Expression and Regulation of Toll-like Receptor 4 in Allergic Disease

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Abstract

The prevalence of allergic diseases is increasing worldwide for unclear reasons. This sudden widespread increase, particularly in children, suggests causes that are not due to genetic makeup of individuals, but rather a change in environment and lifestyle factors. Growing epidemiological and immunological evidence supports the hygiene hypothesis, which states that decreased exposure to immune stimulating-infections in early childhood is the cause of the rise in allergic diseases. Studies have shown that even bacterial components such as lipopolysaccharide (LPS) can have a protective effect. The receptor for LPS is Toll-like receptor 4 (TLR4), an innate immunological receptor, which may play an essential role in regulation of allergic disease. We investigated the expression of TLR4 in the nasal mucosa of allergic and non-allergic children and adults, the effects of LPS on allergic inflammation and the regulation of TLR4 expression. We hypothesized that LPS, through TLR4, could regulate allergic inflammation and that long-term allergic inflammation would limit these responses.

Children and adults undergoing nasal surgery were recruited from the local hospitals. Nasal biopsies from the patients were explanted and cultured *ex vivo* with allergen and LPS. LPS could prevent allergen-induced Th2 cytokine production and inflammatory cell increases in explants from atopic children; this was through induction of Th1 cytokines and IL-10. TLR4 was also detected on $CD4^{+}CD25^{+}$ T cells. LPS did not provide the same protection in adults. Adults, especially atopic subjects, had less TLR4 immunopositive cells; hence, their reduced responsiveness to LPS. This suggests that factors could downregulate TLR4 in allergic adults. The U-937 monocytic cell line

was used as an *in vitro* model to study the regulation of TLR4 by interleukin-4 (IL-4), a T-helper type 2 (Th2) cytokine involved in allergic inflammation. U-937 cells cultured with IL-4 had decreased LPS responsiveness, TLR4 protein surface expression, TLR4 mRNA expression and transcriptional activity of the upstream region of TLR4. This effect was tyrosine kinase and STAT6 dependent. A STAT6 binding site was determined in an area of the TLR4 gene necessary to mediate IL-4's inhibitory effects on TLR4 transcription. IL-4 was also determined to reduce TLR4 expression in PBMCs, especially in $CD4^+$ T cells purified from the blood of children.

As predicted in epidemiological studies and animal studies, LPS was shown to provide anti-inflammatory effects against allergen in human nasal tissue. LPS may directly stimulate regulatory T cells (CD25+CD4+) to produce anti-inflammatory cytokine production. The effects of LPS exposure may be lost due to reduced expression of TLR4 by inflammatory cells, and this may be caused by the allergic inflammation itself. Therefore, development of allergic inflammation can down-regulate anti-inflammatory mechanisms and promote long term chronic inflammation.

Résumé

L'incidence des maladies allergiques i.e. atopiques augmente à travers le monde, pour des raisons qui demeurent inconnues. Cette augmentation marquée, particulièrement chez les enfants, suggère une origine autre que l'hérédité, telle qu'une modification des facteurs liés à l'environnement et au mode de vie. De plus en plus de données épidémiologiques et immunologiques supportent l'hypothèse d'hygiéniste, selon laquelle une diminution de l'exposition à des infections stimulatrices pour le système immunitaire durant l'enfance contribuerait à l'apparition des maladies allergiques. Des études ont montré que certaines composantes moléculaires des bactéries, tels les lipopolysaccharides (LPS), pourraient avoir un effet protecteur. Le récepteur du LPS, 'Toll-like receptor 4(TLR4)', est un récepteur de l'immunité innée jouant un rôle potentiel dans la régulation des maladies allergiques. Nous avons étudié l'expression de TLR4 au niveau des muqueuses nasales d'enfants et d'adultes allergiques et non-allergiques, ainsi que l'effet du LPS sur l'inflammation allergique et la régulation de l'expression de TLR4. Selon notre hypothèse de travail, le LPS, à travers l'activation de TLR4, pourrait réguler l'inflammation allergique, voire réduire l'inflammation propre à la réaction allergique, et ce phénomène pourrait être réprimé par la présence d'une inflammation allergique au long cours.

Des enfants et adultes subissant une chirurgie nasale ont été recrutés dans des hôpitaux locaux. Des biopsies nasales ont été cultivées *ex vivo* en présence d'allergène et de LPS. Nous avons mis en évidence la capacité du LPS à empêcher le développement d'une inflammation de type 'T-helper' 2 (Th2) suite à l'exposition d'allergène dans les tissus provenant d'enfants atopiques. Ce phénomène résultait de l'induction de cytokines

Th1 et de la production d'IL-10. L'expression de TLR4 a également été détectée au niveau des lymphocytes T possédant les marqueurs membranaires CD4 et CD25. Une telle protection par le LPS n'a pas été observée chez les patients adultes. Ceci serait causé par un nombre inférieur de cellules exprimant TLR4, en particulier chez les adultes allergiques. Ces données suggèrent que certains facteurs pourraient diminuer l'expression de TLR4 chez les adultes allergiques. La lignée cellulaire de monocytes U-937 a été utilisée comme modèle *in vitro* pour étudier la régulation de TLR4 par l'interleukine-4 (IL-4), une cytokine de type Th2 impliquée dans l'inflammation allergique. Les cellules U-937 cultivées en présence d'IL-4 ont une sensibilité inférieure au LPS, résultant d'une expression réduite du récepteur à la surface des cellules, d'une diminution de l'expression de l'ARN messager et de la transcription du gène TLR4. Ces effets étaient dépendants d'une activité de type tyrosine kinase et du facteur de transcription STAT6. Un site de liaison pour STAT6 a été mis en évidence dans la région en amont du gène de TLR4, et qui s'avérait nécessaire aux effets inhibiteurs de l'IL-4 sur la transcription de TLR4. L'expression de TLR4 à la surface des cellules mononucléées du sang périphérique des enfants était de plus diminuée en présence d'IL-4, en particulier au niveau des cellules T positives pour le marqueur CD4.

Nous avons montré que le LPS possède des propriétés anti-inflammatoires envers la réponse immunitaire aux allergènes dans les muqueuses nasales humaines. Le LPS pourrait stimuler les cellules T régulatrices CD4+CD25+ à produire des cytokines anti-inflammatoires. Les effets de l'exposition au LPS pourraient être perdus suite à la réduction de l'expression de TLR4 au niveau des cellules inflammatoires, un phénomène pouvant également résulter de la présence d'une inflammation allergique elle-même.

Ainsi, le développement d'une inflammation allergique peut diminuer les mécanismes anti-inflammatoires existants et promouvoir, à long terme, une inflammation chronique.

Table of Contents

List of Diagram, Tables and Figures

Publications during thesis training

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Abbreviations

Abbreviation	Meaning
ADAM33	A disintegrin and metalloproteinase domain 33
ALAS1	Aminolevulinate, delta-synthase 1
AP-1	Activator protein 1
APC	Allophycocyanin
APC	Antigen presenting cells
ARIA	Allergic Rhinitis and Its Impact on Asthma
ATCC	American Type Culture Collection
BALs	Bronchoalveolar lavages
BCG	Bacillus Calmette-Guerin
BCM	Bicarbonate-buffered culture medium
Bp	Base pairs
BrdU	5-Bromo-2'-deoxyuridine
BSA	Bovine serum albumin
CBP	CREB-binding protein
CD	Cluster of differentiation
CD40L	CD40 ligand
C_H	Heavy chain
ChIP	Chromatin immunoprecipitation
CSF-1	Colony stimulating factor-1
Ct	Threshold cycle
Cε	Heavy chain epsilon
DEFB1	Defensin β1
DEPC	Diethylpyrocarbonate
DPP10	Dipeptidyl peptidase X
DTT	Dithiothreitol
ECP	Eosinophilic cationic protein
EDTA	Ethylenediaminetetraacetic acid
EST	Expressed sequence tag

Abbreviation	Meaning
F_{ab}	Antigen binding fragment
Fc	Crystallisable fragment
Fcε	Crystallisable fragment epsilon
FEV_1	Forced expiratory volume in 1 second
FITC	Fluorescein isothiocyanate
FL1	Fluorescent 1
Foxp3	Forkhead Box P3
FSC	Forward Scatter
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GATA3	GATA-Binding Protein 3
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPR154	G protein-coupled receptor 154
HEPES	Hydroxyehtylpiperazine ethanesulfonic acid
HMGB1	High Mobility Group Box Chromosomal Protein 1
HRP	Horseradish peroxidase
HSP	Heat shock protein
hToll	Human Toll
IFN	Interferon
Ig	Immunoglobulin
IgE	Immunoglobulin E
IKK	IκB kinase
IL	Interleukin
IL1RL1	Interleukin 1 receptor-like 1
IL-4Rα	IL-4 receptor alpha-subunit
iNOS	Inducible nitric oxide synthase
IP-10	IFN-γ-inducible 10KDa protein
IPEX	Immunodysregulation, polyendocrinopathy, and enteropathy, X-linked inheritance syndrome
IRAK	IL-1R-associated kinase
IRF	IFN regulatory factor

Abbreviation	Meaning
Iε	Intermediate exon epsilon
IκB	Inhibitors of κ-B
Jak	Janus kinase
LAL	Limulus amebocyte lysate
LARII	Luciferase Assay Reagent II
LBP	LPS-binding protein
LRR	Leucine-rich repeat
LPS	Lipopolysaccharide
LT	Leukotriene
LY 64	Lymphocyte antigen 64
MBP	Major basic protein
mDC	Myeloid dendritic cells
MEM	Minimal essential medium
MFI	Mean fluorescent intensity
MHC	Major histocompatibility complex
MPL	Monophosphoryl lipid A
MyD88	Myeloid differentiation primary response gene 88
NCoA-1	Nuclear receptor coactivator 1
NF-κB	Nuclear factor-kappa B
O_3	Ozone
OCT	Optimal cutting temperature
oligo dT	Oligo deoxythymidylate
PAMP	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cells
PF	Paraformaldehyde
PHF11	PHD Finger Protein 11
PLL	Poly-L-lysine

Abbreviation	Meaning
PNU	Protein nitrogen units
PP2A	protein phosphatase 2A
PPD	purified protein derivative
PRR	pathogen recognition receptors
PTC	Patient Testing Center
QPCR	Quantitative PCR
REL	Reticuloendotheliosis viral oncogene
RIP1	Receptor-interacting protein 1
RLU	Relative light units
RP105	Radioprotective 105 Kda
RPMI-1640	Roswell Park Memorial Institute formulation 1640
RT-PCR	reverse transcription-PCR
SCF	Stem cell factor
SDS	Sodium dodecyl sulfate
SEA	Staphylococcal endotoxin A
SH2	Src-homology domain 2
SHP-1	Src homology-containing phosphatase-1
siRNA	Small interfering RNA
SIRS	Systemic inflammatory response syndrome
SNP	Single nucleotide polymorphisms
SOCS	Suppressor of cytokine signaling
SSC	Side Scatter
SSC	Standard saline citrate
STAT6	Signal Transducer and Activator of Transcription 6
Sε	Switch region epsilon
TAB	TAK1- binding protein
TAK1	TGF-β-activated kinase 1
T-bet	T-Box Expressed in T cells
TBK1	TRAF-family-member associated activator-binding kinase 1

Abbreviation	Meaning
TBS	Tris buffered saline
TCR	T cell receptor
TGF-β	Transforming growth factor-beta
Th	T helper
TIL	Toll/IL-1 receptor-like
TIR	Toll/IL-1 receptor-like
TIRAP	TIR-domain-containing adaptor protein
TLR	Toll-like receptor
TNFRSF9	Tumor necrosis factor receptor superfamily 9
TNF-α	Tumer necrosis factor-alpha
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor protein inducing IFN-β (
TSST-1	Toxic shock syndrome toxin-1
U	International Unit
γ_c	IL-2 receptor common gamma subunit

Chapter 1: Introduction

1.1 Allergy History

John Bostock, an English physician, reported in his own case history in 1819 a syndrome associated with nasal discharge, repeated paroxysms of sneezing, itching of the eyes, difficulty breathing and severe malaise[1, 2]. This is generally accepted as the first complete description of the malady known as hay fever, also known as allergic rhinitis. In the following nine years, Bostock was only able to report 28 other cases of hay fever[2-4]. Over the last two centuries, cases of allergic rhinitis and other allergic diseases have had a remarkable increase. Currently, epidemiological studies have estimated the world-wide average prevalence of allergic diseases at 15-20%[5-7]. Additionally, international comparisons have shown that considerable variation exists between countries in the prevalence of allergic diseases, ranging from about 1% to almost 30% in some surveys[6]. Current estimates for the prevalence of allergic disease in Canada and the United States closely follow the world-wide average.

In 1873, Charles Blackley conducted the first experiments showing that hay fever could be caused by a sensitivity to pollen[2, 8]. Later in 1906, Clemens von Pirquet coined the term *allergy* after noticing that patients who had previously received injections of horse serum or smallpox vaccine had quicker, more severe reactions to a second injection[9]. Allergy comes from the Greek *allos* meaning "other" and *ergon* meaning "reaction," and was used to describe this hypersensitivity reaction. An allergen is defined as any substance, most often eaten or inhaled, that is recognized by the immune system and causes an allergic reaction. Charles Mantoux, shortly after von Pirquet, showed that tuberculin, a glycerin extract from cultures of bacteria that cause tuberculosis,

Mycobacterium tuberculosis, could also cause a hypersensitivity reaction[10]. The Mantoux test, also known as the purified protein derivative (PPD) tuberculin test, is still used today as a diagnostic test for tuberculosis.

Presently, types of hypersensitivity to antigens are classified into four types, in a classification scheme created by Gell and Coombs in 1968[11]. The term *allergy* is most often associated with Type I hypersensitivity, which is mediated by allergen-specific immunoglobulin E (IgE). Allergen-IgE reactions activate the release of inflammatory mediators and lead to an immediate reaction, within seconds to minutes. IgE-mediated diseases include atopic dermatitis, allergic asthma, allergic rhinitis and allergic conjunctivitis.

1.2 Immune System Responses and Receptors

The immune system of an organism is a complex array of cells and mediators that function to distinguish factors of an organism (self) from foreign substances (non-self). The immune system is then activated for the clearance of the non-self factors. There are two main responses of the immune system, called the humoral and cell-mediated immune responses. In humoral immune responses, antibodies are generated specifically for the antigen, and antibodies can neutralize invading microbes that possess the antigen on their surface, or they can flag the microbes for destruction by other cells, such as macrophages. An antigen is any molecule capable of causing the generation of antibodies. Humoral immune responses are important for protection from small extracellular pathogens such as bacteria and viruses and the neutralization of toxins and pathogenic factors but also protection from large extracellular pathogens such as multicellular parasites. In cell-mediated immune responses, antibodies play a lesser role, and rather specific immune

cells are activated, such as macrophages, natural killer cells, and antigen specific cytotoxic T cells. Macrophages are activated to destroy engulfed microorganisms, while natural killer cells and cytotoxic T cells destroy other cell types that have been infected with pathogens. Cell-mediated immunity is important for protection from infections of microbes that can survive in phagocytic cells or that infect non-phagocytic cells. Hence, cell-mediated immunity is important for the removal of virus infected cells, intracellular bacteria, but it also plays a role in clearance of fungal and protozoan infections. Cell-mediated immune responses can also be generated against abnormally growing cells such as tumour cells.

The immune system's ability to discriminate between self and non-self is based on the evolution of cell surface receptors that can recognize specific antigens and other molecules. Two main types of receptors exist, the adaptive immune receptors and the innate immune receptors. Adaptive immune receptors are present on lymphocytes, which have different effector functions depending on the type of receptor. During the maturation of lymphocytes, the adaptive immune receptors are generated by rearrangement of the genetic sequences encoding the receptor. In this fashion, receptors with unique recognition are created on each lymphocyte. B cells, which develop in the bone marrow, have antibodies with transmembrane regions anchoring them to the membranes. This B cell receptor complex can bind to antigen directly, and antibodies form part of an antigen recognition complex. T cells, which originate in the bone marrow but develop in the thymus, have an antigen recognition receptor composed of the CD3 antigen and accessory molecules. T cell receptor complexes bind to allergen, but only when presented by other cell types called antigen-presenting cells. Binding of antigen to

the respective receptors, with appropriate co-stimulatory signals, activates effector cell function and proliferation. B cell effector cells, known as plasma cells secrete soluble cell-free antibodies. Two types of effector T cells exist, T helper cells and cytotoxic T cells. T helper cells produce mediators to activate other cell types, thus helping and coordinating the immune response, while cytotoxic T cells produce toxic proteins to eliminate infected cells in cell-mediated immune responses. Adaptive immune responses are antigen-dependent and this is important to avoid widespread responses that can cause damage to the host. Additionally, after activation by antigen, some cells develop into memory cells. These cells survive after the response to the antigen, and if the antigen is encountered again, the memory cells quickly activate and lead to a strong secondary response to clear the infectious organism.

