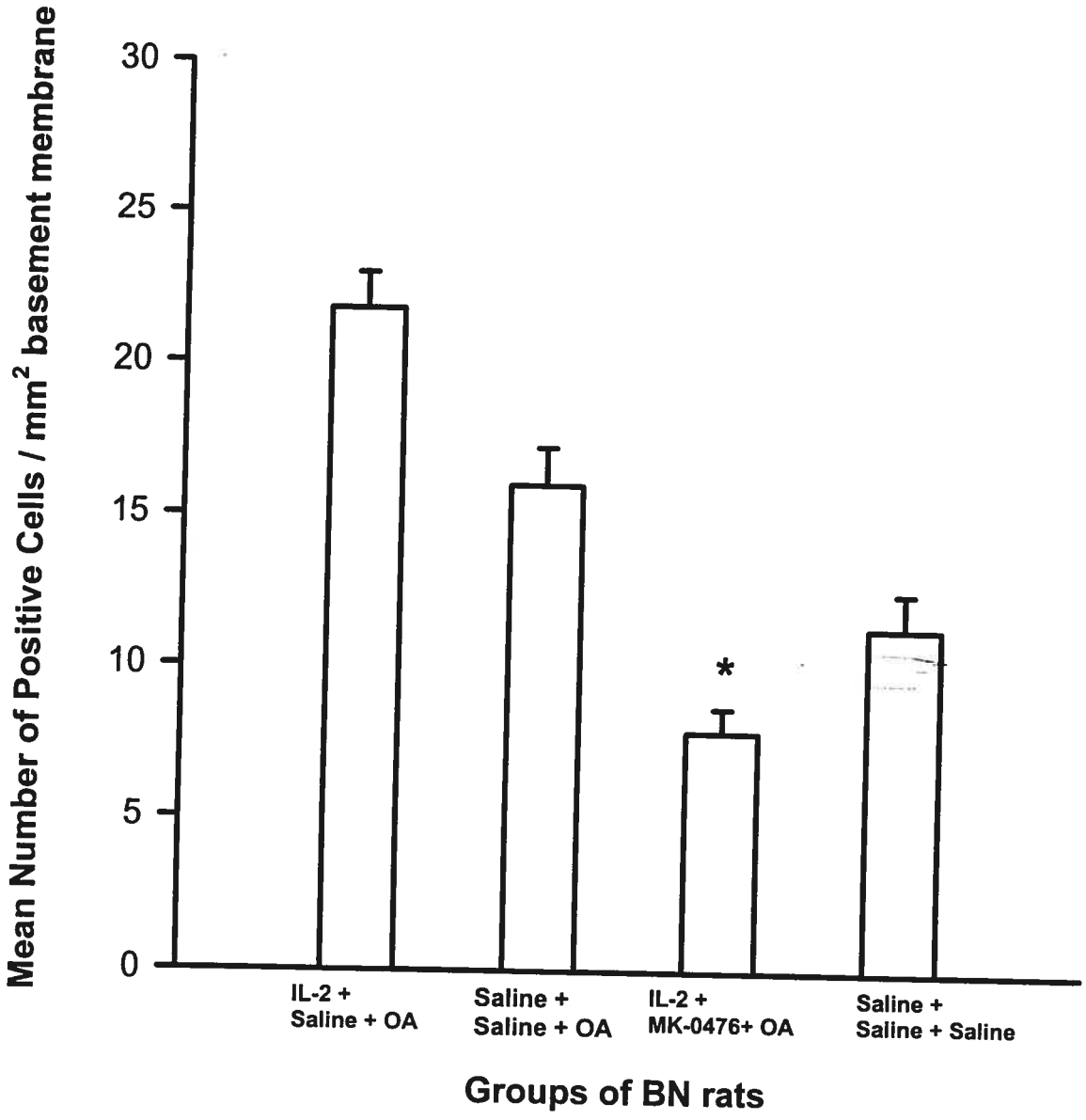


Figure 19A: Effect of IL-2 pre-treatment with or without montelukast on IL-4 mRNA positive cells after OA challenge. Lungs (n=16) were recovered in PBS-Sucrose 8 hours after OA challenge for *in situ* hybridization.

0.05 between IL-2 pre-treated rats that received either saline i.v. or montelukast i.v. prior to and 2 hours following OA challenge

Figure 19B: Effect of IL-2 and Montelukast on IFN-γ mRNA positive cells in the airways

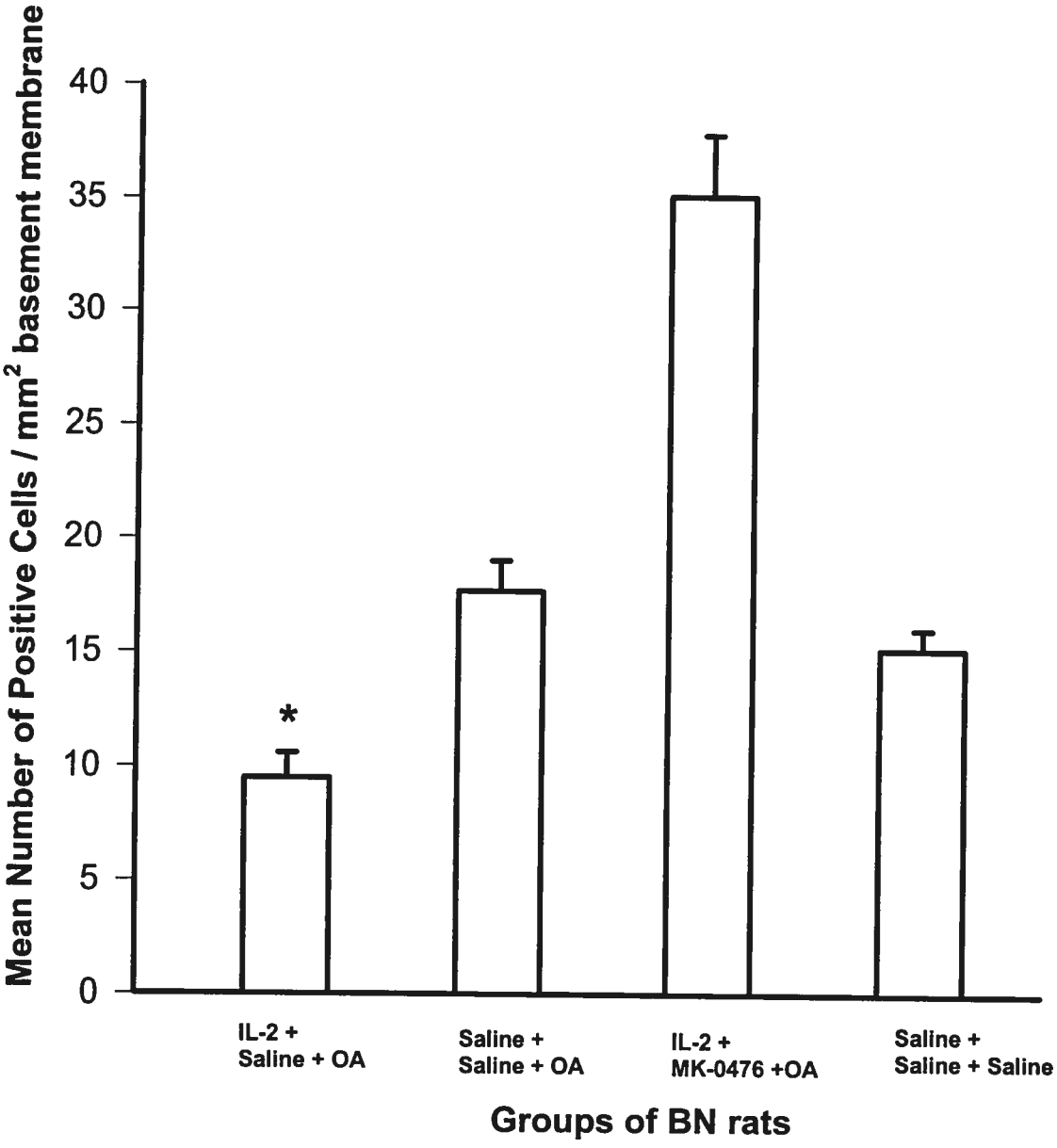


Figure 19B: Effect of IL-2 pre-treatment with or without montelukast on IFN-γ mRNA positive cells after OA challenge. Lungs (n=16) were recovered in PBS-Sucrose 8 hours after OA challenge for *in situ* hybridization.
*$p<0.05$ between IL-2 pre-treated rats that received either saline i.v. or montelukast i.v. prior to and 2 hours following OA challenge

Figure 20: Effect of rhIL-5 on eosinophil progenitor colony formation from rat peripheral blood mononuclear cells