Innate immune receptors are an ancient system of receptors, that are inherited and that recognize conserved molecular patterns on microbes[12]. Innate immune receptors are believed to have evolved to recognize specific molecular determinants present in many pathogens. These molecules are known as pathogen associated molecular patterns (PAMPs) with their binding partners on immune cells known as pattern recognition receptors (PRRs). These are not rearranged like the adaptive immune receptors and are present on many cells types. Many PRRs exist and new members and associated PAMPs are still being discovered. PRRs are not involved in immune memory responses, but are believed to be important in initializing and directing adaptive immune responses (Diagram 1.1).

A model for the immune system's recognition and response functions was proposed in the late 1940s[13-15]. In this model, the immune system can directly

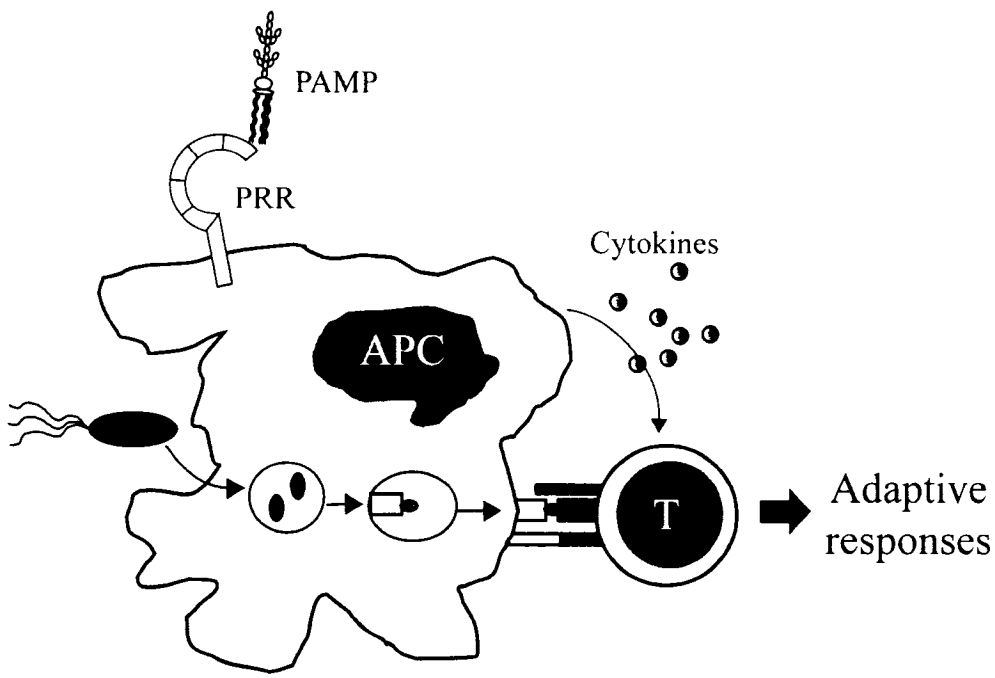

Diagram 1.1. Pathogen associated molecular patterns (PAMPs) and pattern recognition receptors (PRRs) are part of the innate immune system. Recognition of PAMPs by PRRs on antigen presenting cells (APCs) leads them to increase phagocytosis, increase antigen presentation, increased surface expression of co-stimulatory molecules and cytokine production. This leads to activation of T cells to produce cytokines and activate adaptive immune responses.

discern the differences between pathogens and the host, and will only react to pathogenic antigens. This model has been recently challenged, and reformulated. In the innate immune receptor model, proposed by Janeway, lymphocyte activation is controlled by PRRs and PAMPs[12]. PRRs react with PAMPs, and this will activate adaptive immune responses by lymphocytes. PAMPs would be a signal that a foreign antigen is present and an immune response is necessary. The innate immune receptor model was modified by Matzinger, to what is known as the "danger model"[16]. In this model, antigen is presented to lymphocytes by cells known as professional antigen-presenting cells (APCs) only if the host is threatened. Specific danger signals, such as PAMPs or necrotic cells, activate the APCs to increase their ability to activate lymphocytes with antigen. Despite evidence for the "danger model", it is not completely accepted and it is thought that immune responses are not dependent on APCs alone and that danger signal can be recognized by lymphocytes directly.

1.3 IgE, Atopy and Sensitization

In 1865, Charles Blackley showed that a patient was sensitive to grass pollen by scarifying the patient's arm with a lancet and applying an occlusive bandage containing grass pollen to the skin[8]. The patient reacted with a skin reaction presenting swelling (wheal) and redness (flare) around the site of the bandage. This was credited as the first report on immediate skin reactions from allergic patients, and a similar assay is used in the clinic today to test for allergies. In 1921, Carl Prausnitz and Heinz Küstner performed an experiment that showed that wheal and flare reactions caused by allergens could be transferred by serum[17]. Due to its low levels in blood, it took 40 years to

determine that the causative factor was a type of immunoglobulin (Ig) which was named IgE[18, 19].

In 1923, the term atopy, from the Greek word *atopia* meaning "out of place," was used to describe inherited hypersensitivity diseases[20]. Atopy is also sometimes defined as IgE-mediated sensitivity to allergens with a positive wheal and flare reaction when the skin is challenged with allergen. Although possessing a strong genetic component, environmental factors are now thought to be implicated in development of atopic diseases.

Elevated levels of IgE in the serum is recognized as a characteristic of allergy[21, 22]. Units used to quantify IgE were established in 1968 by the World Health Organization[23]. The standard was assigned an arbitrary mass unit, known as an International Unit (U). One unit of IgE was determined to be equivalent to 2.42ng of pure IgE [24]. In normal non-atopic adult subjects, the serum concentration of total IgE ranges from 20kU/L (about 50ng/mL) to 40kU/L (about 100ng/mL) [25]. Levels as high as 375 kU/L (900ng/mL) can be found in adults with allergic disease. Due to large variation of total IgE measurements in patients with and without allergic symptoms, total serum IgE is not used as a predictor of allergic disease[22, 25, 26].

IgE is produced by B cells which are exposed to repeated allergen encounters and appropriate stimulation from T helper cells. IgE binds to receptors called Fcε receptors on the surface of cells, such as mast cells and basophils. When allergen is present, it binds to IgE, activates the receptor and the inflammatory cell produces numerous inflammatory mediators causing an allergic reaction. Inflammatory cells with IgE bound to their Fcε receptors are said to be in their primed state. The process of IgE production

and priming of inflammatory cells is known as sensitization (Diagram 1.2). It is still unclear why allergens, which are presumed innocuous otherwise, cause sensitization.

1.4 Airway Allergic Diseases

Many individuals that experience hypersensitivity to antigen in their upper airways (nasal passages) experience hypersensitivity in their lower airways. This has led to the concept of the united airways disease. In many reports, the fraction of asthmatics with symptoms of rhinitis has been considered to be around 85%[27, 28]. This may still be an underestimate as some asthmatic patients who consider themselves free of nasal symptoms demonstrate evidence of nasal inflammation[29]. Comparison of the pathology of allergic rhinitis and allergic asthma has shown many similarities. One of the main differences between the two airways is the presence of bronchial smooth muscle in the lower airways. Currently, both diseases are considered to be the same syndrome, but in different locations[28, 30], and there is evidence that therapeutic control of rhinitis is beneficial for asthma symptoms such as bronchial responsiveness[31, 32]. In prospective studies, allergic rhinitis has been shown as a risk factor leading to asthma, and the risk factor increases with the severity of the symptoms[33-35]; allergic rhinitics have a 2-3 fold increased risk of developing asthma, while rhinitics with sinusitis have up to a 6 fold increased risk. From this evidence, it would seem that allergic asthma may stem from rhinitis, but the process is not understood. This thesis focuses on nasal inflammation and allergic rhinitis, but the conclusions developed may be extended to allergic asthma and even other allergic diseases.

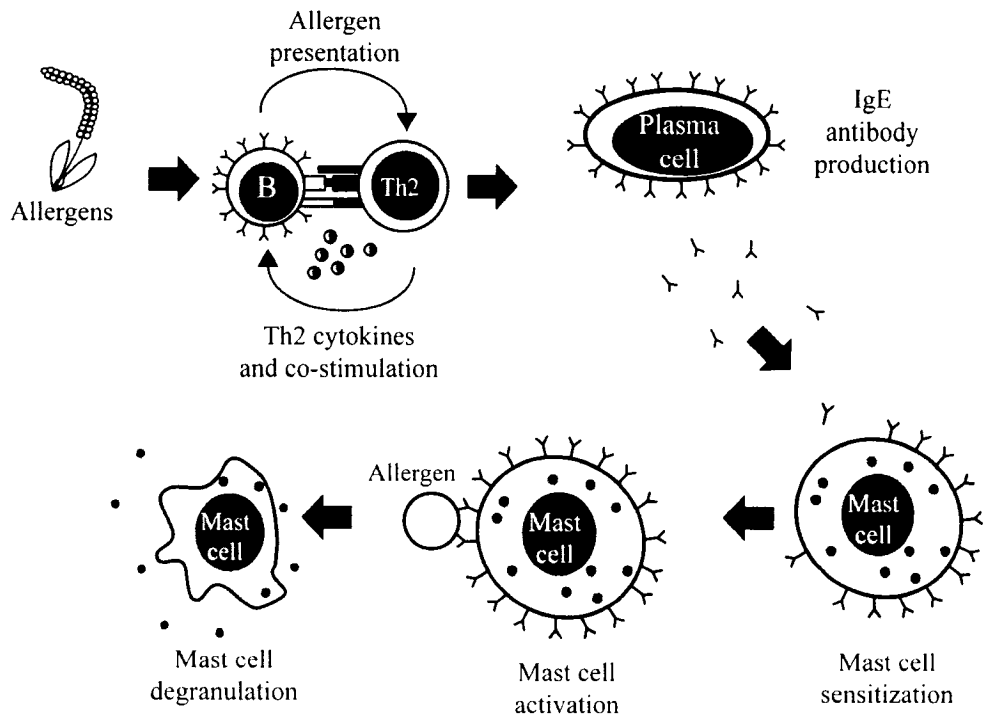

Diagram 1.2. Allergen presentation by B cells can activate Th2 cells to produce cytokines and provide co-stimulatory signals such as CD40L leading to activation of B cells to differentiate into IgE-producing plasma cells. IgE can bind to IgE receptors on mast cells sensitizing them to allergen. Allergen can bind to IgE on mast cells causing cross-linking and triggering of the IgE receptors. This leads to mast cell activation and rapid degranulation of many inflammatory mediators such as histamine and cytokines.

1.5 Allergic Rhinitis Clinical Definition

Rhinitis is known as the inflammation of the mucous membrane of the nose. Symptoms include rhinorrhea, nasal obstruction, nasal itching, and sneezing. Rhinitis may be either non-allergic or allergic in origin. Non-allergic rhinitis is characterized by cloudy (white, yellow or green) nasal secretions and sneezing, and is predominantly caused by virus or bacterial infections.

Allergic rhinitis is clinically defined as a symptomatic disorder of the nose induced by an IgE-mediated inflammation after allergen exposure of the membranes lining the nose. While sharing many symptoms, in contrast to non-allergic rhinitis, nasal secretions are clear and watery. It was originally separated into perennial allergic rhinitis and seasonal allergic rhinitis. Perennial allergic rhinitis is most frequently caused by indoor allergens present throughout the year such as dust mites, molds, insect allergens and animal dander. Seasonal allergic rhinitis is related to outdoor allergens which are only present in the environment for discrete but recurrent periods in the year and include pollens and molds. This definition has proved unsatisfactory because some seasonal allergens in some parts of the world can be perennial, many patients are sensitive to many allergens and present symptoms throughout the year, and there is a wide range of patient responses to perennial allergens that may not persist throughout the year[36]. Allergic rhinitis has been redefined by the Allergic Rhinitis and Its Impact on Asthma (ARIA) organization[36]. Allergic rhinitis is now divided into either persistent or intermittent disease. Intermittent means that symptoms of allergic rhinitis are present less than four days in a week or for less than four weeks in a year. Persistent means symptoms for allergic rhinitis are present for more than four days in a week and for more than four

weeks in a year. In addition, allergic rhinitis is further defined by whether presentation of symptoms is mild or moderate to severe. Mild allergic rhinitis is disease without any sleep disturbance, troublesome symptoms, and impairment of daily activities including school, work, leisure or sports. Moderate to severe is classified as presenting one or more of the preceding conditions absent from mild allergic rhinitis. ARIA redefined allergic rhinitis to correct the discrepancies of the older definitions, to help in standardizing epidemiological studies and to provide a basis for physicians and organizations to develop a local standard of care for their patients.

1.6 Allergic Rhinitis: Type I Hypersensitivity Reaction

The inflammatory mediators of the Type I hypersensitivity reaction in allergic rhinitis have been elucidated using allergen challenge of allergic individuals [37-39]. Two phases occur in this reaction leading to various symptoms (Diagram 1.3). The early phase of the hypersensitivity reaction occurs within minutes. It is as a result of the binding of allergen to IgE primed cells and activation of the Fcε receptor. Triggering leads to the release of preformed mediators such as histamine, cytokines, cysteinyl leukotrienes and prostaglandins from the inflammatory cells into the surrounding tissue. These mediators, especially histamine, are potent proinflammatory mediators causing virtually all of the early phase responses in allergic rhinitis such as sneezing, pruritus, rhinorrhea, vasodilation and nasal congestion[40]. Vasodilation and chemotactic factors lead to influx of lymphocytes, monocytes, macrophages, and granulocytes which coordinate the late phase reaction. The late phase reaction, usually seen 4 to 6 hours following the early phase can last 1 to 2 days. In the late phase, nasal congestion and cellular inflammation becomes more prominent. Maintenance of the late phase

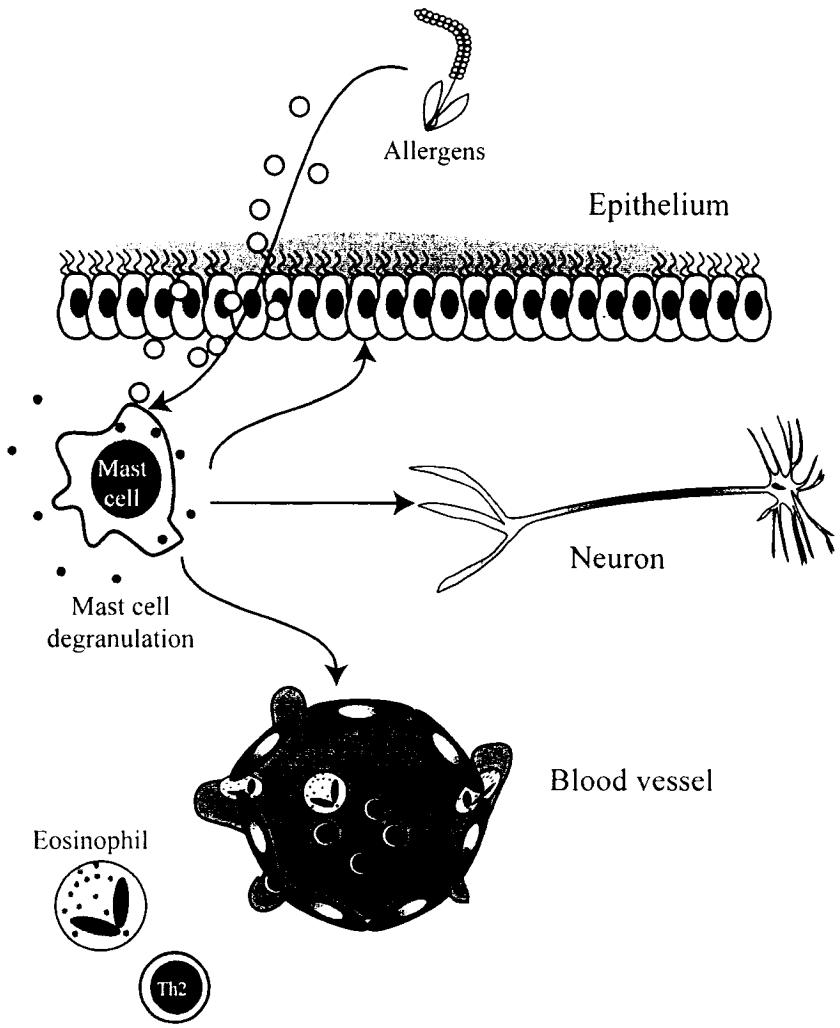

Diagram 1.3. In the early phase of the hypersensitivity response, allergen causes the degranulation of mast cells. Mast cells release many mediators acting on many cell types and cause the symptoms of allergic rhinitis including sneezing, pruritus, rhinorrhea, vasodilation and nasal congestion. Vasodilation, chemotactic factors and activated endothelium lead to influx of inflammatory cells into the tissue. The late phase is coordinated by these infiltrating cells, primarily eosinophils and Th2 lymphocytes.

proinflammatory effects are attributed primarily to infiltrating T cells and eosinophils and the mediators they release[41].