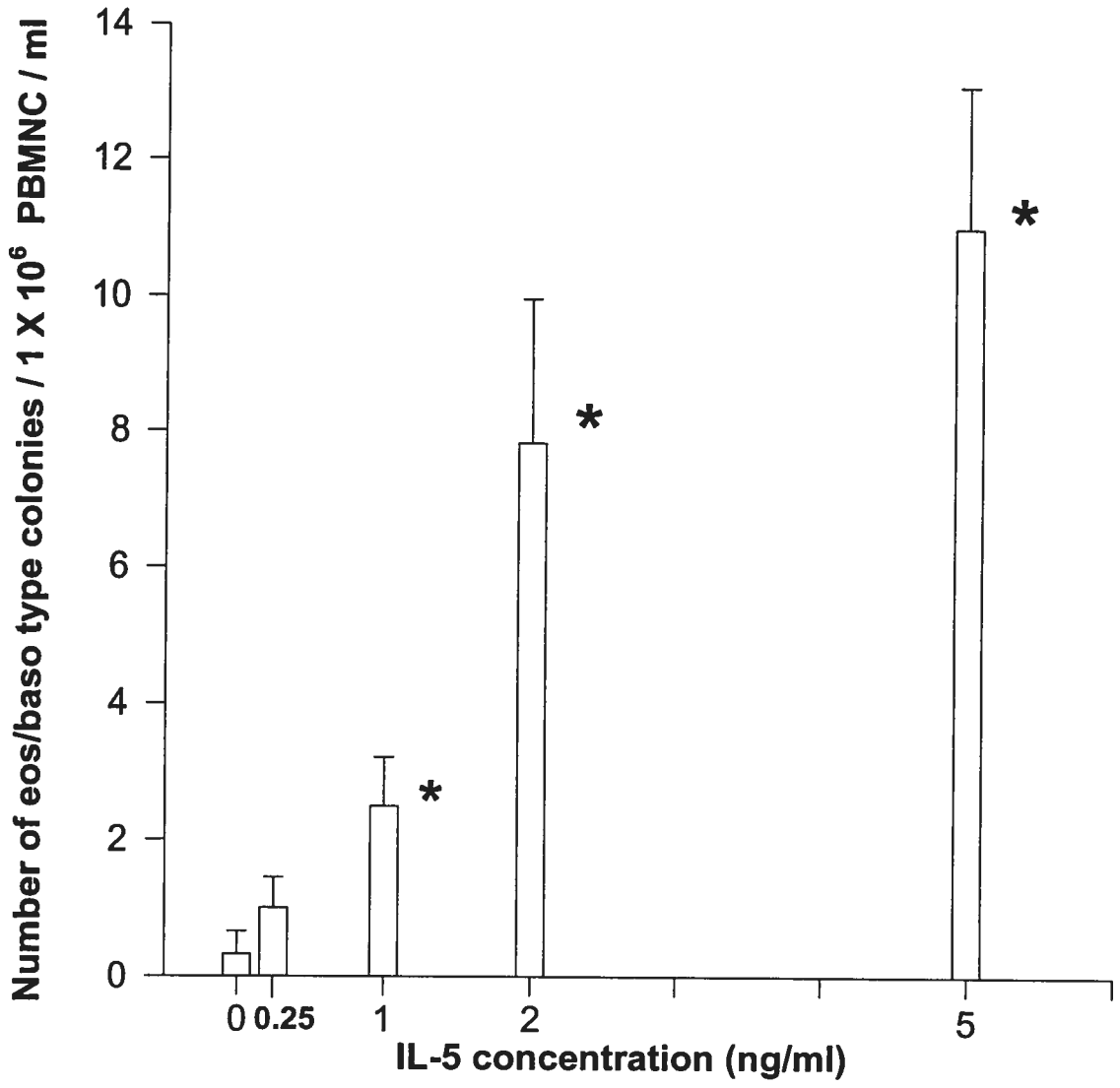


Figure 20: Effect of rhIL-5 on eosinophil/basophil type colony production in rat PBMNC's (n=10). Cells were cultured in medium alone or in different concentrations of rhIL-5 for 14 days at 37°C and colonies were counted by inverted microscopy after May-Grunwald-Giemsa staining. *$p<0.05$ between IL-5 groups (1,2 and 5ng/ml) and control

Figure 21: Effect of ovalbumin sensitization on OA-specific serum IgE levels

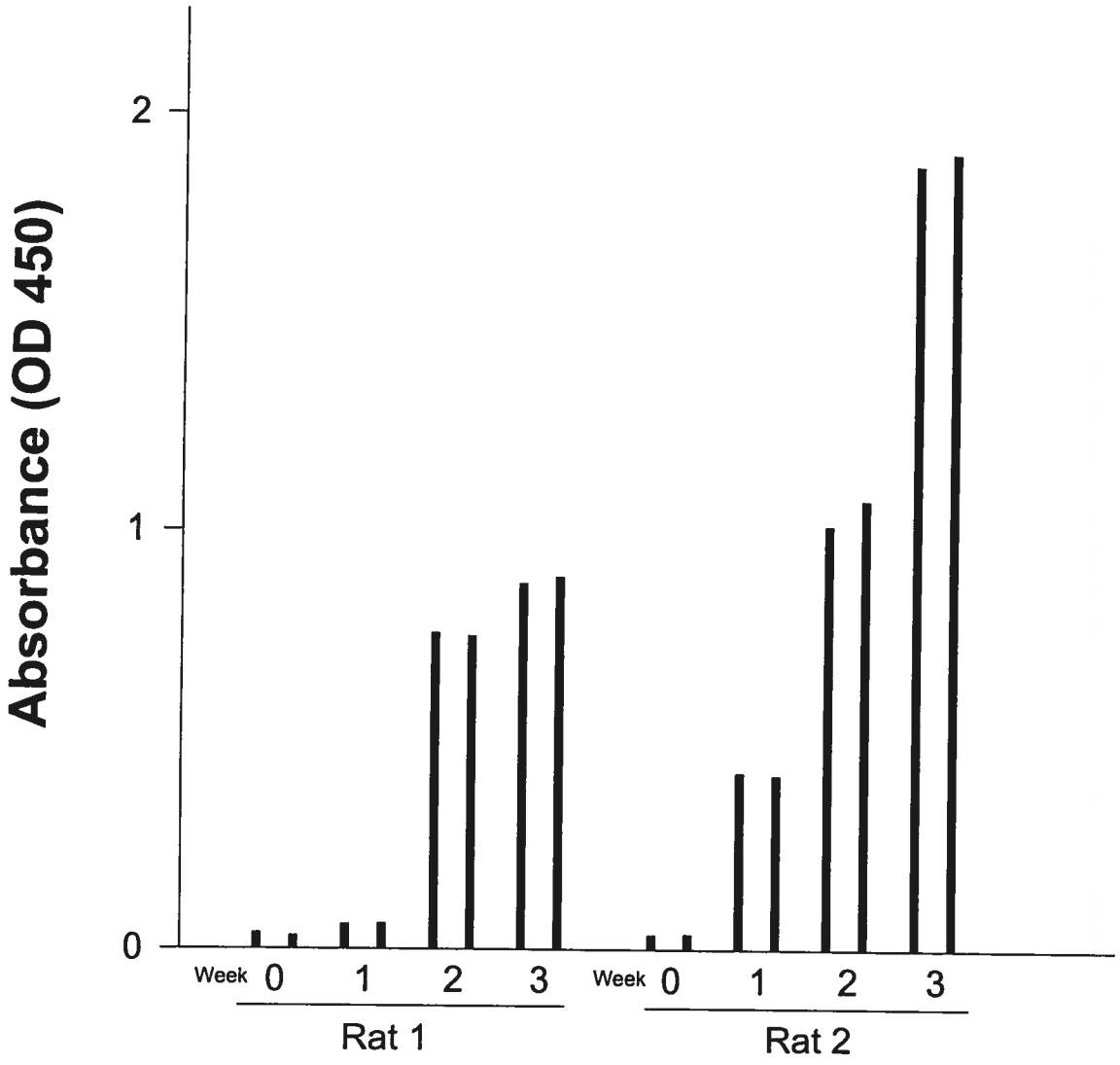


Figure 21: Effect of ovalbumin sensitization on OA-specific IgE levels. Serum was collected in duplicate from 2 actively sensitized rats from week 0 to week 3 following sensitization. OA-specific IgE levels was measured using ELISA. Both animals showed a major increase in OA-specific IgE levels from week 0 to week 3

Figure 22: Effect of IL-5 on airway responsiveness to Methacholine 20 hours after administration