1.7 Allergic Rhinitis: Cellular Inflammation

Sensitization of the nasal mucosa in allergic rhinitis is associated with cellular change within the nasal mucosa[42]. Compared to non-allergic controls, large increases in the populations of lymphocytes, mast cells, granulocytes and macrophages are seen. These cells are either the result of proliferation at the site of inflammation or recruitment from local vasculature. Recruitment of cells from the vasculature involves surface changes in the endothelium that promote adherence of inflammatory cells. Inflammatory cells are then activated to cross the junctions between endothelial cells and through the basement membrane through a process called diapedesis. Cells then migrate towards the site of inflammation. This whole process is mediated by a subset of cytokines known as chemokines that promote migration from zones of low chemokine concentration to zones of higher concentration called a chemokine gradient. It has also been shown that some inflammatory cells can be derived from local stem cells by *in situ* differentiation[43]. Once primed cells are in place and encounter allergen, they respond by producing cytokines and other mediators such as histamine that cause the allergic rhinitis symptoms and the early and late phase of the allergic response. A brief discussion of the cell types and main mediators involved in the allergic inflammation follows.

1.7.1 T Lymphocytes

T cells possess the T cell receptor (TCR) complex which recognizes short peptides derived from antigen complexed with major histocompatibility complex (MHC) I or MHC II. MHC I is expressed on virtually all cells of the body while MHC II is

mainly expressed on professional antigen-presenting cells. MHC I-antigen complexes are recognized by $CD8^+$ cytotoxic T cells, which clear cells infected with virus or bacteria or cells with abnormal proteins such as cancerous cells. MHC II-antigen complexes are recognized by $CD4^+$ T helper cells, and these cells produce many mediators leading to cell-mediated or humoral-mediated inflammatory responses.

$CD4^+$ T helper cells are the main coordinators of inflammation by producing soluble mediators and providing signals through cell adhesion molecules to other cells. They are the cell type found in greatest numbers both in normal and allergic nasal mucosa[42, 44]. T-helper cells were further divided by Mosmman and colleagues into T-helper 1 (Th1) and T-helper 2 (Th2) subsets, based on the cytokines they produced[45]. Not only do the subsets produce different cytokines, they also coordinate different types of immune responses. Naïve Th cells, $CD4^+$ T cells never exposed to antigen, when activated by antigen-presenting cells produce cytokines from both subsets and are known as Th0 cells[46]. If given the proper stimulus, naïve Th cells can differentiate into the biased Th1 or Th2 subsets. The cytokine environment and signals that are received by the naïve T cells induce the differentiation. Bacterial innate immune responses lead to production of IL-12 and interferon-γ (IFN-γ) which drive the development of Th1 cells[47]. Th1 cells mainly produce high levels of IFN-γ, IL-2, IL-12 and lymphotoxin-A (LTA) and are associated with cell-mediated immunity for clearance of intracellular pathogens. These cytokines promote T cell proliferation, macrophage microbicidal activity and antigen presentation, recruitment of phagocytic cells, but are poor initiators of antibody production. Dysregulated Th1 cytokine production can lead to immunopathology and organ-specific autoimmune disease[48]. Conversely, production

of IL-4 early in an immune response directs the development of Th2-biased responses[49]; however, the innate immune response causing this phenomenon, particularly to allergens is not clear. The major Th2 cytokines are IL-4, IL-5, and IL-13 and these have multiple effects on B cells, mast cells and eosinophils and are strongly implicated in atopy and allergic inflammation. Th2 cells can also direct humoral immune responses which are important in responses to large extracellular pathogens, such as parasitic worms.

Th1 and Th2 responses can cross-regulate each other by biasing an immune response one way or another. IL-4 can inhibit dendritic cell and macrophage production of IL-12, a potent stimulator of IFN-γ production by T cells[47]. IL-4 also down-regulates the IL-12Rβ2 chain decreasing responsiveness of Th2 cells to IL-12[50], while IFN-γ can enhance its production[51]. Additionally, IL-4 and IFN-γ can inhibit the T-helper cell production of each other[52].

Both Th1 and Th2 cytokine responses are associated with transcription factors which control gene expression and lineage commitment by promoting transcription of genes and chromatin remodelling [53]. Chromatin remodelling is a process where DNA is made more or less accessible for transcription by modification of DNA and proteins and is coordinated by transcription factors specific for each lineage. One of the main transcription factors associated with Th1 cells is T-Box Expressed in T Cells (T-bet) which controls IFN-γ production[54, 55]. Expression of T-bet is decreased in the airways of patients with asthma[56] concordant with decreased Th1 cytokine production in asthma. Th2 commitment is associated with the transcription factors Signal Transducer and Activator of Transcription 6 (STAT6) and GATA-Binding Protein 3 (GATA3)[57-

59]. STAT6 is activated by IL-4 and IL-13 and it controls transcription of GATA3[57]. GATA3 controls the expression of Th2 cytokines IL-4, IL-5 and IL-13 and other factors[60]. GATA3 and STAT6 have been shown to be increased in the upper and lower airways in asthma and allergies [61-63]. Th1 and Th2 associated transcription factors can further bias cytokine responses by inhibiting gene expression. T-bet can repress Th2 lineage commitment by interfering with GATA3 activity[64]. STAT6 and GATA3 can also inhibit IFN-γ production in Th1 cells[65, 66].

Another subset of T-helper cells are known as Th3 cells. These cells secrete high levels of TGF-β but low levels of Th1 and Th2 cytokines if any[67, 68]. They can suppress both Th1 and Th2 cytokine production and are thought to have a role in mucosal tolerance to antigen but their role in atopy is unclear.

Perhaps of greater interest are the $CD4^+CD25^+$ T-regulatory cells. They produce high levels of IL-10 and are involved in the maintenance of self-tolerance and suppressing immune responses. The expression of CD25 antigen, the IL-2 receptor α-subunit, is induced in T cells upon antigen stimulation, but T-regulatory cells express high levels constitutively[69]. These cells were first discovered in the mid-1990s when depletion of $CD25^+$ cells in mice resulted in a multiple organ autoimmunity[70]. Their population accounts for 5 to 10% of peripheral $CD4^+$ T cells. Production of IL-10 by these can inhibit Th1 and Th2 cytokine production and proliferation $CD4^+$ T cells[71, 72]. A recently described transcription factor, Forkhead Box P3 (Foxp3), is associated with T-regulatory cells [73]. Mutations in Foxp3 in mice lead to lethal autoimmune syndrome as a consequence of deficiency of T-regulatory cells[74, 75]. In humans, mutations in Foxp3 lead to the immunodysregulation, polyendocrinopathy, and enteropathy, X-linked

inheritance (IPEX) syndrome[76]. T-regulatory T cells are present in both atopic and non-atopic individuals, but T-regulatory cells from atopic patients have a significantly lowered activity in inhibiting proliferation and Th2 cytokine production of other cells, especially during allergen exposure[77, 78].

$CD8^+$ cytotoxic T cells, although present in the nasal mucosa, are mainly associated with cell-mediated immune responses against viral infections. Some studies have shown that $CD8^+$ T cells can produce IL-4 and stimulate B cells to produce IgE and produce IL-5 and induce tissue eosinophilia[79, 80]. $CD8^+$ T cells may not play as great a role in controlling allergic inflammation as Th2 cells, but they may help promote and maintain it.

1.7.2 B Lymphocytes and IgE Production

B cells are also central for mediating atopic diseases, primarily due to their production of IgE. B cell development occurs in the bone marrow and is dependent upon the production of antibodies also known as immunoglobulin protein. Immunoglobulins are tetramers composed of two heavy chains and two light chains that are bound by disulfide bridges to form a three-dimensional "Y" shaped structure[81-83]. The heavy chains are larger and form the tail of the "Y". The two arms of the "Y" are where the light chains and heavy chains are linked by more disulfide bonds. Both the heavy chain and light chain have constant regions and variable regions at their amino-termini. The variable regions are formed by gene segments brought together by genomic recombination. Only B cells that are successful in rearranging both the heavy chain and light chain genes survive and proliferate to produce antibody. Successful B cells also

express a surface form of the immunoglobulin that they produce which is associated with accessory chains and forms a B cell receptor complex for activation of the cell by antigen.

Digestion of immunoglobulins with papain yields three 50 kDa fragments[84]. Two fragments bind to antigen and are called F_{ab} (antigen-binding fragment), while the third fragment forms protein crystals readily and is called F_c (crystallisable fragment). The F_c portion is made up of constant regions of the two heavy chains (C_H) and mediates binding to Ig receptors. There are five immunoglobulin C_H classes in humans, called isotypes, based on the F_c portion expressed. Cμ encodes for the IgM isotype, Cδ encodes for IgD, four Cγs encode for the IgGs, two Cαs encode for the IgAs and Cε encodes for IgE. Immunoglobulin mRNA is produced by transcription of the variable region and the constant region found downstream of it. When a B cell encounters an antigen and adequate stimulation from other cells, it is capable of changing its constant region gene transcribed by a process of isotype switching (Diagram 1.4). The C_H genes for each isotype are arranged in a tandem array on chromosome 14q32 downstream of the heavy chain variable region. Specialized sequences of DNA called switch region sequences are found surrounding the C_H genes[85]. IgE isotype switching is initiated by germline transcription from a promoter upstream of Cε. This transcript, named IεCε RNA, contains an extra intermediate exon (Iε), the switch region Sε and Cε. After transcription of IεCε RNA, the Cε gene is translocated downstream of the variable region of the heavy chain, by intrachromosomal rearrangement and any intervening DNA is removed. Once the Cε is downstream of the variable region, IgE mRNA production can occur. IεCε RNA transcription and splicing is essential for IgE isotype switching to take place[86, 87]. IεCε transcription depends on the Th2 cytokines, IL-4 or IL-13 with the aid of trimeric

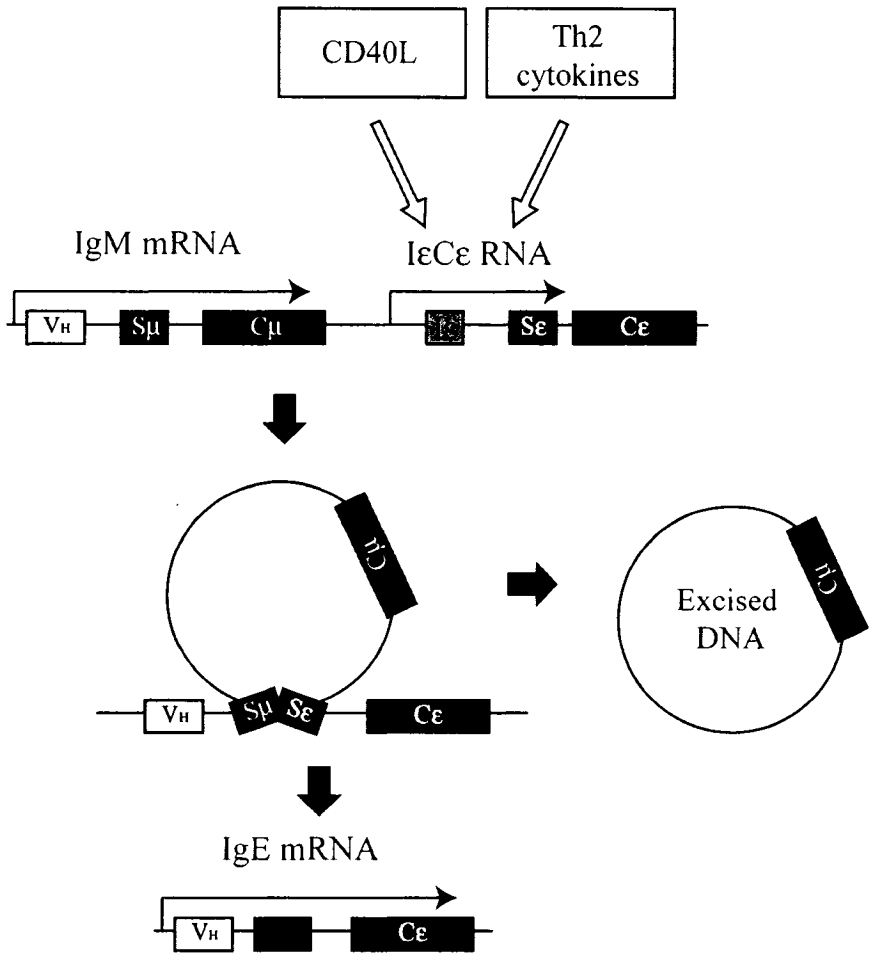

Diagram 1.4. Th2 cytokines and CD40L cause isotype switching in B cells. IεCε RNA is transcribed and the switch regions of IgM (Sμ) and IgE (Sε) are brought together bringing the constant region of IgE (Cε) downstream of the variable region of the heavy chain gene (V_H). After the intrachromosomal rearrangement IgE mRNA can be produced and any intervening DNA is removed forming circular DNA.

CD40 ligand (CD40L)[88, 89]. Isotype switching and IεCε RNA transcription usually occurs in lymphoid organs, but they can also occur in the nasal mucosa upon allergen challenge [90]. IgE production is also increased in the nasal mucosa upon allergen exposure[90] and blockade of IL-4 by antisense oligonucleotides or neutralizing antibodies can reduce both IgE mRNA transcription and IεCε RNA transcription[91, 92].

Immature naïve B cells with functional BCR migrate out to secondary lymphoid organs. Following antigen stimulation, B cells either become memory B cells or plasma cells. These cells are able to secrete immunoglobulins based on their BCR, but the secreted immunoglobulins do not have the transmembrane region found in the BCR. Memory B cells retain their ability to recognize antigen and provide a high source of immunoglobulins when stimulated[93]. Plasma cells are terminally differentiated cells that produce large amounts of immunoglobulins[94, 95]. Plasma cells are no longer able to proliferate or switch isotype from the current one they are secreting, and are relatively short lived[95].

B cell numbers are lower than T cells in normal nasal mucosa[42, 44] and do not increase significantly upon allergen challenge of patients with rhinitis in the nasal mucosa, but increase in numbers in the blood [96].

1.7.3 Antigen-presenting Cells

Professional antigen-presenting cells take up antigen from the surrounding environment, process it into smaller fragments that fit in the groove of the MHC II and then present the resulting complex at the cell surface[16]. The MHC II-antigen complex is recognized by the TCR of $CD4^+$ T cells. Co-stimulatory molecules on antigen-presenting cells are also necessary for $CD4^+$ T cell activation. Co-stimulatory signals

mainly include B7 family proteins, such as B7-1 (CD80) or B7-2 (CD86) interacting with CD28 on T cells, and members of the tumour necrosis factor receptor family such as CD40. The most common APCs include B cells, macrophages, and dendritic cells.

The role of B cells as APCs in the nasal mucosa is not as clear as their role in antibody production. It is thought that transmembrane immunoglobulin allows B cells to bind to antigen and selectively present this antigen to T cells[97]. It is possible that, although not part of the primary immune response, B cells in tissues promote secondary exposure to allergens and induce T cells to produce Th2 cytokines. *In vitro* stimulation of B cells with antigen can induce them to stimulate Th2 cell IL-4 synthesis[98]. In addition, B cells stimulated with IL-4 and CD40L can be induced to produce IL-13 and IgE, potentially promoting Th2 inflammation[99].

Macrophages take up antigen by phagocytosis which can be non-specific or antibody-mediated through antibody receptors. Macrophages also express MHC II following activation. They are seen as scavenger cells that phagocytose antigen and are involved in clearance of small extracellular pathogens. In some circumstances, they can suppress adaptive immune response through mediators, such as IL-10, which can inhibit Th cell and dendritic cell function[100, 101]. In addition, macrophages promote cell-mediated responses by inducing Th1 cell production of IFN-γ[102, 103]. Thus, their role in allergic disease is unclear.

A clearer role can be seen for dendritic cells as they are the only cells capable of inducing a primary humoral or cell-mediated immune response[104-106]. High numbers of dendritic cells can be found in the epithelial surfaces of the upper airways[107]. Most dendritic cells in the periphery are functionally immature and cannot present antigen to T

cells efficiently. They can however present antigen to activated T cells for secondary immune responses; therefore, immature dendritic cells in the periphery may be involved in T cell memory responses. After taking up antigen in the periphery, immature dendritic cells migrate via the lymphatic system to lymphoid tissue. In the lymphoid tissue, dendritic cells change their phenotype and start expressing B7 family receptors, increasing their ability to activate T cells[108]. Dendritic cells express many PRRs and are highly responsive to PAMPs. When dendritic cells are exposed to antigen without PAMP stimulation or low level stimulation, they can promote Th2 cell differentiation, while in the presence of PAMPs, they can induce Th1 cell differentiation[109]. After allergen sensitization, dendritic cell numbers increase in the airways[110, 111]. These cells are mature and may be important in interacting with primed T cells to induce them to produce Th2 cytokines in the presence of allergen, or for migrating to lymphoid tissue to promote greater lymphocyte reactions. In human blood, two types of dendritic cells exist, the myeloid dendritic cells(mDCs) that are derived from monocytes and the plasmacytoid dendritic cells (pDCs) that develop from lymphoid origins[112, 113]. In humans, mDCs produce a large amount of IL-12 and preferentially induce Th1 development, while pDCs produce lower amounts of IL-12 and preferentially induce Th2 development[112, 114]. In mice, the opposite is seen where mDCs produce low IL-12 and induce Th2 differentiation and pDCs produce high IL-12 and induce Th1 differentiation[113, 115]. This may show a difference that exists between humans and mice or that the different dendritic cell lineages are not restricted in the Th phenotype that they induce and have multiple functions; pDCs have been shown to play an important role in tolerance to harmless antigen[116] and production of Type I IFNs after exposure

to viruses[117]. Stimulation of pDCs with PAMPs, such as CpG oligonucleotides, can also induce the generation of $CD4^+CD25^+$ T regulatory cells[118]. In allergic diseases, dendritic cells may play a prominent role in both inducing primary and secondary recall responses to allergens through activation of T cells by antigen presentation.

1.7.4 Mast Cells

Mast cells are one of the major inflammatory cells present in the nasal mucosa [44, 119]. They originate from stem cells in the bone marrow. Mast cell precursors migrate from the bone marrow and localize to their site of action in various tissues. Mast cells in tissues are of two major types depending on the serine proteases they produce. Tissue mast cells can produce either tryptase, chymase, carboxylpeptidase and cathepsin G (MC_{TC}) or just tryptase (MC_T)[120, 121]. In the nasal mucosa, MC_T are found close to or within the epithelium, while MC_{TC} are found nearer to the nerves and blood vessels[122].

Mast cells express the high affinity IgE receptor FcεRI at their surface, which is a tetramer composed of an α chain, a β chain and two γ chains[123]. The constant region, the crystallisable fragment of IgE (Fcε) can bind to this receptor, effectively priming the mast cell for allergen. Allergen can cross-link IgE-FcεRI complexes bringing them into proximity of each other and initiating rapid signal transduction cascades in the cytoplasm of the mast cells[124]. Signal transduction cascades involve tyrosine phosphorylation, inositol phospholipid production, and entrance of calcium within the mast cells[124]. These lead to mast cell activation and rapid degranulation of granules of stored preformed inflammatory mediators. Preformed mediators include mainly histamine, serine proteases, IL-4[125] and tumor necrosis factor-α (TNFα)[126]. Histamine has immediate effects on surrounding cells to produce the immediate affects of an allergic

response[40]. IL-4 and TNFα promote cellular inflammation by activation of many cell types. At the same time, mast cell activation causes immediate production of the inflammatory lipid mediators prostaglandin D2 and leukotrienes C_4 (LTC_4). The lipid mediators can cause bronchoconstriction and enhance vascular permeability[121, 127]. Shortly after degranulation, mast cells also start producing other cytokines: granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-5 and IL-13[128, 129], which promote the late phase of the allergic reactions by promoting eosinophil development and survival. Mast cells also express CD40L and may play a role in IgE isotype switching[130].

1.7.5 Basophils

Although basophils are granulocytes that express FcεRI, Th2 cytokines and histamine, they are believed to be a separate lineage from mast cells[131]. Basophils develop in the bone marrow and represent less than 1% of peripheral blood leukocytes. Basophils produce many mediators, similar to mast cells, such as histamine, LTC_4, IL-4, and IL-13[132]. Conversely, PGD_2 and IL-5 are not produced by basophils[121]. Increased numbers of basophils are seen in the tissue following allergen exposure in the late phase of allergic reactions[132, 133]. Due to their low levels in the blood, it has been difficult to ascribe a physiologic function for the basophil, but they may promote late phase inflammation by Th2 cytokine production and may also promote IgE class switching by their expression of CD40L[130].

1.7.6 Eosinophils

Eosinophils possess granules that stain with acidic aniline dyes such as eosin. These cells are rarely found in the blood or nasal tissue in non-allergic subjects, but tissue

eosinophilia is associated with allergic diseases and acute allergen exposure[122, 134]. Eosinophils develop and mature in the bone marrow from stem cells and are released to the peripheral blood as mature cells. These cells can also develop in the tissue from resident stem cells[43]. Eosinophils are characterized by their expression of cytotoxic granular proteins including major basic protein (MBP) and eosinophilic cationic protein (ECP). MBP is directly toxic to respiratory epithelial cells and causes bronchial hyperresponsiveness and bronchodilation[135-137]. Eosinophils also contain lipid bodies involved in lipid mediator production such as LTC_4[138]. Eosinophils can produce a large number of cytokines including transforming growth factor-β (TGF-β)[139], GM-CSF[140], IL-4[141], IL-5[140], and TNF-α[142], although their contribution in cytokine production is much less than Th2 cells. They also express the FcεRI[143] and CD40L[144] and can participate in IgE reactions.

1.7.7 Epithelial Cells and Goblet Cells

The epithelial cells of the nasal tissue form a barrier protecting the inner mucosa from the exterior environment. Allergic nasal mucosa is marked by areas of focal denudation, atrophy and squamous cell metaplasia, possibly as a result of inflammation and production of cytotoxic proteins such as MBP[135]. This condition is improved by local intranasal glucocorticoid treatment, suggesting that inflammation has a role in this pathology[145]. Loss of the integrity of the epithelial layer may promote exposure of the mucosa by allergen and promote further inflammation. Aside from its barrier functions, epithelial cells can also play a role in the inflammatory process by producing cytokines and chemokines to recruit and activate cells. Cytokines produced by epithelial cells include IL-1β, IL-6, IL-11, IL-13, IL-16, GM-CSF, and TGF-β[146]. Epithelial cells can

also produce many chemokines, such as eotaxin-1(CCL1)[147, 148], to recruit cells from the blood for inflammatory reactions in the tissue[146].

Goblet cells are unicellular mucous-producing cells found above the basement membrane interspersing the epithelial cells. Roughly 10000/mm^2 goblet cells can be found in normal nasal mucosa[149]. These cells produce the protective mucous layer over the epithelial cells which aids in pathogen and allergen clearance. It is not clear whether the numbers of goblet cells change during allergic rhinitis as reports in the literature are conflicting[150]. Increased numbers of goblet cells were reported in nasal smears from allergic patients during grass pollen season[151, 152], while in persistent rhinitis, goblet cell numbers did not change[153]. The mucin proteins, the glycoprotein component of mucous fluid, are inducible by Th2 cytokines[154-156] and are increased in airway allergic disease.

1.8 Major Th2 Cytokine Functions

Cytokines are a family of small glycosylated proteins, ranging from 8 kDa to 30 kDa that are involved in many aspects of allergic rhinitis. Cytokines act through specific cytokine receptors on the surface of target cells. Cytokines usually act on cells near the location where they were secreted or even on the cytokine producing cell, but they can also act at distant locations. Allergic rhinitis is believed to be driven by an imbalance between Th1(IFNγ, IL-2, IL-12) and Th2(IL-4, IL-5, IL-9, IL-13) cytokines, with a predominant Th2 cytokine response[37, 157-159]. There may also be involved a loss or regulatory mechanisms such as loss of activity of T-regulatory cells[77, 78]. Current attempts at immunotherapy have as aim to reestablish the balance by increasing Th1 cytokines and immunosuppressive cytokines to counteract increased Th2 cytokines. The

following is a brief discussion of the roles of the major Th2 cytokines IL-4, IL-5 and IL-13 and their involvement in allergic diseases.

1.8.1 Interleukin-4

IL-4 was discovered in 1986 as a factor capable of inducing B cell, T cell and mast cell proliferation[160]. IL-4 is present on chromosome 5 (5q31.1), the so-called Th2 cytokine gene cluster, alongside IL-3, IL-5, IL-9, IL-13 and GM-CSF [161, 162]. IL-4 is mainly produced by T cells, particularly Th2 cells, but is also produced by mast cells[125], basophils[132] and eosinophils[141]. IL-4 is essential in promoting the commitment of T helper cell precursors to produce the Th2 subset of cytokines and is important for the development of humoral mediated immunity[163-165]. IL-4 increases expression of MHC class II molecules on B cells resulting in increased allergen presentation capacity to Th2 cells[166]. On the vasculature, IL-4 promotes expression of VCAM-1 on endothelium, thereby allowing for recruitment of eosinophils and other inflammatory cells such as T cells, monocytes, and basophils from the blood into sites of inflammation[167]. IL-4 can regulate production of eotaxin, a potent eosinophil chemoattractant, by fibroblasts, endothelial and epithelial cells[168-170]. IL-4 also induces isotype switching, a process leading to the production of IgE by B cells, and after switching occurs, IL-4 potentiates IgE production. Furthermore, IL-4 enhances the IgE-mediated response by upregulating IgE receptors on inflammatory cells within the airway such as mast cells[171, 172]. IL-4 can also stimulate mucus cell secretions and metaplasia[154].

IL-4 mediates its actions through the IL-4 receptor composed of two subunits, IL-4 receptor α-subunit (IL-4Rα) and the IL-2 receptor common γ subunit(γ_c)[173, 174]

(Diagram 1.5). The γ_c is also part of the IL-2, IL-7, IL-9 and IL-15 receptors. Binding of IL-4 to the receptor activates Janus kinases (Jaks) which phosphorylate tyrosine residues of the intracellular domain of the receptors. STAT6 is recruited to the receptor and is phosphorylated by the Jaks. STAT6 does so by binding to phorphorylated tyrosine residues through a specialized protein domain called Src-homology domain 2 (SH2). IL-4Rα is associated with Jak1[175] and the γ_c is associated with Jak3 [175, 176] and both can phosphorylate STAT6. Phosphorylated STAT6 homodimerizes through SH2, migrates to the nucleus, binds to STAT6-response elements and once there it regulates transcription of many inflammatory genes[177]. Many genes activated by STAT6 are important for allergic inflammation, and STAT6 deficient animals have defective IL-4 responses[58].

1.8.2 Interleukin-13

IL-13 was first described in 1993, as a cytokine produced after activation of T cells[178]. The IL-13 gene is found only 12kbp from the IL-4 gene on chromosome 5q31.1 [179]. IL-13 has a 70% sequence homology with IL-4 and binds a heterodimer composed of the IL-4Rα chain and an IL-13Rα chain, either low affinity IL13Rα$_1$ or high affinity IL13Rα$_2$[180, 181]. Due to the redundancy in IL-4Rα binding, both IL-4 and IL-13 exhibit some degree of functional overlap. Similarly to IL-4, over-expression of IL-13 within the lungs results in IgE production, eotaxin, inflammation, mucus hyper-secretion, eosinophilia and upregulation of VCAM-1[146, 182]. However, in animal models of antigen challenge, IL-13 can cause airway hyperreactivity to contractile agonists[156]. The expression of IL-13 is more widespread than IL-4[183], and it is suggested that IL-13 has effects on other processes such as fibrosis and airway

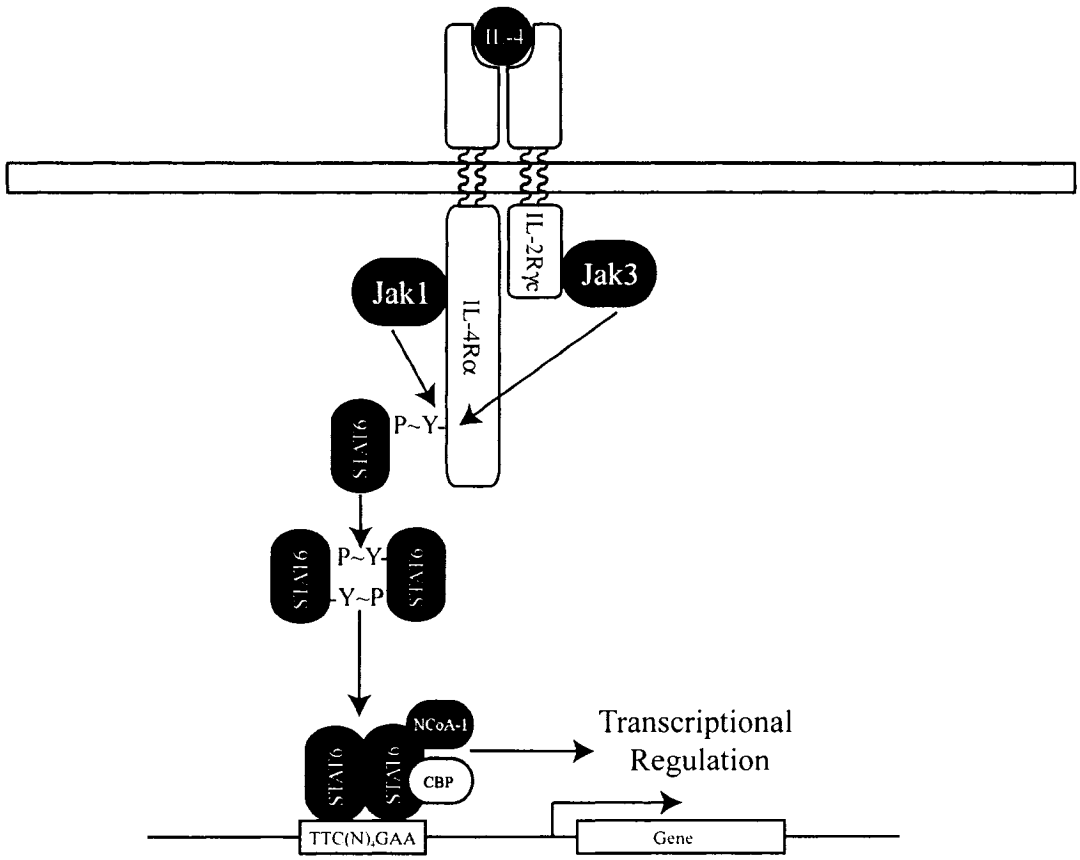

Diagram 1.5. The major pathway activated by IL-4 through the IL-4 receptor is the Jak-STAT pathway. The IL-4 receptor is composed of the IL-4 receptor α-chain (IL-4Rα) and the IL-2 receptor γ common subunit (IL-2Rγc). IL-4Rα is associated with Janus kinase 1 (Jak1) and IL-2Rγc is associated with Jak3. When activated, these phosphorylate tyrosine (Y) residues on IL-4Rα. Phosphorylated tyrosines recruit STAT6 to the receptor and Jaks phosphorylate this protein. Phosphorylated STAT6 can homodimerize which leads to its activation, translocation to the nucleus and binding to DNA through special sequences. STAT6 can associate with CREB-binding protein (CBP) and nuclear receptor coactivator 1 (NCoA-1) to regulate transcription by chromatin remodeling and activation of RNA polymerase.

hyperreactivity. Like IL-4, IL-13 induces tyrosine phosphorylation cascades through its receptor. IL-13 activates JAK1 through IL-4Rα chain, leading to activation of STAT6, but not JAK3 as the IL-13Rα subunits do not associate with γ_c. IL-13$R\alpha_1$ can associate with JAK2 and Tyk2 for its signal transduction[184]. IL-4 can also bind to the IL4Rα-IL13$R\alpha_1$ complex to mediate its effects[185]. Both IL-4 and IL-13 can activate insulin receptor substrate-phosphatidylinositol 3'-kinase pathways for cell survival and proliferation through this receptor[186]. The IL-13$R\alpha_2$, has a short intracellular region that is thought to downregulate the actions of IL-13 by acting as a decoy receptor[187]. IL-13$R\alpha_2$ has also been shown to inhibit IL-4 activation of STAT6 by interaction of its cytoplasmic domain with the IL-4 receptor[188]. A recent report has shown that IL-13 $R\alpha_2$ may indeed initiate signal transduction by binding IL-13, and its activation leads to production of TGF-β in macrophages[189].