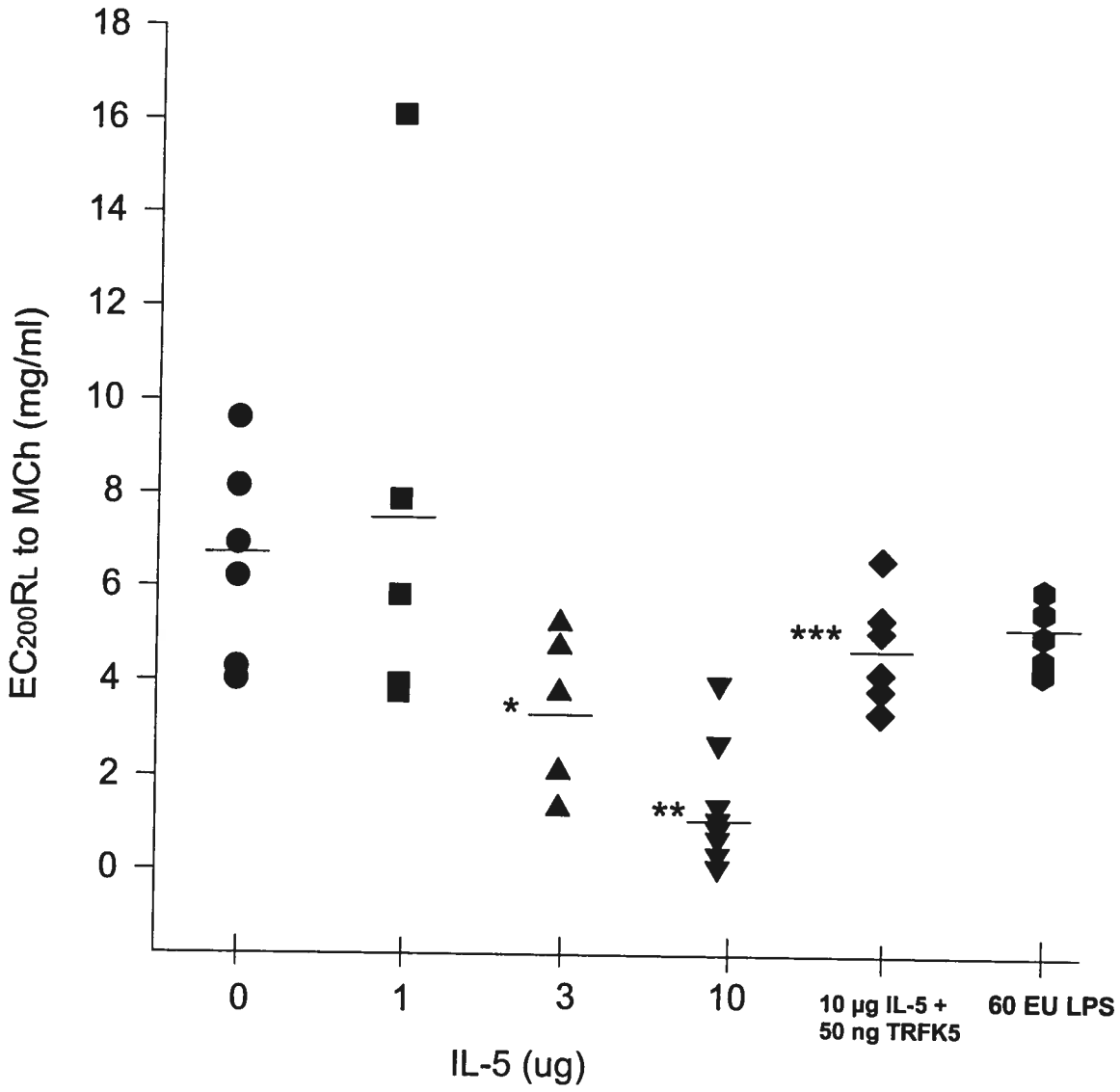


Figure 22: Effect of IL-5 on airway responsiveness to Mch 20 hours after administration. Rats (n=26) received either 1µg, 3µg, 10µg of IL-5 or BSA (control) intra-tracheally followed by Mch challenge 20 hours later. 6 rats were given 50 ng of anti IL-5 antibody intra-tracheally immediately after receiving 10µg of IL-5. 5 rats were given 60 EU of LPS 20 hours prior to Mch challenges. Doubling doses of nebulized Mch was given untill baseline R_L doubled. $EC_{200}R_L$ was calculated as the amount of Mch necessary to double R_L. Bars represent the means of each group. *p<0.05 between IL-5 treated (3µg) and BSA-treated (control). ** p<0.01 between IL-5 treated (10µg) and BSA-treated (control). *** p<0.05 between IL-5 treated (10µg) and 10µg IL-5 with 50ng TRFK5.

Figure 23: Effect of 10µg rhIL-5 on airway responsiveness to Methacholine 30 minutes and 72 hours after administration

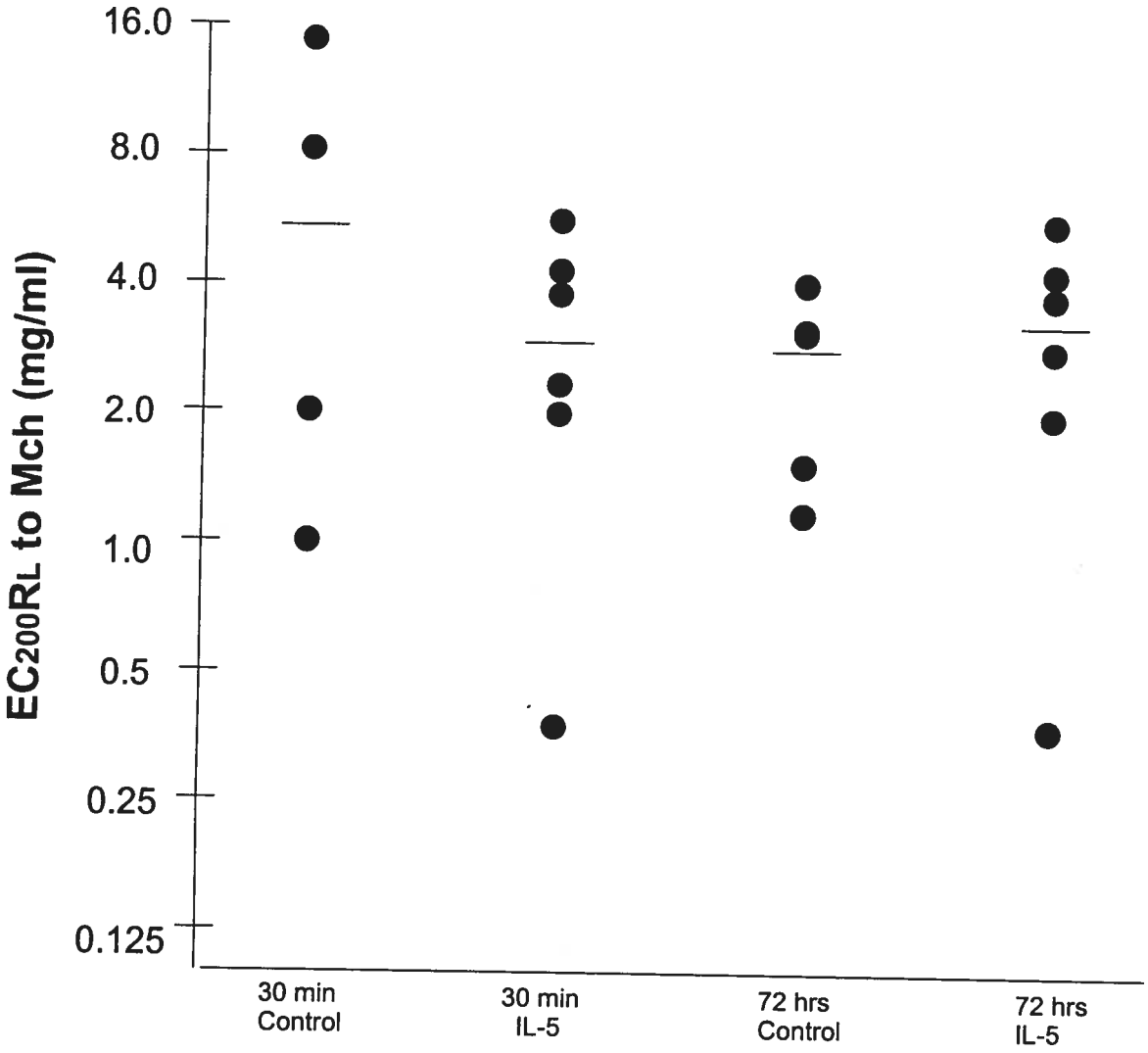


Figure 23: Effect of 10µg of IL-5 on airway responsiveness to Mch 30 min. and 72 hours after administration (n=22). Rats received either 10µg of rhIL-5 or BSA (control) intra-tracheally followed by Mch challenge 30 min. or 72 hours later Doubling doses of nebulized Mch was given until baseline R_L doubled. $EC_{200}R_L$ s calculated as the amount of Mch necessary to double R_L. Bars represent the mean of each group

Figure 24A: Effect of pre-treatment with rhIL-5 on Early Airway Response

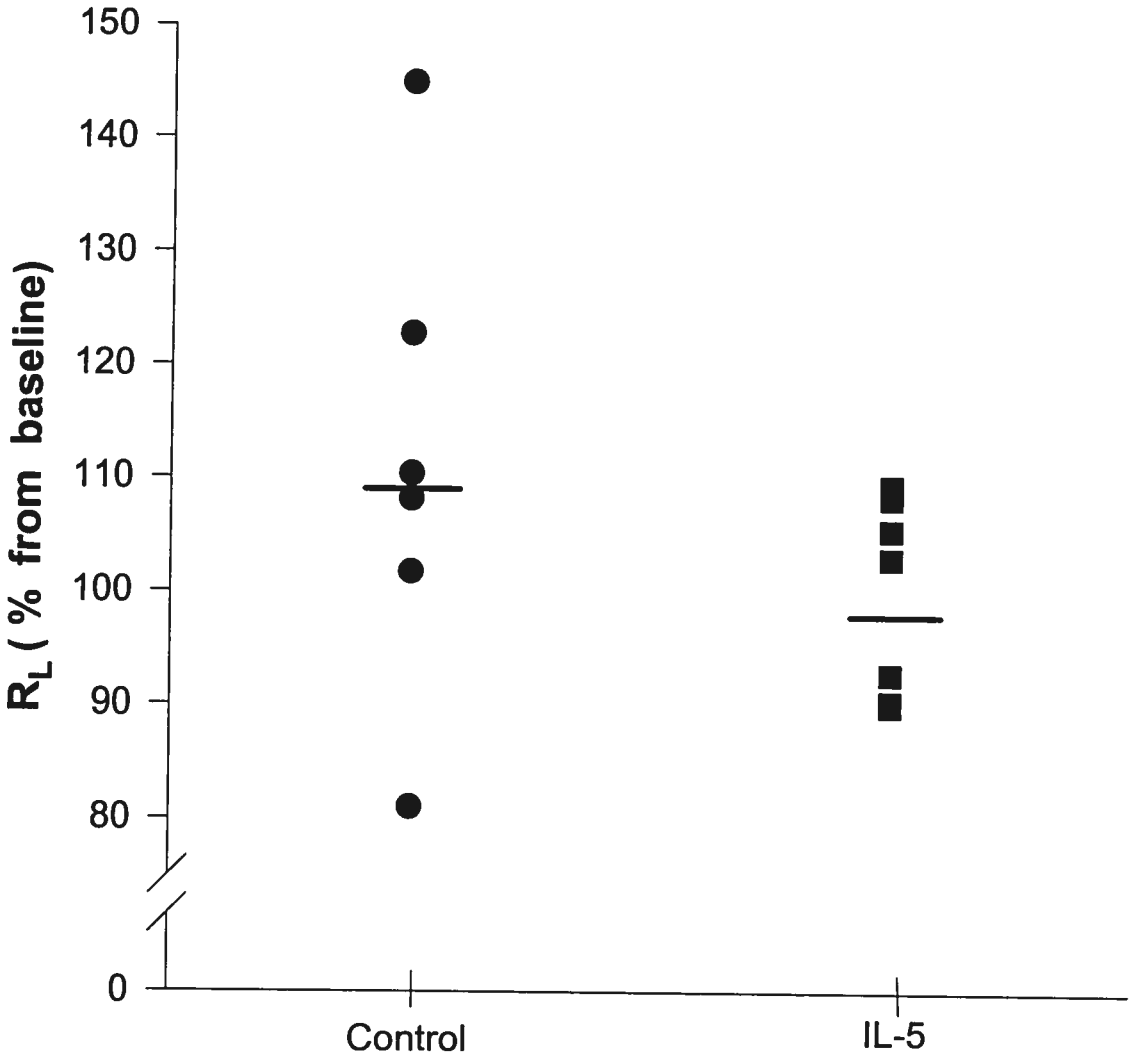


Figure 24A: Effect of pre-treatment with rhIL-5 on the ER after antigen challenge. Rats (n=23) were sensitized to OA and received either 3µg of rhIL-5 or BSA intra-tracheally. Twenty hours later, rats were challenged with OA and R_L was measured for 8 hours. The ER was calculated using the highest R_L value during the first hour after antigen challenge. Bars represent the means of each group.