1.8.3 Interleukin-5

Interleukin-5 was cloned as a factor causing the growth and differentiation of eosinophils [190-192]. IL-5 is the most important Th2 cytokine associated with eosinophils, and it can regulate most aspects of eosinophil behavior. Mice that are genetically engineered to express high levels of IL-5 have life-long eosinophilia in their lungs[193]. In addition, intravenous injections of antibodies blocking IL-5 can decrease asthmatic sputum eosinophilia[194]. IL-5 promotes survival by blocking eosinophil apoptosis[195]. IL-5 can also activate eosinophil secretion, cytotoxicity and chemotaxis[196]. IL-5 activates eosinophils to upregulate integrin receptor expression, which promotes adhesion to VCAM-expressing endothelial cells and eosinophil accumulation in the tissue[197, 198]. IL-5 is produced by T helper cells, cytotoxic T

lymphocytes and mast cells, but a major source of IL-5 is the eosinophils themselves [199].

The receptor for IL-5 interacts with a heterodimeric receptor composed of an α-subunit and a β-subunit shared with GM-CSF and IL-3 receptors[200]. IL-5 signal transduction, like IL-4 and IL-13, involves tyrosine phosphorylation cascades involving JAK2 and STAT5a and STAT5b and activation of Lyn and Syk tyrosine kinases[201, 202].

1.9 Possible Reasons for Increasing Prevalence in Allergic Diseases

1.9.1 Genetic Predispositions

Although many processes and mediators of allergic inflammation and Type I hypersensitivity have been characterized, no clear explanation exists for the large increases seen in disease prevalence. In 1916, a study was published showing that 50% of patients with allergic rhinitis and asthma had a positive family history for these diseases [203]. Yet, such a rapid increase in the number of people with allergy over the past 50 years denotes changes in the environment or lifestyle and not a pure genetic cause. Nevertheless, the sensitivity to allergen and the severity of the allergic disease may depend on the genetic susceptibility of the individual. A number of genetic studies, using linkage analysis, fine mapping studies and gene expression studies have implicated a number of potential genes leading to a predisposition for atopy. Almost all genes involved in the development of the IgE-mediated inflammatory cascade have been linked with the development of atopy and asthma (for recent review see [204]). For example, the IL-4 gene cluster containing the IL-4, IL-5 and IL-13 genes[205, 206] and the high affinity IgE receptor [207] are highly linked to the development of atopy. Genetic studies

have also become so sophisticated that genes are being linked to atopic disease before a clear role in disease is determined. Some examples of recent genes discovered include arginase[208], a disintegrin and metalloproteinase domain 33 (ADAM33)[209], PHD Finger Protein 11 (PHF11)[210, 211], dipeptidyl peptidase X (DPP10)[212], G protein-coupled receptor 154 (GPR154)[213] and defensin β1 (DEFB1)[214]. What role and how great a role these and other genes play in the development of atopy is still undetermined but their study may show them as potential therapeutic targets and give a clearer understanding of how allergies develop. Many genes discovered in the genetic studies are associated with single nucleotide polymorphisms (SNPs), which provide a greater or lesser susceptibility to disease. For example, SNPs found in STAT6 have been associated with allergic disease and asthma and an increased production of serum IgE[215-217].

1.9.2 Pollution

It has long been speculated that pollution plays a large role in the increased prevalence of atopic diseases. The Industrial Revolution began in England during the late 18^{th} century and the beginning of the 19^{th} century and was associated with the first massive chemical pollution in human history. Since then pollution has become a global problem and its increase may be associated with increasing atopic diseases. It is thought that chronic chemical damage to the epithelial barriers of the body could facilitate the entry and sensitization to allergens. Animal models have shown allergic sensitization can be increased by SO_2 and diesel exhaust[218, 219]. In humans, exposure of the nasal mucosa to diesel exhaust can also increase IgE levels[220, 221]. A strong association was also determined between allergic rhinitis to cedar pollen and locations close to heavy

traffic[222], but in other studies the same association was not seen[223]. NO_2 present in car exhaust was associated with an increased prevalence of asthma and allergic rhinitis[224]. In one study, ozone (O_3) was able to increase the sensitivity to allergen doses[225], but other studies were not able to reproduce this finding[226, 227]. Although pollution may exacerbate the effects of allergens, it is thought that it is not the underlying cause of the development of atopy. In addition, the variation of the results obtained between epidemiological reports suggests the need of large global longitudinal studies to study pollution and its link to allergic diseases[228].

1.9.3 Tobacco Smoke

Tobacco smoke is a complex mixture of various particles and organic compounds that have many harmful health effects. Parental, particularly maternal smoking is associated with an increased wheezing and asthma in children[229]. With respect to allergic sensitization, atopic smokers compared to non-smokers also have increased levels of IgE[230]. Mixed results have been obtained for passive smoke exposure to be a risk factor for sensitization of children to indoor allergens, with some studies showing a link and others not[229, 231, 232]. Maternal smoking during pregnancy has been shown to be a risk factor for sensitization and wheezing during childhood [233, 234], but other studies have not found this association[235]. It is difficult to discern how great a factor tobacco smoke plays in allergic disease and more studies are required.

1.9.4 Obesity

Obesity may also be a risk factor for atopy but discrepancies are seen in the epidemiological reports. In a European study, men and women with a high BMI (>30) had a higher incidence of wheeze and shortness of breath compared to subjects with a

lower BMI (20-25)[236]. BMI was not associated with allergic rhinitis, total IgE or specific IgE in this study. In children, no association is seen between BMI and atopy, regardless of sex[237]. In a recently conducted study using male conscripts in Sweden, asthma was shown to be associated with a high BMI, but allergic rhinitis was not[238]. Contrary to the European studies, in a longitudinal study conducted in New Zealand, BMI was positively associated with atopy and IgE levels in women but no association was seen in men[239]. Supporting evidence shows that obese women with atopy versus atopic non-obese women have three times higher total and specific IgE levels[240].

1.9.5 Family Size and Day Care

There is much evidence demonstrating that family size has a role to play in decreasing atopy. The number of older and younger siblings is inversely related to the occurrence of atopic diseases and specific IgE in children, adolescents and adults[241-244]. This relationship is strongest for atopic eczema[245] and hay fever[246, 247], but weaker for protection from asthma[246, 248, 249]. Reasons for this difference either lie in the definition of asthma used for the studies or that the development of some forms of asthma may be different from other atopic diseases[250, 251]. In addition to increased number of siblings, young children who are exposed to day cares also have reduced atopic diseases[252, 253].

1.9.6 Hygiene Hypothesis

Many epidemiological studies have found a strong inverse relationship between infections in early life and the subsequent development of atopic diseases[250]. One of the first studies that linked the two was conducted in 1976, looking at serum IgE levels, in white population and Métis Indians from Saskatchewan in Canada[254]. Gerrard et al.

found that the prevalence of asthma and eczema was significantly higher in the white population compared to the Métis population; in contrast, a higher prevalence of helminth infestations and untreated viral and bacterial infections were seen in the Métis population compared to the white population. Soon after, in another study, Anderson reported that in Papua New Guinea, respiratory infections in children occurred more frequently where asthma prevalence was low[255]. The hygiene hypothesis also follows from the sibling and daycare effect mentioned earlier. Increased contact between children promotes childhood infections, and this would be protective against atopy. These pioneering studies and the numerous others that followed led to what is known as the hygiene hypothesis, first proposed in 1989[247]. It postulates that in the absence of common environmental immunological challenges, an infant's immune system may not develop properly, leading to unregulated responses to allergens. More recent studies have directly correlated the level of sanitations with the incidence of allergic diseases[256]. Additionally, prevention or cure of certain infections has shown to increase the risk of allergic diseases. For example, a pneumococci vaccination program in South Africa led to an increased incidence of atopy[257].

1.10 Animal Model Support for the Hygiene Hypothesis

Largely due to recently accumulating immunological evidence to support the epidemiological studies, the hygiene hypothesis is slowly becoming the most plausible hypothesis to explain the temporal and regional differences in the prevalence of atopic diseases. A number of animal models of infection and antigen sensitization have been created to test the hygiene hypothesis. In studies using mice infected with attenuated *Mycobacterium bovis*, also known as the Bacillus Calmette-Guerin (BCG) used for

vaccination against tuberculosis, a suppression of allergen-induced airway eosinophilia by 60-70% with a decrease in IL-5 production by T cells and decreased airway reactivity was observed[258, 259]. Peritoneal injections of mice with heat-killed lactobaccilus reduced IgE production in mice fed allergen with an increase in IL-12 production[260].

1.11 Mechanisms of the Hygiene Hypothesis

Two main mechanisms have been proposed to form the immunological basis of the hygiene hypothesis[261]. The first mechanism is based on the competition of anti-infectious immune responses with allergen immune responses. As the burden of infectious material is less, antigen-presenting cells are more likely to present allergen to T cells. Allergen specific T cells would increase due to activation and proliferation, and these would compete with other T cells for homeostatic factors.

The second mechanism is based on loss of suppressor mechanisms that would prevent or control allergic inflammation [261, 262] (Diagram 1.6). Exposure to bacterial products may promote development of Th1 cytokine production and responses, which would prevent overactive Th2 immune responses. Some of the regulation could also be mediated by $CD4^+$ $CD25^+$ T regulatory cells, a population of cells which could be increased by exposure to infections to maintain homeostasis. $CD4^+$ $CD25^+$ T cells are present in both allergic rhinitis patients and non-allergic individuals, and are capable of suppressing both Th1 and Th2 cytokine production[78, 263]. However, *in vitro* experiments have shown that while $CD4^+$ $CD25^+$ T cells from non-atopic individuals retain suppressive ability, this is reduced in atopic $CD4^+$ $CD25^+$ T cells, particularly during the pollen season[77, 262]. Similarly, during birch-pollen season, $CD4^+$ $CD25^+$ T cells from allergic patients are defective at down-regulating IL-13 and IL-5 production,

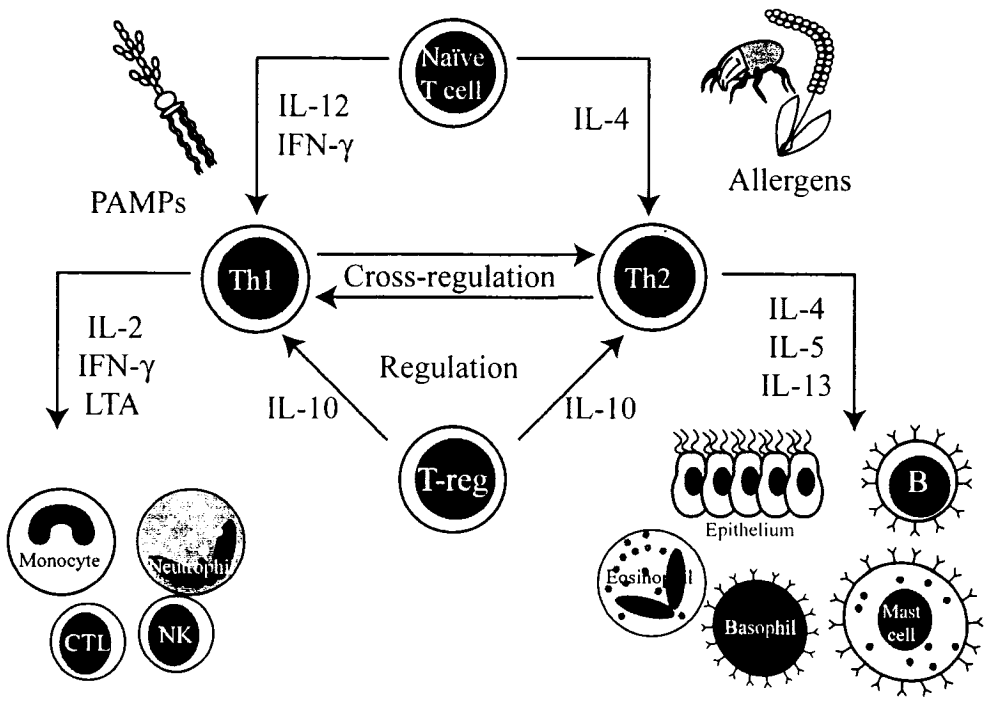

Diagram 1.6. The hygiene hypothesis proposes that exposure to infections and pathogen associated molecular patterns (PAMPs) helps the proper development of regulatory mechanisms, such as Th1 cytokines and T-regulatory cytokines, against the development of allergic disease. Without this regulation, Th2 cytokines are produced which cause the development of IgE sensitization and promote allergic inflammation by activation and priming of effector cells.

while still retaining their ability to inhibit IFN-γ production[78]. The mechanism by which the activity of $CD4^+$ $CD25^+$ T cells is differentially regulated in atopic and non-atopic individuals remains to be determined.

1.12 Lipopolysaccharide

The hygiene hypothesis has been extended not only to include to bacterial and viral infections but also exposure to bacterial components, such as lipopolysaccharide (LPS); numerous epidemiological and animal model studies haves shown that LPS can be protective against the development of atopic diseases. The work of this thesis focuses largely on the actions of LPS in atopic disease. This PAMP is a potent inducer of inflammatory reactions and may play a role in inhibiting the development of atopic disease. The following is a summary of the studies performed on LPS and its effects in the airways, its involvement in sepsis, its structure and its receptor.

1.12.1 Airway Exposure to LPS

Several epidemiological studies have shown that LPS can be protective against the development of allergic diseases. The first direct *in vivo* evidence that LPS exposure from the domestic environment protected against allergen sensitization was reported by Gereda et al[264]. In a pool of children aged between 9 and 24 months, concentrations of house-dust endotoxin and allergen were measured. A significant amount of LPS was found in common house dust, and these levels did not vary much throughout the year [264]. Allergen sensitization was measured by skin-prick testing. It was found that sensitized children lived in homes with significantly lower concentrations of endotoxin, compared to non-sensitized infants. Additionally, IFN-γ positive $CD4^+$ T cell numbers were positively correlated with house-dust endotoxin concentrations, suggesting that

indoor endotoxin exposure enhanced Th1 immunity and may protect from allergen sensitization. In a second study, children aged between 5 to 10 year olds were recruited and living-room floor dust was analyzed for endotoxin[265]. Allergen-specific IgE levels were quantified in this study, and it was determined that sensitization to allergen was negatively correlated to endotoxin levels. In addition, higher levels of endotoxin were associated with less severe sensitization to one antigen and were protective against sensitization to more than one allergen. Increased endotoxin levels in house dust reduced the risk of atopic eczema, but the risk of respiratory infections and bronchitis was increased[266]. Results from the first two studies were confirmed by Braun-Fahrlander et al. in a study involving children aged 6 to 13 years of age[267]. High endotoxin levels, in mattresses, were once again correlated with decreased occurrence of hay fever, atopic asthma and sensitization to allergens. Interestingly, peripheral blood leukocytes isolated from the children from low endotoxin households produced more TNF-α, IFN-γ, IL-10 and IL-12 compared to children from high endotoxin households after stimulation with LPS or Staphylococcal Enterotoxin B. The authors speculated that exposure to high levels of endotoxin resulted in a generalized tolerance to immunological stimulus.