Figure 24B: Effect of pre-treatment with rhIL-5 on the Late Airway Response

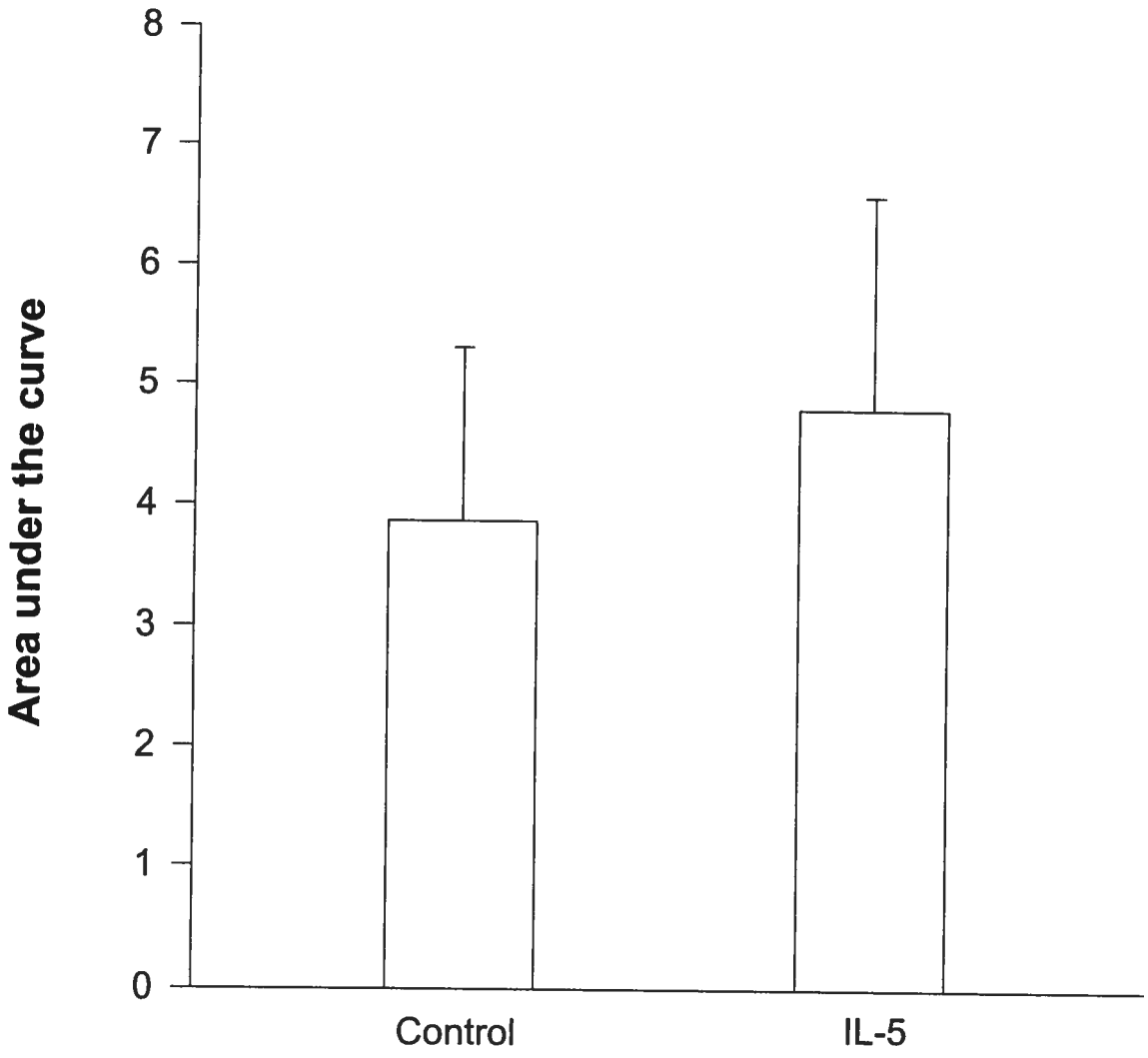


Figure 24B: Effect of pre-treatment with rhIL-5 on the LR after antigen challenge. Rats (n=23) were sensitized to OA and received either 3µg of rhIL-5 or BSA intra-tracheally. Twenty hours later, rats were challenged with OA and R_L was measured for 8 hours. The LR was calculated as the area under the curve for R_L values obtained from 4 to 8 hours after antigen challenge. Bars represent the means of each group

Figure 25: Effect of rhIL-5 on lung resistance 20 hours after antigen challenge

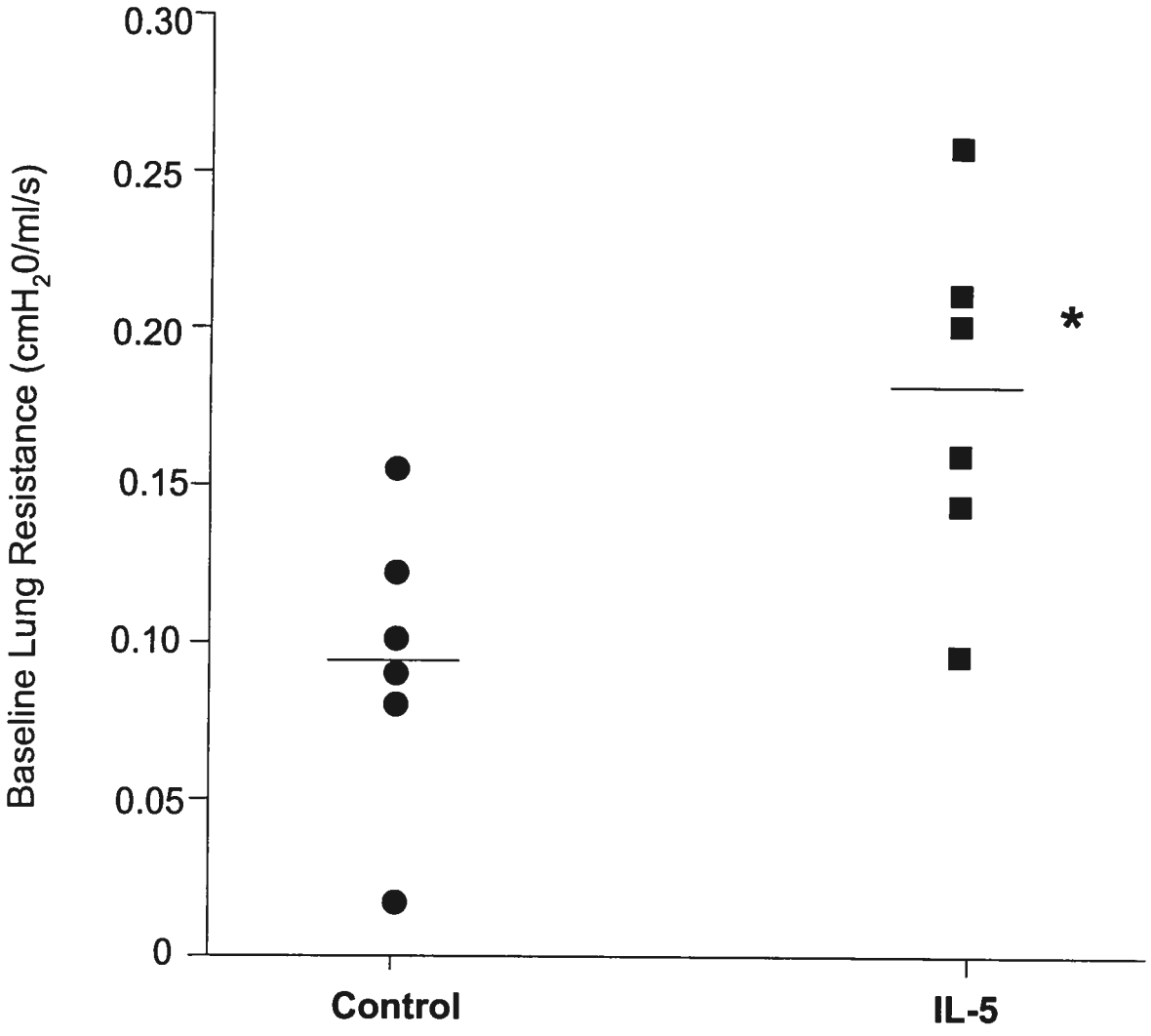


Figure 25: Effect of rhIL-5 on lung resistance 20 hours after antigen challenge. Rats (n=12) were given 3 µg of rhIL-5 or BSA and challenged with OA 20 hours later. R_L was measured 20 hours after antigen challenge in both groups of rats. Bars represent the mean of each group. *$p<0.05$ between IL-5 treated/ challenged and control groups.