Although endotoxin seems to be protective for the development against atopic diseases in children, it may be a risk factor for occupational asthma in adults[268]. Occupational asthma is thought to be caused by abnormal agents in the workplace such as dusts, chemicals, fumes or vapours and is not necessarily IgE-mediated. In addition to household dust, LPS can be found in high concentrations in air pollution[269], organic dusts [270], and even cigarette smoke[271]. Concentrations of endotoxin in the air are strongly associated with acute decreased airflow in cotton workers[272] and swine

farmers[273]. Endotoxin and grain dust can even cause airflow obstruction in previously unexposed individuals[274-276] and increased neutrophil levels in the airways[277]. Additional reports have also shown that endotoxin levels in households may be related to clinical severity of asthma[275, 278, 279].

In experimental settings, when human subjects are exposed to pure endotoxin by inhalation, they experienced acute clinical effects such as fever, shivering, joint pain, malaise, increased blood and airway neutrophil levels, dry cough, dyspnea, reversible bronchial obstruction and a impairment in lung function and forced expiratory volume in 1 second (FEV_1)[268, 275, 280, 281]. Considerable variability is seen in the magnitude of these symptoms in reported responses to LPS challenges. In one study of 77 naïve subjects exposed to endotoxin, only half the subjects reported fever, about a third felt chest tightness, and exposure caused only a small dose-related decrease in FEV_1 and a slight increase in bronchial hyperresponsiveness[276]. In another study, normal subjects had no brochial hyperresponsiveness upon LPS challenge[282]. Subjects with asthma also seem to respond more than patients without asthma[282-284]. Kline and colleagues recruited 72 healthy, non-allergic, and non-smoking subjects, aged 18 to 50 years of age to study variable responses to LPS[269]. Based on the FEV_1 following inhalation of increasing doses of LPS and *in vitro* production of IL-6 and IL-8 from peripheral blood monocytes and alveolar macrophages, three phenotypes were determined: sensitive, intermediate and hyporesponsive. This variability observed may depend on genetics of the individuals but also other extrinsic factors. From the different epidemiological studies, it seems that endotoxin may be protective against allergen sensitization in early

life and the development of atopy, but it may be detrimental, can cause non-allergic asthma for some patients and may exacerbate already established asthma.

Animal models and *in vitro* experiments have been performed to try and replicate the results of epidemiological studies. In a study by Tulic et al., airborne exposure of endotoxin to 8-10 week old rats one day before intraperitoneal OVA exposure or 4 days after the OVA exposure protected against the development of ovalbumin specific IgE[285]. When LPS exposure to the rats occurred 6 to 10 days after sensitization with OVA, OVA challenge caused a greater lung cellular influx and increased vascular leakage compared to rats not exposed to LPS but sensitized to OVA. In this model, LPS could also reduce bronchial hyperresponsiveness to methacholine challenges and eosinophilia caused by OVA. In a mouse model of allergic asthma, LPS administration suppresses IgE-mediated and mast cell-dependent allergen responses, pulmonary inflammation, airway eosinophilia, mucus production and airway hyperresponsiveness[286]. The effects of LPS seemed to be independent of IL-12, IFN-γ but did require the presence of inducible nitric oxide synthase (iNOS). In addition to a role for nitric oxide in diminishing airway hypereactivity, IL-10 may play a role in diminishing airway inflammatory events in these models[287]. In another study, cockroach antigen sensitized mice were administered different doses of LPS before antigen challenge[288]. In this model, airway hyperresponsiveness and eosinophil levels and Th2 cytokines levels in the lungs decreased and neutrophils and Th1 cytokine levels increased. In contrast to these studies, Wan and colleagues showed that mice exposed to airborne endotoxin before ovalbumin sensitization and 5 days during ovalbumin sensitization had enhanced IgE expression and increased eosinophils in bronchoalveolar

lavages (BALs) [289]. Low doses of LPS have also been shown to promote Th2 responses to allergen while high doses of LPS promote Th1 responses[290]. When low doses of endotoxin were given to sensitized mice during challenge, IL-5 expression and eosinophilia were promoted[291]. In addition, it has been proposed that endotoxin in low doses is necessary for the development of optimal Th2 responses to an allergen in mice[290, 292]. Taking all these studies together, it can be seen that the effect of LPS on allergic responses can vary depending on dose and timing with respect to sensitization.

In animal models, exposure to LPS before allergen sensitization is protective, suggesting the evolution of anti-inflammatory mechanisms in early life. To investigate this phenomenon in humans, Tulic and colleagues used a human *ex vivo* tissue culture model to determine the responses of non-atopic children(aged 3.6 ± 0.7 years old) to LPS[293]. Tissue was resected from the inferior turbinate of the nasal passages and incubated with different doses of LPS for 24 hours. In this model, as the tissue is incubated outside the body, systemic infiltrates are eliminated as responses to challenges are solely derived from tissue constituents. LPS caused a large increase in macrophages, mast cells, elastase positive cells and surprisingly large increases in T cells. The increase in T cells was due to proliferation and was LPS dose dependent. Numbers of IL-2, IFN-γ and IL-12 mRNA positive cells were also increased within the tissue. In addition, many of the proliferating T cells were determined to be $CD4^+CD25^+$ cells, and IL-10 mRNA positive cells were increased within the tissue after LPS stimulation. It is possible that non-atopic children have LPS sensitive $CD4^+CD25^+$ T regulatory cells that produce IL-10 upon endotoxin exposure. These naturally occuring cells could be able to provide anti-

inflammatory signals to protect against the development of Th2 inflammation in the tissue.

1.12.2 LPS and Sepsis

LPS can have strong widespread effects when present in the vasculature. It is one of the agents capable of causing the signs and symptoms of sepsis. Systemic inflammatory response syndrome (SIRS) is defined as a response with two or more of the following: increased or decreased temperature, increased or decreased leucocyte count, tachycardia, and rapid breathing[294-296]. Sepsis is defined as SIRS combined with a documented infection.

Infections that cause sepsis include Gram positive and Gram negative bacteria, fungi, viruses and parasites. Usually the infections are contained locally by the immune system, but if this system fails, a systemic response to the infection occurs. Inflammatory responses and neuroendocrine responses lead to generalized inflammation, coagulation, dysfunction in the circulatory system, acute organ dysfunction, multiple organ dysfunction syndrome and eventually death. Severe sepsis is defined as sepsis with at least one sign of organ hypoperfusion or dysfunction, and septic shock is severe sepsis with refractory arterial hypotension[296].

LPS causes effects on multiple cell types, but adoptive transfer experiments in mice have shown that cells that mediate the toxic effects of LPS during sepsis are of hematopoietic origin[297]. More specifically, macrophages seemed to be the primary cell type mediating these effects[298]. One of the major products produced by endotoxin-activated macrophages is TNF-α. Administration of high doses of TNF-α can cause many of the toxic effects of LPS including fever, vascular leakage, blood

coagulation, and widespread tissue injury[299, 300]. In addition to TNF-α, LPS can induce the production of many different proinflammatory cytokines including IL-1, IL-6, IL-8, IL-12 and many lipid mediators[301, 302]. These cytokines can activate production of multiple proinflammatory factors such as nitric oxide and chemokines[303]. Additionally, LPS induced cytokine cascades can promote the production of Th1 cytokine production from T cells, to stimulate a cell-mediated immunity for clearance of the pathogen.

Many efforts have been made to determine agents capable of reducing the toxic effects of LPS. The anti-inflammatory cytokine, IL-10 can be induced by LPS, and can limit the activation caused by LPS and prevent toxicity[304, 305]. Some studies have been performed on other cytokines, particularly the Th2 cytokines IL-4 and IL-13. *In vitro*, IL-4 can inhibit LPS-induced human monocyte production of IL-1, TNF-α, IL-6, IL-10 and IL-12 [306-310]. *In vivo,* IL-4 has been extensively studied as an agent capable of preventing sepsis. One report showed that mice given recombinant IL-4 were capable of surviving lethal sepsis-causing doses of *Pseudomonas aeruginosa*[311]. IL-13 is also protective against sepsis, and can reduce LPS-induced IL-6, IL-8, IL-1β and TNF-α[178, 312, 313]. Although the mechanisms have not been determined, IL-4 and IL-13 activation of STAT6 may play a role, as STAT6 deficient mice have uncontrolled production of cytokine and chemokine production when given LPS[314]. Corticosteroids have also been shown to downregulate the LPS responses[315].

1.12.3 LPS Structure

Bacteria can be differentiated into two groups based on their ability to retaining the violet dye/iodine complex, in a procedure called the Gram stain[316]. Gram negative

bacteria have two cell membranes, an interior cell membrane composed of a phospholid bilayar and a secondary outer cell membrane. The outer cell membrane is composed of proteins and a lipid bilayer. The inner leaflet of the bilayer is composed of phospholipids but the outer membrane is mostly composed of LPS also known as endotoxin [317]. LPS contributes to the integrity of the outer membrane and protects the cell against bile salts and antibiotics[318].

In 1879, Louis Pasteur discovered that patients with sepsis had microbes in their blood [319]. Soon after, Richard Pfeiffer associated the term endotoxin as an agent responsible for causing the fever and shock in animals injected with heat-killed *Vibrio cholerae*[320]. By using chemical and biochemical analysis, it was concluded that endotoxin was a glucolipid complex[321]. Finally, in the 1960s and early 1970s the endotoxin component structures were determined, consisting of lipid A[322], the core sugars (R antigen) and the distal O-antigen polysaccharide (O antigen)[323](Diagram 1.7). Lipid A portion causes the actions of endotoxin[324], while the R- and O-antigens are immunogenic and cause the production of antibodies, useful for antibody-dependent phagocytosis. Most studies on the structure and production of LPS have been performed using *Escherichia coli*. *E. coli* lipid A consists of a disaccharide moiety attached to four branched fatty acids by ester linkages (Diagram 1.7)[325]. The core oligosaccharide attaches to one of the sugars in the disaccharide. Attached to the core is the O antigen, a large polymer consisting of repeating oligosaccharide subunits. The O polysaccharide maintains the hydrophilic nature of LPS molecule.

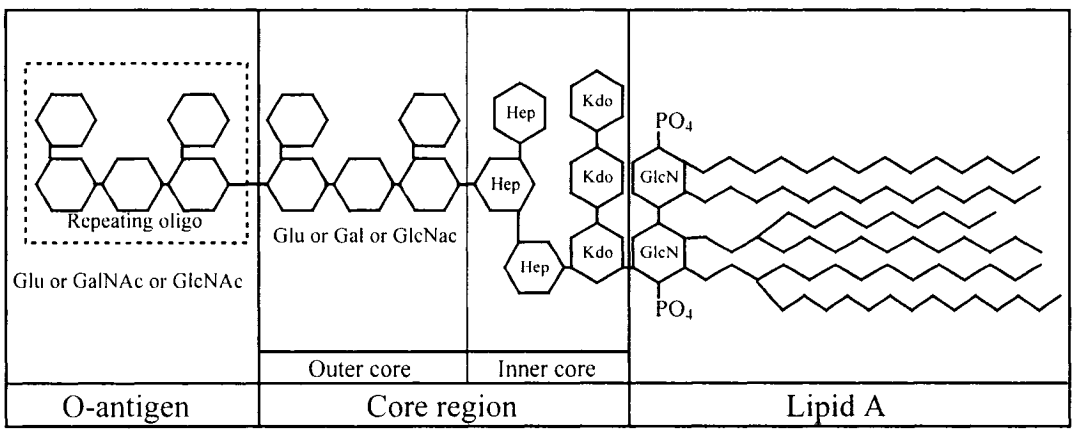

Diagram 1.7. LPS is composed of lipid A, a core region and an O-antigen. Lipid A is composed of a disaccharide of biphosphorylated glucosamine(GlcN). Fatty acids are linked to the disaccharide, two of which are branched. One the GlcNs of the disaccharide is linked to the core region. The inner core is a short chain 6-7 sugar residue oligosaccharides consisting of keto-deoxyoctulonate (Kdo) and heptulose (Hep). The outer core is composed of more sugars including glucose (Glu), galactose (Gal) and N-acetyl-glucosamine (GlcNac). The O antigen is a large polymer consisting of repeating oligosaccharide subunits made up of 3 to 5 sugars, usually Glu, GlcNac and and N-acetyl-galactosamine(GalNac). The O antigen can be highly branched with individual chains varying up to 40 subunits of sugars.

1.13 Toll-like Receptor Proteins

1.13.1 TLR4 Discovery

In 1965, the first steps to determine the identity of the LPS receptor were laid out. It was discovered that LPS functioned in an antigen-independent manner not requiring T cells or antigen presentation to mediate its effects [326, 327]. A spontaneous mutation occurred in the C3H strain of mice forming a substrain designated as C3H/HeJ. These mice are tolerant to LPS and have a strong susceptibility to infections[328, 329]. The mutation was identified to affect a single genetic locus called *Lps* [330, 331]. Another spontaneous mutation that caused hyporesponsiveness to LPS was discovered in another strain of mouse, the C57BL/10ScCr strain[332]. After crossing C3H/HeJ and C57BL/10ScCr strains, complementation, the acquisition of LPS sensitivity, was not observed, suggesting that LPS sensitivity in both mouse strains resided in the *Lps* locus.

Parallel research in fruit flies in the early 1980s leading up to the late 1990s gave headway into identification of the gene at the *Lps* locus. The Toll (meaning "great" in German) gene was discovered as part of a set of 12 genes that are involved in establishing dorsoventral polarity in embryogenesis of the fruit fly, *Drosophila melanogaster*[333-335]. Toll was later found to play an important role in innate immune reactions to bacteria and fungi[336-338]. *Drosophila* Toll cytoplasmic domain was found to have high homology with the interleukin-1 receptor[339]. By homology searching, the first mammalian Toll-like protein was cloned and designated as Toll/IL-1 receptor-like (TIL) and was thought to have developmental functions like in fruit flies[340]. In 1997, by comparing the sequence of Toll to a human expressed sequence tag (EST) database, Medzhitov et al., discovered and cloned a human Toll (hToll) analog [341]. The hToll

was also capable of activating nuclear factor-κ B (NF-κB) in a biochemical assay, but no ligand was discovered. Soon after in a similar process, Rock et al. compared fruit fly Toll to EST databases and determined 5 genes with sequences similar to Toll, that were designated Toll-like receptors (TLRs) 1 to 5[342]. In this screen TIL was found to be TLR1 and hToll was found to be TLR4.

The identity of the *Lps* locus was only cleared up in genetic studies of the late 1990s. Before then, a plasma protein was shown to bind to LPS and enhance the sensitivity of mononuclear cells to LPS[343-346]. LPS-binding protein (LBP) was produced constitutively by the liver and was inducible by LPS challenge. In 1990, another molecule essential for LPS sensing was discovered, called CD14[347]. CD14 could be associated with the plasma membrane of cells by a glycosylphosphatidylinositol anchor, but it does not span the membrane and it was not clear how signal transduction could be triggered by LPS to the interior of the cells. In 1998-1999, through laborious genetic screening and physical mapping of the *Lps* locus in mice, the Tlr4 gene was discovered and was shown to be the LPS receptor[348-350].

1.13.2 TLR Family and Ligands

The TLR family is an ancient family of proteins with homologs in insects and even in plants[351]. Even if ligands have not been ascribed to them, TLRs play a role as PRRs in the innate immune systems leading to responses against pathogens (Table 1.1). Ten TLRs are present in humans, but mice have 13[352]. They can associate to form dimers, and thus can increase the number of potential ligands that they can bind to. Some TLRs, especially TLR2 and TLR4, have been shown to bind to several ligands. TLRs are expressed in both lymphoid and nonlymphoid tissues, and the pattern of expression varies

with the cell type, tissue and species studied[353]. TLRs share several common features including an extracellular domain made up of multiple leucine-rich repeats (LRRs), a single membrane spanning domain and a highly conserved cytoplasmic signaling domain homologous to the IL-1R called a Toll/IL-1R (TIR) domain. The extracellular domain LRRs form a horseshoe-shaped solenoid with a broad hydrophobic surface for binding of ligands[354]. Due to a high conservation of the TIRs in the cytoplasmic domain, IL-1R and TLRs use common adaptor proteins for signal transduction cascades. Some TLRs can bind to endogenous ligands and it is thought that these can serve as danger signals to mobilize the immune system against tissue damage, but may also contribute to autoimmune diseases[355].

Table: 1.1 Mammalian Toll-like receptors and their ligands:
Table summarized from references [353, 355-357] with additional information.

Toll-like receptor	Ligands and potential ligands	Reference
TLR1 (with TLR2)	Tri-acyl lipopeptides (bacteria)	[358]
	Soluble factors (*Neisseria meningitides*)	[359]
TLR2	Peptidoglycan (Gram-positive bacteria)	[360-362]
	Lipoprotein/lipopeptides (various pathogens)	[363-368]
	Lipoteichoic acid (Gram-positive bacteria)	[362, 369]
	Lipoarabinomannan (mycobacteria)	[370]
	Modulin (*Staphylococcus epidermidis)*	[371]
	Glycoinositolphospholipids (Trypanosomes)	[372]
	Glycolipids (*Treponema*)	[373]
	Porins (*Neisseria*)	[374]
	Zymosan (fungi)	[365]
	Lyso-phosphatidylserine (Schistosoma)	[375]
	Atypical LPS	[376, 377]
	Heat shock proteins (endogenous)	[378, 379]
	HMGB1(endogenous)	[380]
TLR3	Double-stranded RNA (virus)	[381]
	mRNA (endogenous)	[382]
TLR4	Lipopolysaccharides, lipid A (Gram-negative bacteria)	[348, 350, 360, 383-387]
	Taxol (plant)	[388-390]
	Protein F (respiratory syncytial virus)	[391]
	Aspergillus fumigatus hyphae	[392]
	HSP60 (*Chlamydia*)	[393, 394]
	Envelope proteins (viruses)	[395]
	Heat shock proteins (endogenous)	[378, 379]
	HMGB1 (endogenous)	[380]
	Fibronectin extra domain A (endogenous)	[396]
	Fibrinogen (endogenous)	[397]
	Lung surfactant protein A (endogenous)	[398]
	Minimally modified-low-density lipoprotein (endogenous)	[399]
	Heparan sulfate (endogenous)	[400, 401]
	Hyaluronan fragments (endogenous)	[402, 403]
	Saturated fatty acids (endogenous)	[404]
TLR5	Flagellin (bacteria)	[405, 406]
TLR6 (with TLR2)	Di-acyl lipopeptides (mycoplasma)	[407, 408]
	Peptidoglycan, zymosan	[409]
TLR7	Single-stranded RNA (viral and synthetic)	[410, 411]
	Imidazoquinolines (antiviral agents)	[412]
	Guanine nucleoside analogs (antiviral agents)	[413]
TLR8	Single-stranded RNA (viral)	[410]
	Poly(G)-containing DNA oligonucleotides	[414]
TLR9	CpG DNA (bacteria and Herpes simplex virus)	[415-417]
	DNA (endogenous)	[418-421]
TLR10	Unknown	
TLR11/12 (mice)	Uropathogenic *Escherichia coli*	[422]
	Profilin-like molecules from protozoa	[423]
TLR13 (mice)	Unknown	
LY 64 (RP105)	LPS	[424]

1.13.3 TLR4 Expression and Signal Transduction

TLR4 is expressed on many different cell types including monocytes[425], macrophages[425], immature dendritic cells[426], myeloid dendritic cell [113], mast cells[427, 428], neutrophils[425] and eosinophils[429]. B cells express low levels of TLR4[430] but express another member of TLR family called RP105[424], which can recognize LPS, associate with TLR4 and transduce signals to the interior of the cells[431]. Low levels of TLR4 mRNA have been shown on T cells by PCR[432], but mouse CD4+CD25+ T regulatory cells have been shown to express high levels of TLR4[433]. TLR4 is also expressed on structural cells including intestinal epithelial cells[434], pulmonary epithelial cells[435, 436], nasal epithelial cells[437], endothelial cells[438], vascular smooth muscle cells[439], airway smooth muscle cells[440] and fibroblasts[441]. Expression levels are variable on the different cell types with cells of the immune system expressing the highest levels. In some cells, such as intestinal epithelial cells, TLR4 is found mostly associated with the Golgi apparatus and expressed at almost undetectable levels on the cells surface in normal conditions[442, 443].

The LPS receptor complex is very sensitive, being able to recognize most types of lipid A molecules at picomolar concentrations[444]. TLR4 is physically associated with the protein MD-2 in its extracellular domain, and MD-2 is essential for binding of LPS and activation of TLR4[445-447]. MD-2 associates with TLR4 in the endoplasmic reticulum or the cis Golgi, and then the TLR4/MD-2 complex moves to the cell surface[448]. The current model of LPS cell activation involves either direct binding of LPS to CD14 on the surface of cells, binding of LPS to the soluble form of CD14 (sCD14) or binding of LPS to LBP which transfers LPS to CD14[347, 449, 450]. CD14

also has many LRRs forming a horseshoe-like solenoid[451]. Membrane bound CD14 or sCD14 then associates with the TLR4/MD-2 complex on the surface of cells causing LPS to bind to TLR4 and activate signal transduction[452, 453].

Signal transduction involves association of proteins through domains, phosphorylation of proteins by kinases and protease degradation of inhibitors (Diagram 1.8)[356, 357]. TLR4, like other TLRs and the IL-1R possess a TIR domain. Upon binding of its ligand, TLR4 dimerizes and undergoes conformational changes that are transmitted to the interior of the cell membrane. Conformational changes cause the association of the TIR domain of TLR4 to the TIR domain of the adaptor protein myeloid differentiation primary response gene 88 (MyD88)[454-456]. The N-terminal portion of MyD88 possesses a death domain which can, when MyD88 is activated by TLR4, recruit the serine/threonine kinase IL-1R-associated kinase 4 (IRAK4) through its own death domain[454, 455]. IRAK4 phosphorylates and activates IRAK1[457]. IRAK1 can autophosphorylate residues in its N-terminus which enables association with another adaptor protein called TNF receptor-associated factor 6 (TRAF6)[458]. IRAK1 and TRAF6 disengage from the receptor and interact at the plasma membrane with a complex consisting of the serine/threonine kinase TGF-β-activated kinase 1(TAK1) and the two adaptor proteins TAK1- binding protein 1 (TAB1) and TAB2[459]. TAB2 links TAK1 to TRAF6 and TAB1 functions as an activator or TAK1[460, 461]. TRAF6 interaction with the TAK1 complex induces phosphorylation of TAB2 and TAK1 which then translocates with TRAF6 and TAB1 to the cytoplasm.

The main signal transduction pathways activated by LPS lead to activation of the transcription factor NF-κB. The NF-κB family consists of five members including

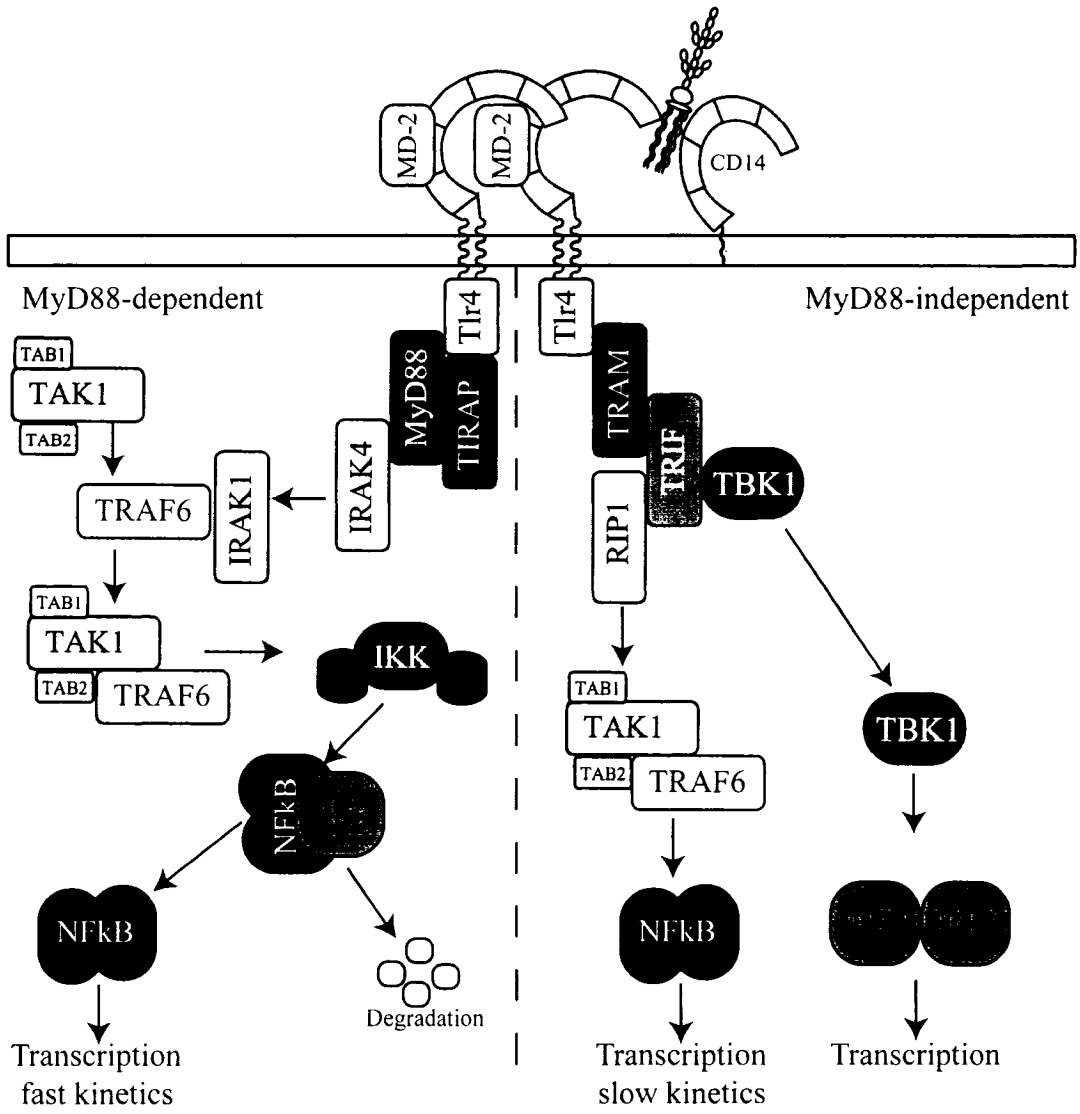

Diagram 1.8. TLR4 like other TLRs is a transmembrane receptor containing a leucine rich repeat domain in the extracellular portion that forms a horseshoe shaped solenoid. TLR4 is associated with MD-2. CD14 binds to LPS and transfers it to TLR4-MD-2 complex to initiate signal transduction events. TLR4 signal transduction involves two major pathways. The MyD88-dependent pathway requires the MyD88 adaptor protein, causes activation of kinases ultimately leading to phosphorylation of IκB and liberation of NF-κB to cause transcription of genes. The MyD88-independent pathway also leads to NF-κB activation but with slower kinetics and activation of IRF3 which transcribes genes such as IFN-β.

reticuloendotheliosis viral oncogene (REL), p65 (REL-A), REL-B, p50 and p52. These proteins homo and heterodimerize, and once active, they associate with NF-κB binding sites in the genome to promote the transcription of genes. NF-κB proteins are usually sequestered in the cytoplasm by association with members of the inhibitors of κB (IκB) family. IκB releases NF-κB when it is phosphorylated by the IκB kinase (IKK) complex which is composed of IKK-α, IKK-β and IKK-γ proteins[462, 463]. When IκB is phosphorylated, it is degraded by the 26S proteasome. In the TLR4 pathway, activated TAK1 phosphorylates IKK, ultimately leading in NF-κB activation, translocation to the nucleus and transcriptional activation of LPS response genes. The TIR-domain-containing adaptor protein (TIRAP) is an adaptor protein similar to MyD88 and plays an essential role in TLR4 signaling [464, 465]. The role which TIRAP plays in signal transduction is unclear, but it can associate with TLR4, with MyD88 and is thought to be involved in recruitment of MyD88 to TLR4[357, 464].

MyD88-deficient mice are unresponsive to IL-1 and microbial components that activate TLR2, TLR5, TLR7 and TLR9[356, 357, 466]. In these mice, some functions are still maintained in response to LPS, albeit at slower kinetics, showing the existence of a MyD88-independent pathway[467]. The MyD88-independent pathway can induce genes such as the IFN-β and IFN-γ-inducible 10KDa protein (IP-10 or CXCL10)[468] and cause the maturation of dendritic cells[469]. The LPS activation of the MyD88-independent pathway involves activation of IFN regulatory factor 3 (IRF3)[470]. IRF3 is activated by phosphorylation, which leads to its homodimerization and localization to the nucleus, and it is involved in transcription of IFN-β and IP-10. The TRAF-family-member associated activator-binding kinase 1 (TBK1), which are related to IKK, is

believed to cause the phosphorylation of IRF3 in TLR4 signal transduction[357, 471, 472]. Two other adaptors, TIR-domain-containing adaptor protein inducing IFN-β (TRIF)[473] and TRIF-related adaptor molecule (TRAM) [474] have TIR domains and participate in the MyD88-independent pathway, although the precise mechanism is unknown. TRAM can associate with TLR4 and may form a bridge to recruit TRIF [475]. The MyD88-independent pathway also involves activation of NF-κB, but by slower kinetics than the MyD88-dependent pathway. This pathway also involves TRIF and involves physical association of the TRIF N-terminus to TRAF6[476, 477]. Recent evidence points to interaction of receptor-interacting protein 1 (RIP1) with TRIF to interact with TRAF6 and TAK1 to activate NF-κB [478, 479].

1.13.4 TLR4 regulation

Infectious agents can affect TLR4 levels. *Mycobacterium avium* can decrease TLR4 mRNA expression in macrophages[480]. Respiratory syncytial virus can upregulate TLR4 and sensitize airway epithelial cells to endotoxin[481]. Hepatitis C virus can also induce mononuclear cell TLR4 expression[482]. In areas endemic for schistosomiasis, infected children were shown to have decreased responses to LPS[444]. In contrast, in an animal model, helminth infections were shown to upregulate TLR4 levels on mucosal T cells[483]. Even bacterial products can modulate TLR4 levels. LPS itself can lead to an internalization of the TLR4/MD-2 complex[484], but has no significant effect on the level of expression in human monocytes[485]. In contrast, LPS can induce upregulation of TLR4 mRNA expression in human vascular endothelial cells and airway smooth muscle cells [440, 486]. Other bacterial products, such as toxic shock syndrome toxin-1 (TSST-1) or staphylococcal endotoxin A (SEA) can upregulate both

TLR4 and MD-2 mRNA and surface expression[485]. It would seem that most infections can lead to an upregulation of TLR4.

Cytokines can also play a role in TLR4 regulation. IL-1β and TNF-α can induce the expression of TLR4 mRNA in airway smooth muscle cells[440]. Colony stimulating factor-1 (CSF-1) can enhance LPS responsiveness of macrophages[487]. The Th1 cytokine IFN-γ can prime human monocytes and macrophages to respond to LPS by upregulating their levels of expression of TLR4[488]. It seems that Th2 cytokines and factors also have an effect on TLR4. As mentioned above, IL-4 and IL-13 can both inhibit LPS responsiveness and are protective against the development of sepsis (section 1.12.2). It has been shown that the Th2 cytokine IL-4 decreases surface expression of TLR4 on human peripheral blood monocytes[314, 489]. Protein expression was affected but the mechanism for this downregulation was not determined. The interleukin 1 receptor-like 1 (IL1RL1), is a receptor expressed by fibroblasts, mast cells and Th2 cells but not Th1 cells[490]. Triggering of IL1RL1 leads to downregulation of TLR4 in mice[491, 492], and in humans[493]. The ligand for IL1RL1 has recently been determined to be IL-33, which has a significant role to play in inducing Th2 cytokines[494, 495].

Few studies have been performed on the transcriptional regulators of TLR4. The promoter and transcription factor involved in basal transcription have been described by Rehli and colleagues[496]. Upstream of the promoter, an IFN response factor (IRF) binding site and an Ets binding site were found to be constitutively associated with PU.1 protein and IFN consensus sequence binding protein. These proteins are thought to drive basal transcription for TLR4 in myeloid cells. In another study, Ishida et al.

demonstrated that TLR4 can be downregulated by reactive oxygen species in hypoxic conditions[497]. This was caused by decreased translocation of the transcription factor activator protein 1 (AP-1) to the nucleus and binding to an AP-1 binding site upstream of the TLR4 promoter.