Figure 26: Effect of rhIL-5 on the airway responsiveness to Methacholine 20 hours after antigen challenge

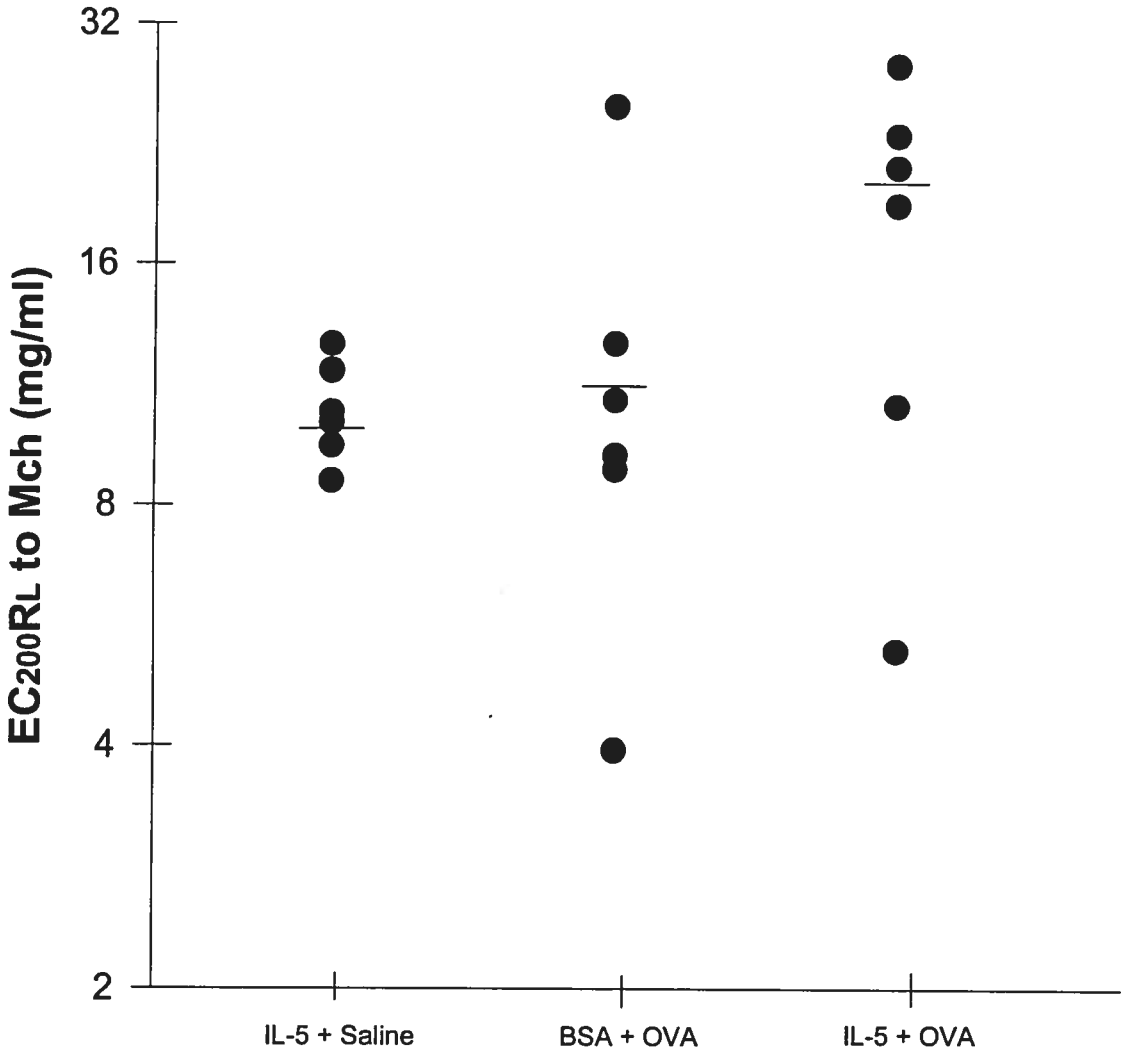


Figure 26: Effect of rhIL-5 on the airway response to Mch 20 hours after antigen challenge. Rats (n=19) were given 3 µg of rhIL-5 or BSA and challenged with saline or OA 20 hours later. Twenty hours after OA or saline challenge rats were given exponentially increasing doses of nebulized Mch until baseline R_L doubled. $C_{200}R_L$ was calculated as the amount of Mch necessary to double R_L. Bars represent the mean of each group.

Figure 27: Effect of rhIL-5 on CD4/CD8 Lymphocyte ratio in the blood 8 hours after antigen challenge

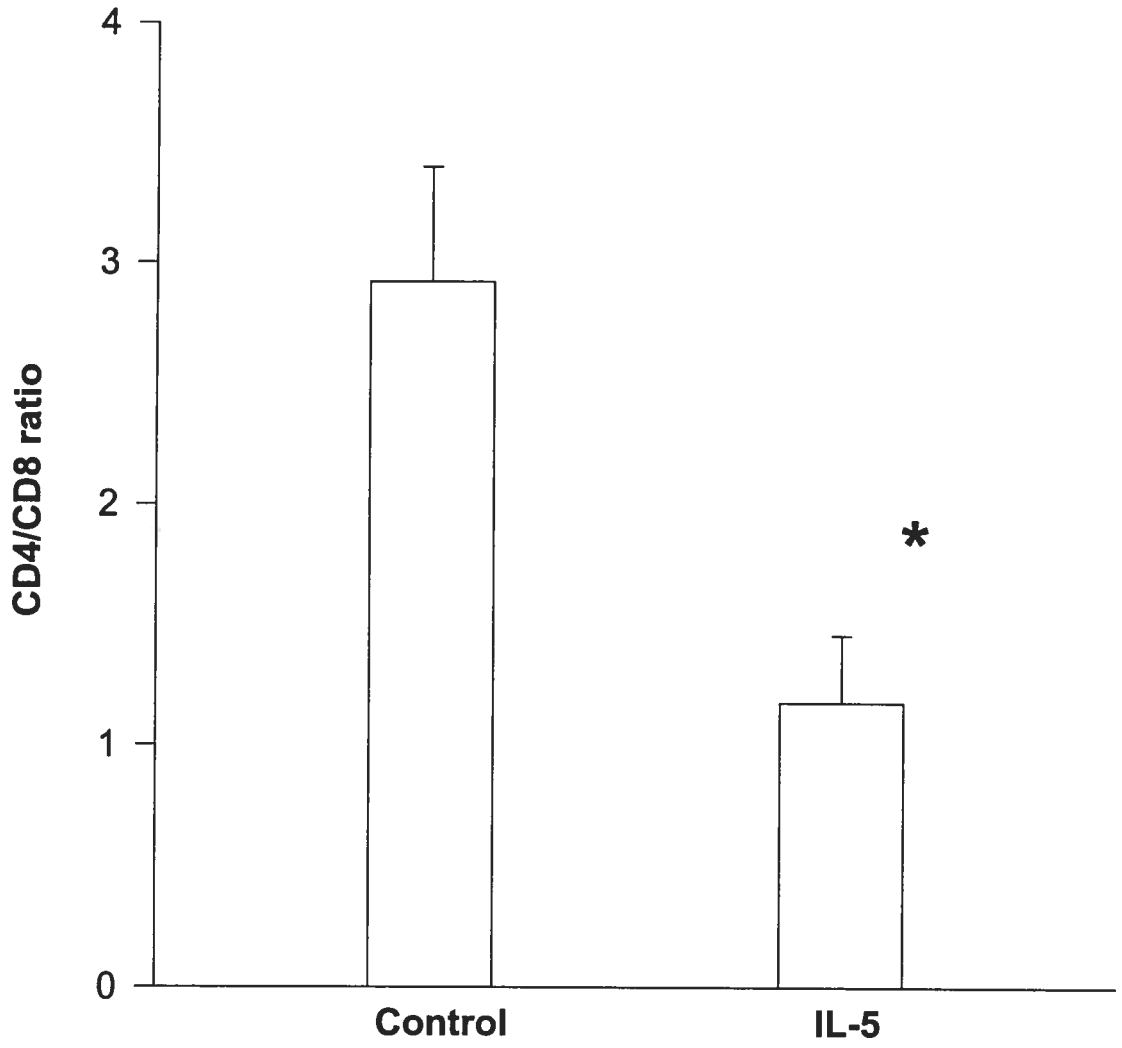


Figure 27: Effect of rhIL-5 on lymphocyte subsets in the blood 8 hours after antigen challenge. Rats (n=14) were sensitized with OA and received either 3µg of rhIL-5 or BSA intra-tracheally.Twenty hours later, rats were challenged with OA. Peripheral blood was recovered 8 hours after challenge and analyzed by flow cytometry for the ercentages of $CD4^+$ (helper) and $CD8^+$ (suppressor/cytotoxic) lymphocytes. The CD4/CD8 ratio is presented for 7 rats in each group. *p<0.05 between IL-5 treated/challenged and control groups

Discussion

The initiative for my doctoral thesis began by reflecting on a paper by Renzi *et al.* that assessed whether the increase in airway narrowing by pre-treatment with IL-2 in BN rats was mediated by an increase in leukotriene production (11). Cysteinyl-LT synthesis was evaluated by measuring the different leukotriene metabolites in the bile by radioimmunoassay after purification by high-pressure liquid chromatography. Bile has been previously shown to be the major route of excretion of cys-LTs (302,303,304). Although IL-2 pre-treatment increased the early and late airway responses after OVA challenge and enhanced airway inflammation, cys-LTs were lower in IL-2-treated rats at baseline and during the late response compared to controls, and this effect could not be explained by changes in cys-LT production or metabolism. Studies in humans, primates, and sheep that have looked at the release of mediators from inflammatory cells have shown that cys-LT retrieval from the bile or from the lungs increases during the late response and that selective antagonists of cys-LTs block the late response (302,305,306,307). Therefore, it is undeniable that cys-LTs have a role in the airway response to allergen. I sought to explain these observed discrepancies in the IL-2 pre-treated BN rat model.

We began our investigation in this animal model by studying the effects caused by IL-2-mediated T cell activation. Although there is strong evidence that the allergic airway response can be modulated by T lymphocytes, it is unclear which pathways are used. By designing an animal model protocol that produced a state of heightened T cell activation, I hoped to see by what mechanism cell mediated immunity may increase the late response. IL-2 is an important T cell growth factor that is released by T lymphocytes in response to antigen presentation and leads to lymphocyte activation, proliferation and cytokine production (11). IL-2 and its receptor are increased in the lungs of asthmatic subjects (308). IL-2 is currently used in cancer therapy to activate immune effectors, such as T lymphocytes (309). Activated T lymphocytes are present in the airways of patients with asthma and have been shown to correlate with asthma severity as evidenced by the inverse correlation between $CD25^+$(IL-2)/ $CD4^+$ cells and peak expiratory flow rates (310). Not only are there increased numbers of "activated" $CD25^+$ (IL-2 receptor) bearing T helper cells in the peripheral blood of resting mild atopic asthmatics compared to normal controls, this difference is even more pronounced during the development of exercise-induced bronchospasm (310). In T cell cultures, IL-2 not only induced CD25 (IL-2R) but also induced surface

expression of several other activation-associated glycoproteins including OX40, LFA-1, B7.1, B7.2, TCR, and CD4. This pleiotrophic activation led to subsequent antigen reactivity in assays for T-cell proliferation (311). IL-2 also stimulates T lymphocytes from asthmatic subjects to release mediators that increase eosinophil proliferation and survival (312). Its administration to human subjects causes eosinophilia through production of IL-5 (313). IL-2 has been shown to increase inflammatory cells in the lung lavage, namely macrophages, neutrophils and lymphocytes (11), therefore, it is capable of inducing pleiomorphic inflammation.

This inflammation can have a multitude of effects on the airways and the lungs, therefore our first experiment was to test their sensitivity to leukotrienes. In previous studies performed on the BN rat, Renzi *et al* showed that IL-2 pre-treatment caused an increase in the number of inflammatory cells present in the BAL (10) and an increased airway response to ovalbumin (10). Since the LR in BN rats and in humans is related to LTs (10), one might assume that leukotrienes would also be increased but as Renzi *et al.* showed, cys-LTs were not increased in IL-2 pre-treated rats despite a LR (11). This observation could be the result of the airways being more sensitive to leukotrienes. We tested this theory by assessing the airway responsiveness to LTD_4 in IL-2 pre-treated and control rats. Our results show that rats pre-treated with IL-2 require a significantly lower mean dose of LTD_4 to double baseline R_L when compared to controls ($p< 0.05$) **(Figure 14)**. We conclude that up-regulation of immune function with IL-2 increases the airway responsiveness to LTs. The precise mechanism by which IL-2 alters the responsiveness to LTD_4 is unclear. We have previously found a significant increase in BAL eosinophils after pre-treatment with IL-2 compared to untreated control animals that were challenged (10) or unchallenged with antigen (OA) (11). In the experiments reported here, eosinophils were also increased in IL-2 pre-treated rats after OA challenge when compared to controls. An increase in eosinophils in the airways may lead to the release of more mediators such as eosinophil cationic protein (ECP) (312) that have the potential to affect the airway responsiveness to LTs. The role of eosinophil-derived proteins in bronchial asthma is linked to their ability to injure the respiratory epithelium. (314,315,316). This cytotoxic effect may then result in the loss of the functional integrity of the epithelium and the exposure of mucosal structures and sensory nerve endings, allowing the development of increased sensitivity of the airways to nonspecific bronchoconstrictor agents. Another cause of increased sensitivity to LTs is the potential effect

caused by IL-2 on Cys-LT_1 receptors. Therefore our next experiment was to study the Cys-LT_1 receptor in our model.

The cysteinyl leukotrienes are known to cause airway smooth muscle contraction, which is a direct function of airway responsiveness. The Cys-LT_1 receptor is expressed in several tissues, including human lung, human bronchus, and on human peripheral blood leukocytes including eosinophils, neutrophils, monocytes, and T lymphocytes (317). It has been shown that airway smooth muscle cells express a higher level of cytokine receptor mRNA and surface protein in the atopic asthmatic sensitized state (318). IL-2 upregulates the production of cytokines that can increase surface receptors (319). It is possible then that IL-2 increases the expression of the Cys-LT_1 receptors on the surface of airway smooth muscle cells hence an enhanced sensitivity to LTD_4. Although oligonucleotide primers for the Cys-LT_1 receptor gene have not been successfully synthesized at this time, future studies would include studying the expression of this important receptor. However, IL-2 may also be exerting an effect at different points along the signal transduction pathway of the Cys-LT_1 receptor and not just specifically on the absolute number of receptors. Activation of these receptors, which are coupled to G proteins, induces calcium release that results in airway smooth muscle contraction (320). Nabata *et al.* have shown that vascular smooth muscle cells pre-incubated with IL-2 had an increase in intracellular Ca^{+2} and in DNA synthesis when exposed to angiotensin II (321). The Cys-LT_1 receptor has been shown to have a calcium mobilization response to the individual Cys-LTs. This signal appears to be the result of Ca^{+2} release from intracellular stores (322). Therefore, it is possible that IL-2 may potentiate the effects of LTD_4 on the airways by increasing Cys-LT_1 receptor expression on airway smooth muscle and/or increasing the activity of the signal transduction pathway.

In order to assess whether the cys-LTs were involved in the increased LR after pre-treatment with IL-2, we administered the Cys-LT_1 receptor antagonist, montelukast, at the time of OA challenge in rats pre-treated with IL-2. The IL-2 induced LR was completely inhibited by montelukast **(Figure 15)**. These results show that LTs are not only involved in the LR but also in the increased airway response to OA after pre-treatment with IL-2. The basis for Cys-LT activity in the lung may be due to the site where its receptor is located. The recently cloned Cys-LT_1 receptor has been detected in human lung smooth-muscle cells and lung macrophages (321,322). The identification of the Cys-LT_1 receptor in the lung is

consistent with the anti-bronchoconstrictive actions of Cys-LT_1 receptor antagonists such as montelukast (322). It is therefore likely that IL-2's effects on the LR occur through either an expansion of Cys-LT_1 receptor expression in the lungs or an increase in the sensitivity of the receptor to LTs as described above. Besides the Cys-LT_1 receptor, airway function can be modulated by countless other inflammatory pathways. Our next experiment studied cellular and cytokine inflammation in the airways and lungs of our animal model.

Studies on the mechanisms of the LR have linked its pathophysiology to several types of inflammatory factors such as IgE production, Th1 and Th2 cytokine expression, and cell influx (323). However, the precise mechanism by which a LR occurs remains to be determined. We studied BAL inflammatory cells and the expression of Th1 and Th2 cytokine mRNA in the lungs of rats in order to determine if any of these factors were affected by IL-2 treatment or by inhibition of the Cys LT_1 pathway with montelukast. We found no statistically significant difference in neutrophils, macrophages and lymphocytes in the BAL between control and experimental groups of animals **(Figure 16)**. Animals challenged with OA and pre-treated with IL-2 showed a significant increase in BAL eosinophils compared to the group of animals unchallenged and receiving saline ($p<0.05$) **(Figure 16)**. The former group of animals also showed a LR, while the latter did not. This data supports results from previous studies indicating that eosinophils play an important role in mediating a LR (324). Montelukast administration inhibited the IL-2-mediated LR without affecting the total number of cells or differential in the BAL 8 hours after OA challenge. Although there was a decrease in the number of BAL eosinophils in the group of animals that were pre-treated with IL-2 and received montelukast, this difference was not statistically significant **(Figure 16)**. Montelukast has been shown to have numerous effects on eosinophils. Virchow *et al* (325) showed that eosinophil transmigration across cultured human umbilical vein endothelial cells can be blocked by montelukast. Moreover, LT receptor antagonists increased basal rates of eosinophil apoptosis and reversed GM-CSF-induced eosinophil survival (326). This is evidence of an autocrine cysteinyl leukotrienes pathway that supports eosinophil survival in response to a range of survival stimuli. Montelukast has been shown to reduce the percentage of eosinophils in the sputum and in the peripheral blood of mild asthmatics, but there was no significant correlation between this decrease and the observed increase in peak expiratory flow (PEF). This suggests that montelukast has anti-inflammatory effects on

the airways of patients with asthma and that its bronchodilatory effect is not solely dependant on a decrease in airway eosinophilia (327). These results suggest that airway inflammation may be present without any impact on airway tone (if LT activity is inhibited). However in a mouse model of asthma, montelukast had inhibitory effects on inflammatory cell infiltration in the bronchoalveolar lavage along with a reduction in bronchial hyperresponsiveness (328). These inhibitory effects of montelukast suggest a more important role for Cys-LTs in the animal model of allergic asthma.

We continued our investigation of this role by studying cytokine expression, specifically Th1 and Th2, in the lungs of our animal model. We assessed cytokine mRNA expression in the lung by using SQ-PCR and found that rats pre-treated with IL-2 had a significant increase in IL-4 and IL-5 mRNA (Th2) **(Figure 17A/17B)** and lower IFN-γ mRNA (Th1) **(Figure 17C)** expression after OA challenge when compared to animals given IL-2 and receiving montelukast at the time of challenge ($p<0.05$). Densometric analysis allowed us to compare cytokine levels between groups in our study but their values cannot be compared with other published literature. Therefore, the baseline levels of IL-4, IL-5 and IFN-γ in our control groups reflect only the expression of these cytokines in our animal model. These values indicate that even without IL-2 treatment, control animals express IL-4, IL-5 and IFN-γ in their lungs; findings not unexpected since these cytokines are important mediators in numerous immunological pathways. However, the differences in cytokine expression between control groups are not significant.

The change in the balance between Th1 and Th2 cytokine expression was also confirmed by *in situ* hybridization. *In situ* hybridization tests revealed that cells expressing IL-4 mRNA were more predominant **(Figure 18A)** and cells expressing IFN-γ mRNA were less predominant **(Figure 18B)** after pre-treatment with IL-2 and OA challenge. Interestingly, these changes are similar to those described previously in the BN rat or in humans when a LR occurs (329). IL-2 effector functions have been linked to Th2 cytokine activity, specifically Youn *et al* (330) showed that transgenic mice which have expression of a chimeric cytokine receptor (extracellular: IL-2Rβ chain, cytoplasmic tail: IL-4Rα) dramatically enhanced Th2 responses (IL-4, IL-5, and IgE production) upon *in vitro* TCR stimulation or *in vivo* antigen challenge. This augmented Th2 effector function is sufficient for establishment of antigen-induced airway hyperresponsiveness on a normally disease-resistant background (C57BL/6). The addition of montelukast at the time of OA challenge lead to a predominance of cells expressing the Th1 cytokine, IFN-γ and less

cells expressing IL-4 and IL-5 mRNA as described in rats that do not develop a LR (301). Previous studies showed a similar effect by montelukast on Th2 cytokines. Stelmach *et al.* (331) demonstrated that montelukast contributes to inhibition of allergic inflammation by decreasing serum IL-4 levels in correlation with improvements in clinical parameters (symptoms score, FEV_1), bronchial hyperresponsiveness in children with moderate asthma. A separate study by Henderson *et al* (332) showed that montelukast inhibited IL-4 and IL-13 mRNA in lung tissue and protein in BAL fluid in OA-treated mice. These data demonstrate that montelukast can reverse the effects of IL-2 and OA challenge on Th1-Th2 cytokine mRNA expression.

Many studies have shown the important role of cytokines in asthma (333). IL-4 can lead to selective recruitment of inflammatory cells such as mast cells and eosinophils as well as inducing the production of IgE from B cells (334). IL-5 is involved in recruiting and activating eosinophils, cells that release mediators with the potential of causing several changes that are found in asthma such as bronchoconstriction, inflammation and epithelial cell desquamation (327). Th2 cytokines have been shown to be increased in the lungs of atopic asthmatics (335). Venkayya *et al* (336) showed that Th2 cytokines might induce AHR by acting directly on resident airway cells. Th2-lymphocyte-conditioned medium administered to the airways induced AHR within 6hr in naive mice. However, AHR was not induced when mice lacked the IL-4 receptor alpha subunit or Stat6, suggesting a critical role for IL-4 and/or IL-13. The induction of AHR occurred in the absence of inflammatory cell recruitment or mucus production. IFN-γ is a Th1 cytokine that may inhibit certain effects of Th2 cytokines (318). The role that cys-LTs play in modulating the balance between Th1 and Th2 cytokines has not been previously assessed. A recent study by Hasday *et al.* showed that asthmatics with high cys-LTs production in the BAL 24 hours after segmental ragweed challenge also had increased IL-5, IL-6, and TNF-α production compared to low cys-LTs producers (337). This indicates that cys-LTs production may have a direct relationship with cytokine production. Recently, numerous studies have pointed to an important link between cytokines and the Cys-LT pathway. For example, Thivierge *et al.* (338) showed that IL-5 upregulated the Cys-LT_1R expression on a differentiated eosinophil cell line. Consequently, receptor function on these eosinophils is also augmented, namely enhanced responsiveness and chemotactic response to LTD_4. This same group showed a similar relationship between cytokines and the Cys-LT

pathway in monocytes and macrophages (339). However, in this case, the Th2 cytokines, IL-4 and IL-13, upregulated the expression and function of the cys-LT_1 receptor. These cells also showed enhanced responsiveness and chemotactic activity to LTD_4, indicating a possible mechanism through which IL-4 and IL-13 can contribute to the pathogenesis of asthma. Leukotriene D_4 has also been shown to influence cytokine production from alveolar macrophages (AM) (340). LTD_4 primes AM by causing an increase of mRNA for MIP-1α and TNF-α and when further stimulated with LPS resulted in a release of MIP-1α, TNF, and NO. This is evidence of LTD_4's ability to potentiate the production of proinflammatory mediators by AM during immunologic stimuli. Moreover, it has recently been established that the cys-LT_1 receptor is not only expressed on the smooth muscle cells of the airways, but that there is also a high expression of the receptor on peripheral blood lymphocytes (PBL) (320), suggesting that cys-LTs have an effect on T lymphocyte function. The Cys-LT_1 receptor has also been found on human mast cells. Mellor *et al.* (341) not only identified the receptor on mast cells but also showed that the receptor responds to cys-LTs and the pyrimidinergic ligand, UDP. The threshold of responsiveness to these agonists was lowered after priming of the mast cells with IL-4 for 5 days. This enhanced sensitivity was accomplished without altering Cys-LT_1 receptor mRNA or surface protein expression, suggesting the likely induction of a second receptor with Cys-LT_1-like dual ligand specificity. Evidence of such a receptor has not been observed yet, but this theory could explain how rats in our study are more sensitive to LTs after IL-2 treatment without an increase in LTs production. The results presented here suggest that cys-LTs are involved in mediating cytokine production after OA challenge because montelukast was able to change the profile of Th1 and Th2 mRNA expression. The mechanism by which the LT pathway affects cytokine mRNA production has not been explored.

In this animal model, it is clear that LTs along with inflammatory cells and cytokines play an important role in mediating a bronchopulmonary response. Allergen challenge of sensitized animals causes the activation and recruitment into the airways of a variety of cells, including eosinophils, lymphocytes, mast cells, and neutrophils (342, 300, 343). These inflammatory cells are involved in the physiological and pathological changes that are present in atopic asthma (296). The mechanism by which these changes occur are not clear. However results from this study so far suggest that the cytokine IL-5 may be an important modulator of allergic inflammation. Studies to date have shown IL-5 to be increased

in the blood and lungs of patients with asthma (344) and that administration of IL-5 to patients with allergic bronchial asthma increases cholinergic responsiveness and the number of activated eosinophils in the airways (7). Studies in animals have shown that administration of IL-5 to sensitized guinea pigs enhances antigen-induced hyperresponsiveness (345) and that IL-5 neutralizing antibodies inhibit the late phase response after antigen challenge (346). Although there is convincing evidence that IL-5 is important in asthma and in animal models of asthma, no study has studied the effects of IL-5 alone in a nonasthmatic animal model, namely in animals that do not respond to sensitization and antigen challenge with early and late-phase responses. We performed this study in the SSN substrain of BN rat that does not develop an ER or LR after antigen challenge (285, 347) in order to assess whether IL-5 has the capacity to transform non-responsive BN (SSN) rats into those with the characteristics encountered in atopic asthma.

We first determined whether recombinant human IL-5 (which has 71% homology with rat IL-5 (346)) had effects on rat blood cells *in vitro*. Experiments performed on rat PBMNC showed that rhIL-5 caused a concentration-dependent proliferation of eosinophils from progenitors at a dose ranging from 1 to 5 ng/ml **(Figure 19)**. We also determined if the BN rat model was actively sensitized to OA by measuring levels of OA-specific IgE for 1 to 3 weeks following sensitization. Rats showed a significant increase in serum OA-specific IgE for at least 3 weeks following OA sensitization **(Figure 20)**. We then determined whether intra-tracheal administration of rhIL-5 had effects on airway responsiveness to Mch. An increase in airway responsiveness to Mch occurred 20 hours after intra-tracheal administration of rhIL-5 at a dose of 3 µg or more **(Figure 21)**. Recombinant human IL-5 did not affect airway responsiveness 30 minutes or 72 hours after intra-tracheal administration **(Figure 22)**. The fact that airway responsiveness to Mch occurred at 20 hours and not at 30 minutes or 72 hours following IL-5 administration suggests that IL-5's effects on the airway is mediated thru an indirect pathway. It has been shown that in guinea pigs, inflammatory cell numbers in the tissues peak at or before 24 hours following IL-5 administration (298) and return to baseline over 6 days. Results obtained from lung lavage in our animals would concur with this study. We found a dose-dependent increase in the total cellular return, which consisted mainly in neutrophils, from the lung lavage. We measured the level of endotoxin in the rhIL-5 given to animals, and to be certain that this low level of endotoxin was not affecting the results we

administered the same dose of LPS intra-tracheally to animals and measured their airway response to Mch and inflammatory cells in the BAL 20 hours later. Data collected from animals given LPS did not differ significantly with controls, therefore we were confident that the effects of rhIL-5 were not due to endotoxin contamination. We also performed additional experiments confirming the direct effect of rhIL-5 by using TRFK5, an IL-5 neutralizing antibody. Addition of 50ng of TRFK5 to 10µg of rhIL-5 significantly reduced airway responsiveness and cell influx after OA challenge when compared to the groups that received only 10µg of rhIL-5. The type of cell influx in our IL-5-treated animals was particularly interesting. We decided to furthur understand the discrepancy between our results and published literature.

Our cell influx experiment showed that IL-5 caused an increase in BAL neutrophils 20 hours post-administration, but not in eosinophils as has been described in guinea pigs (298). This discrepancy may be due to the inherent differences between the two animal models. BN SSN rats may not have had an increased number of eosinophils in lung lavage because of technical considerations. Van Rensen *et al.* (348) administered IL-5 intravenously and by inhalation to mild asthmatics and showed an increase in blood eosinophils without any effect on eosinophil mobilization in the lungs or on bronchial responsiveness in either group. Moreover, eosinophil degranulation and mediator release may have already taken place 20 hours after IL-5 administration preventing us from measuring their presence by a regular Wright-Giemsa stain. However, we obtained the same results when we employed MBP staining which adds more probability to the conclusion that eosinophils were not increased in lung lavage 20 hours after IL-5 administration. Another reason for this discrepancy is that perhaps eosinophils are unavailable for recruitment into the lungs in this strain of rat. Until now, experiments assessing whether IL-5 is involved in asthma have employed animal models that develop allergic responses after antigen challenge and have shown that the physiological response can be increased by IL-5. It is possible that in "atopic" animal models, eosinophils or their progenitors are readily available to be recruited into the lungs whereas in BN SSN rats these cells are not available. Certain studies claim that eosinophils do not play an important role in bronchoconstriction. *In vitro* studies of the human smooth muscle response to IL-5 showed enhanced responsiveness independant of eosinophils (349). Several studies over the last year have shown that certain therapies that reduce eosinophil numbers do not lead to clinical improvement of asthma (350). Researchers are now questioning the role of eosinophils in allergic inflammation. Other

inflammatory cells may have more important roles than previously thought; such as neutrophils which release products (oxygen radicals, proteases, and cationic proteins) that have the potential for altering airway function by causing tissue injury (351). Therefore, although the SSN strain of BN rat may not respond to IL-5 with increased eosinophil accumulation into the airways, increased responsiveness to Mch 20 hours post-IL-5- administration may have still occurred through neutrophil degranulation.

Having studied the effects of administering IL-5 alone in our animal model, we decided to continue studying the effects of IL-5 on animals challenged with antigen, which causes higher IgE production. Could IL-5 cause a physiological change in the lungs of these animals perhaps leading to an ER, LR, or AHR? Pre-treatment of guinea pigs with IL-5 has been shown to induce lung eosinophilia and bronchoconstriction after antigen challenge (8). As well, administration of a monoclonal anti-IL-5 antibody to sensitized guinea pigs inhibits both BAL eosinophilia and airway responsiveness after OA challenge (352). We assessed the effects of pre-treatment with rhIL-5 on the ER **(Figure 23A)**, LR **(Figure 23B)**, and AHR in BN SSN rats that do not have a physiological response after sensitization and OA challenge. Intra-tracheal administration of 3µg of rhIL-5, 24 hours prior to OA challenge, did not affect the ER, LR, or cellular influx after challenge when compared to controls receiving the same amount of BSA. It may be that in the predisposed subject, intra-tracheal IL-5 is capable of increasing all the physiological changes that occur after antigen challenge, but that several crucial factors that are necessary for an atopic airway response may be lacking in the normal rat. These include the presence of inflammatory cells, certain chemokines (eotaxin, MCP-4) or of other cytokines (GM-CSF) (353,354). Although these animals did not produce an ER or LR after IL-5 treatment and Ag challenge, their lungs could still be susceptible to surface changes.

We measured these surface changes by calculating the animals' lung resistance and AHR 20 hours after Ag challenge. We chose these parameters becauses physiological changes that occur after antigen challenge also include airway obstruction and increased cholinergic airway responsiveness. We first tested the efficacy of our sensitization protocol by giving a group of non-sensitized rats a 3 µg dose of IL-5 followed 20 hours later by an OA challenge. Lung resistance was not significantly different from the control group of animals that were sensitized and given BSA instead of IL-5. However, pre-treatment of sensitized rats with IL-5 increased baseline lung resistance measured 20 hours after OA challenge

(Figure 24). IL-5 had no effect on airway responsiveness to Mch **(Figure 25)**. The changes in airway resistance can be linked to various immunological events including T cell activation, IL-4 production, IgE synthesis and mediator release by effector cells such as eosinophils and mast cells (355). In our study, we found a significant decrease in the $CD4^+/CD8^+$ T cell ratio in the blood between IL-5 pre-treated/challenged and control animals 8 hours after antigen challenge **(Figure 26)**. The difference is mainly due to an increase in $CD8^+$ cytotoxic/suppressor T cells in the blood of challenged animals. A decrease in the helper/suppressor ratio in the blood has been described during the LR in humans (296). It has been postulated that $CD4^+$ lymphocytes are recruited into the lungs after antigen challenge in the subjects that develop a LR. This hypothesis has been predicated upon the finding of increased $CD4^+$ cells in lung lavage after antigen challenge (355). We have previously reported a decrease in the helper/suppressor ratio in the blood of BN rats that develop a LR (300). Interestingly, pre-treatment with IL-5 induced the same changes in the CD4/CD8 ratio as those previously reported in BN rats with LR. However, it is not clear how IL-5 modulates the CD4/CD8 T cell ratio in the blood of the rats. The absence of a LR in the IL-5 pre-treated rats would suggest that changes in blood lymphocyte subsets is not sufficient in mediating a LR. Although blood lymphocyte subsets were not significantly different between groups, we were interested in studying the possible changes in cytokine production from these lymphocytes.

In our search to find a possible mechanism by which IL-5 could increase R_L 20 hours after OA challenge, we assessed lung Th1 and Th2 cytokine mRNA expression. We found increases in both IL-4 and IL-5 mRNA expression after antigen challenge but no effect on the Th1 cytokine IFN-γ mRNA expression. Increases in Th2 cytokines are associated with increases in lung resistance after antigen challenge in humans (356). Lee *et al* measured cytokine levels by ELISA in the serum of acute asthmatics and found higher levels of IL-4, IL-5, and IL-13 (356). They also associated higher levels of IL-5 and lower levels of IFN-γ with severe airway obstruction. Therefore, it is possible that the effects in R_L encountered in OA challenged BN rats are explained by changes in these cytokines. These cytokines may act by recruiting and/or degranulating inflammatory cells in the lungs following antigen challenge. Our next experiment measured the levels of an important inflammatory cell, eosinophils, in the BAL of our animals.

IL-5 administration followed by allergen challenge has been shown to increase eosinophil numbers in lung tissue and increase AHR (7). However, in our study there was no observed increase in AHR to methacholine following IL-5 treatment/OVA challenge. It is possible that the increased R_L in IL-5 pre-treated rats masked the effects on AHR. Perhaps other factors are necessary for AHR to occur after antigen challenge in the BN SSN rat. Factors such as increased $CD4^+$ T cell activation, higher IgE levels, the presence of more eosinophils, cytokines such as TNF-α, GM-CSF, immunoglobulins, and chemokines such as eotaxin and RANTES may be necessary for AHR to occur. In addition, certain characteristics of cells that constitute normal lung tissue i.e. epithelial cells and smooth muscle cells may also be lacking in the BN SSN rat for AHR to occur after antigen challenge.

Conclusion

We have confirmed previous results in rats and in guinea pigs that show up-regulation of cellular immunity with IL-2 increases the airway response to antigen. Moreover, IL-2 does not increase LT production in this animal model following antigen challenge, but it can enhance the sensitivity of the airways to Cys-LTs. Further investigation led to the discovery of a link between LTs and Th1/Th2 cytokine production. Taken together, the first part of my thesis shows a link between cell-mediated immunity and leukotrienes in an animal model of asthma.

We then proceeded to assess whether one important cytokine could reproduce all the changes that are found in asthma by assessing the effects of IL-5 in BN SSN rats that do not develop ER and LR after sensitization and antigen challenge. We found that intra-tracheal administration of rhIL-5 to rats that do not have the characteristics of atopic asthma will cause some but not all of the cellular and physiological changes that are found in atopic asthma. As suggested in a recent publication (357), therapeutic strategies directed against IL-5 alone may be insufficient to revert the airway response to normal in atopic asthmatics. Although preliminary data from the first clinical trials give rise to skepticism about the efficacy of anti-IL-5 treatment regarding the improvement of lung function of asthmatic patients, further studies with a better defined profile of the target population may provide encouraging results.

A synthesis of the results from both parts of thesis leads to the general conclusion that asthma is a heterogeneous disease. On one hand, Cys-LTs are clearly an important mediator in the asthma

phenotype, with our data showing that it can cause the late response and promote Th2 cytokines. But on the other hand, IL-5, considered a key mediator in asthma, according to our data has limited impact on asthma development. Therefore, the overall message is that there is no single cause of asthma, rather multiple interacting proinflammatory mechanisms, each with varying levels of consequence on the airways.

Future Perspectives

The discovery of the processes underlying the inflammatory response in the lung and their control mechanisms is a long and winding road that many scientists take. This project has managed to shed light on parts of this road, however much work remains. In order to add more weight to the theories, future studies are needed to quantify the expression of the Cys-LT_1 receptor in our animal model. Moreover, a complete analysis of other important inflammatory markers such as chemokines (particularly CCR3), and cytokines (ex: IL-13, IL-8) must be done. It would also be important to see if our results (namely immunomodulation of Th1 and Th2 cytokines) could also be duplicated in asthmatics currently being treated with montelukast. The cytokine Interleukin-5 was once considered an important mediator due to its effects on eosinophils. However unsuccessful anti-IL-5 therapies have persuaded scientists that it is not the magic bullet for curing asthma. If we acknowledge that the causes of asthma are multifactorial, a multiple anti-cytokine therapy, including anti-IL-5, is a potentially effective means to combat the disease. While this study adds to the wealth of knowledge on immunomodulatory function, specifically in asthma, the scientific community continues to strive to identify specific targets for therapeutic measures that are designed to impede or abolish the allergic inflammatory cascade.

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