1.13.5 Association of TLR4 with Atopy

TLRs are part of the innate immune system and are passed on to progeny with little if no genetic change. Genetic studies have been conducted to determine whether specific single nucleotide polymorphisms in the TLR4 gene are associated with atopy. If polymorphisms are present in the gene, they may affect the expression of TLR4, the ability of TLR4 to recognize LPS or to initiate signal transduction events. Two polymorphisms, an A to G substitution at nucleotide 896 (Asp^{299} to Gly^{299}) and a C to T substitution at nucleotide 1196 (Thr^{399} to Ile^{399}) have been associated in causing reduced function of TLR4[498-500]. Although no association was seen with asthma and the Asp^{299} to Gly^{299} substitution, this polymorphism is highly associated with worse atopy severity scores[501]. Other polymorphisms have shown no link with atopic symptoms[502]. More studies need to be performed to better understand if there is a link between atopy and TLR4 polymorphisms, but these studies are difficult to perform due to the large part that environmental factors play in allergy development.

Lauener et al. have directly compared the expression of TLR4 mRNA between children from a farming environment versus children from a non-farming environment[503]. TLR2 and CD14 but not TLR4 levels were significantly higher in children raised on farms. However, in this study, children were not compared based on their level of atopy or levels of exposure to TLR ligands. In a recent study, levels of

TLR4, have been compared between allergic and non-allergic mothers and the cord blood from their children[504]. It was found that maternal allergies were associated with significantly lower levels of TLR4 mRNA and protein surface expression in maternal blood and cord blood samples. No studies have been performed to compare TLR4 levels in allergic and non-allergic adults.

1. 14 Rationale

Atopic diseases are associated with the production of Th2 cytokines and inflammatory cells, and are believed to be caused by a lack of stimulation in early life. According to the hygiene hypothesis, LPS can trigger maturation of the immune system, promoting Th1 immune responses and T regulatory immune responses to counterbalance the Th2 immune response. Previous studies have shown that LPS can cause the production of Th1 cytokines and inflammatory cell proliferation in the nasal mucosa of non-atopic children[293]. In addition, large amounts of T cells that expressed CD25 and IL-10 were present after stimulation of the nasal tissue of the children with LPS. Whether these factors or cells play a role in controlling allergen-induced responses in children needs to be determined. Children may have the plasticity to respond to LPS and to develop anti-inflammatory reactions against allergens; it is possible that adults have lost these anti-inflammatory mechanisms during the development of allergic inflammation. Th2 cytokines and STAT6 can reduce LPS induced sepsis, and long-standing Th2 inflammation in atopic adults may reduce LPS responsiveness and its potential protective effects.

1.15 Hypothesis

Firstly, it is hypothesized that LPS could prevent allergen induced Th2 inflammation within the nasal mucosa of children with atopy and this Th2 regulation is provided either by induction of Th1 cytokines or IL-10(Diagram 1.9). In addition, the responses of children would be different from adults who are allergic. Secondly, it is hypothesized that Th2 cytokines, such as IL-4, have a role in modifying the responses to LPS through the downregulation of TLR4 via STAT6(Diagram 1.10).

1.16 Aims

1.16.1 General Aims:

The general aim of this thesis was to examine the local inflammatory events that occur in the nasal mucosa of children in the presence of allergen and LPS. This is followed by a comparison between atopic adults and atopic children. If a difference is observed, then an investigation into possible mechanism would be performed.

1.16.2 Specific aims:

The specific aims of this thesis were:

1. To assess the effects of LPS on *ex vivo* allergen challenge, using explanted nasal mucosal tissue, from atopic children, and then to measure the inflammatory cell numbers and mRNA for inflammatory cytokines. If Th1 cytokines or IL-10 are produced in the mucosa, the anti-inflammatory effects of these will be evaluated by incubating the tissue with neutralizing antibodies.

2. To compare the effects of LPS in adults and children, to determine whether adults with atopy have lost protective anti-inflammatory mechanisms present in children. To determine whether LPS can cause proliferation of CD3 positive T cells in the nasal

mucosa of children, to determine if these cells also express TLR4 and can produce IL-10, and to determine if a similar population exists in atopic and non-atopic adults.

3. If levels of TLR4 are different between adults and children, or between atopic and non-atopic adults, first to determine if IL-4 can affect the LPS response and the expression of TLR4. Second, if IL-4 can affect TLR4 levels, to determine whether associated transcription factors, such as STAT6 play a role in regulating the expression of TLR4. Finally, to determine if Th2 cytokines can affect the levels of TLR4 expression in monocytes and T cells from children.

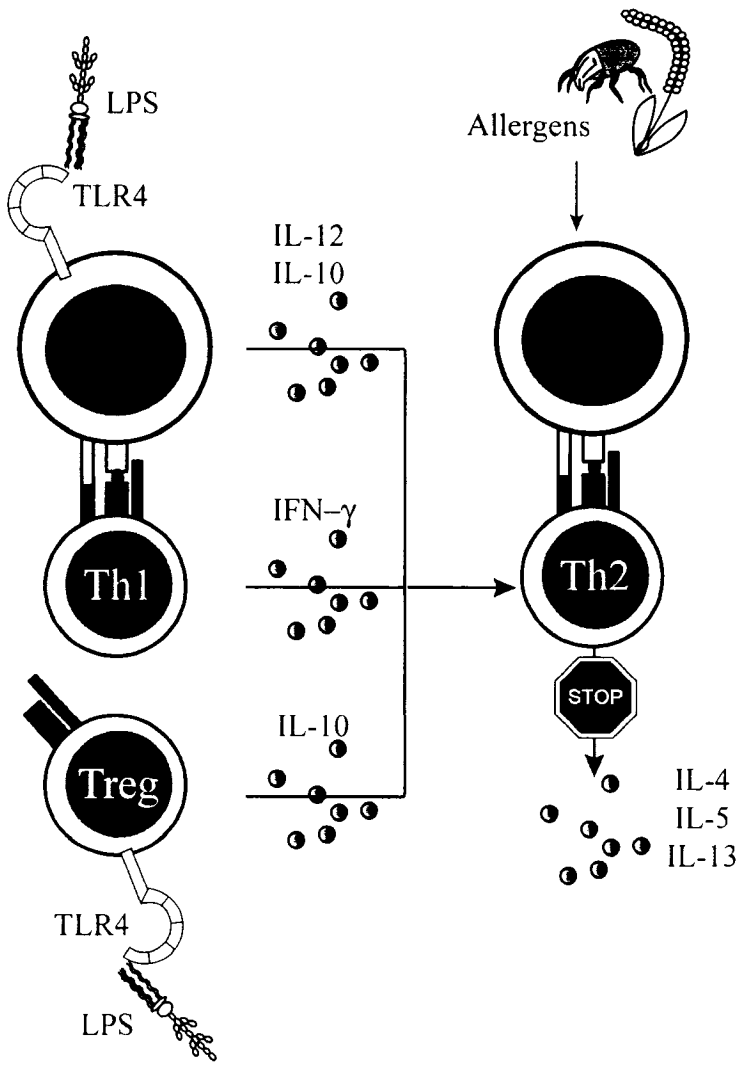

Diagram 1.9. It is hypothesized that LPS can prevent allergen-induced Th2 cytokine production by inducing the production of IFN-γ, IL-10 and IL-12. This action would be by activation of TLR4 present on antigen presenting cells, but also directly on T-regulatory (Treg) cells in the nasal mucosa of children. These mechanisms may be absent in adults.

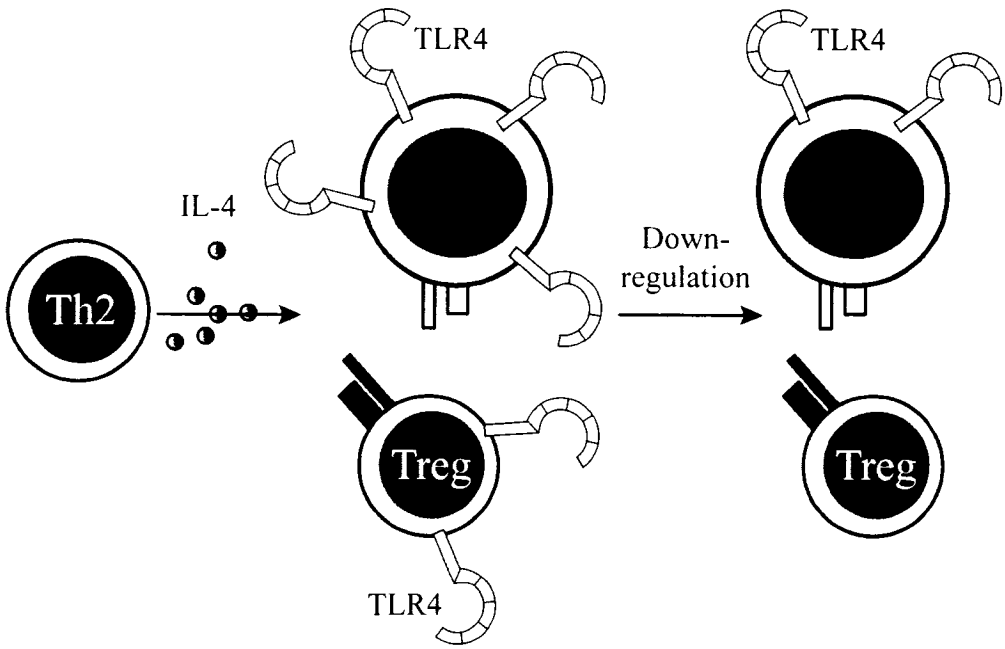

Diagram 1.10. It is hypothesized that IL-4 plays a role in modifying the response to LPS by downregulation of TLR4 via the activation of STAT6. This would affect many cell types including monocytes and T cells in children and adults.

Chapter 2: Materials and Methods

2.0 Summary of Experimental Design

To address the questions put forward in this thesis, two major studies were conducted. In the first study, human subjects were recruited, and immune responses in nasal biopsies from the subjects were investigated. Inflammatory responses were a result of *ex vivo* exposure of the nasal tissue to specific allergens and bacterial LPS. Inflammatory cell numbers and cytokine mRNA expression was measured. Tissue was also incubated with antibodies that block cytokine activity to determine how inflammatory responses are regulated. Inflammatory responses were compared between atopic and non-atopic individuals and between children and adults. Additionally, TLR4 expression in the tissue was measured and was localized to certain cell types in children. The experiments performed in the first studies include culture and stimulation of nasal explants, immunohistochemistry, *in situ* hybridization and fluorescent immunohistochemistry.

In the second study, TLR4 regulation was investigated. Firstly, an *in vitro* cell model was used to observe the effects of IL-4 on TLR4 transcription and protein expression. The experiments involved cell culture, real-time quantitative PCR (QPCR), flow cytometry and the luciferase assay. Secondly we investigated the mechanism by which IL-4 mediated these effects. These experiments involved real-time QPCR, protein overexpression, luciferase assays and chromatin immunoprecipitation. Finally, we wanted to determine whether comparable effects were seen in cells taken from the peripheral blood of children. In this last part, experiments involved density gradient centrifugation of cells, cell culture and flow cytometry. The experiments conducted in the

studies yielded enough data to allow us to answer the proposed hypothesises and to form a conclusion.

2.1 Nasal Mucosa Explant Model Experiments

2.1.1 Patient Characteristics

Twenty-two children and seventeen adults were recruited from the local hospitals. Fifteen atopic children (3.2±0.4 years old) and seven non-atopic children (3.8±0·7 years old) of both sexes were recruited from the ear, nose, and throat clinic of the Montreal Children's Hospital (Montreal, Canada). Atopy was confirmed by skin-prick testing, where drops containing common allergens including housedust mite, grass pollen, ragweed extract, and cat and dog antigens were placed on the forearm of the subject and scratched into the skin using a pointed tool. A wheal and flare reaction was deemed positive if greater than 5mm in diameter. Non-atopic children had no history of asthma or other allergic diseases and had negative wheal and flare upon skin-prick testing. Ten adults with intermittent allergic rhinitis (40.4±5.3 years old) and seven nonatopic adults (35.2±6.8) were recruited from the Notre-Dame Hospital (Montreal) and the Jewish General Hospital (Montreal). Nasal mucosal tissue (approximately 5mm in diameter) was resected from the inferior turbinate of adults and children undergoing tonsillectomy, adenoidectomy, nasal septal surgery, or sinus surgery. All children and adults were asymptomatic at the time that the samples were taken; biopsies were also done outside of the ragweed season. Individuals were excluded if they had had any viral or bacterial infections during the month before surgery or were taking anti-inflammatory medication regularly. Informed consent was obtained from parents of children or from adult participants before the procedure. The study was approved by the ethics committees of

the McGill University Health and Research Centre and the Centre hospitalier de l'Université de Montréal.

2.1.2 Tissue Culture Media

Freshly prepared bicarbonate-buffered culture medium (BCM) was used for the transport, culture and challenge of the explanted mucosal tissue. This media was specially designed to culture resected biopsies of animal airways[505, 506] and human tissue[507]. Basic BCM is composed of minimal essential medium (MEM) powder, Earl's salts, L-glutamine and sodium bicarbonate in ultra-purified distilled water. 20mL/L of 50X MEM amino acid solution, 10mL/L of 100mM sodium pyruvate, 10mL/L of 100X MEM vitamin solution, 0.1μg/mL of bovine insulin, 0.1μg/mL vitamin A, 0.1μg/mL of hydrocortisone and 50μg/mL of gentamycin were added to the basic BCM solution. After mixing, the pH of the solution was adjusted to a pH of 7.25 (physiologic pH) and then was filter sterilized with a vacuum. Each biopsy sample was placed in bicarbonate-buffered culture medium immediately after excision and transported to the Meakins-Christie Laboratories for culture. The time between removal of tissue and culture of nasal mucosa was less than 1 hour in all cases.

2.1.3 Nasal Explant Culture and Stimulation

Explanted biopsies were cut in evenly sized pieces and each piece was placed on a 30mm filtered-well insert (Millicell CM, Millipore Corp) with a 0.4μm pore diameter within a six-well culture plate containing 2mL of BCM. The filter-well insert allowed the tissue to be bathed in BCM but not to be immersed. As the nasal epithelium is normally in contact with atmospheric air, use of the filtered-well inserts more closely mimicked *in vivo* conditions. Each well contained either BCM, BCM with an allergen

specific for the individual patient's atopy, BCM with 0.1μg/mL of LPS from *Escherichia coli* serotype O26:B6 (Sigma-Aldrich) or BCM with allergen combined with LPS. The dose of LPS used was determined as the optimum dose for the nasal explant model in a previous report[293]. All tissue cultures were incubated at 37°C in a tissue and cell culture incubator in 5% CO_2 and 95% air for 24 hours.

Ten of the fifteen atopic children had a positive skin prick test for house dust mite allergen (*Dermatophagoides farinae*), two were positive to ragweed allergen (*Ambrosia artemisiifolia*) and two were positive for dog dander allergen and one to cat allergen. All atopic adults were sensitive to ragweed allergen. The amount of allergen used for stimulation was a concentration of 2000 protein nitrogen units (PNU)/mL for *D farinae*, 1000 PNU/mL for ragweed, and 10 PNU/mL for dog or cat allergen.

Depending on the method used for purification of LPS, significant levels of contaminants may be included, and these may have effects on other TLRs[508-510]. A purity of greater than 99.9% can be achieved by performing phenolchloroform-petroleum ether extraction followed by electrodialysis and conversion of the LPS into a uniform salt[511, 512]. A subset of biopsies from three atopic children and from three atopic adults was cultured with highly purified LPS from *E. coli* serotype R515 (Alexis Biochemicals). This preparation is a strong activator of TLR4 but has no effect on other TLRs, and was used to show that effects were mediated by TLR4 alone.

To examine the functional role of specific cytokines, in four experiments, samples of atopic mucosa from children were also incubated with neutralizing antibodies. Antibodies used were specific at blocking the effects of interleukin-12 (0.9μg/mL), interleukin-10 (0.03μg/mL), and IFN-γ (0.06μg/mL). The anti-IL-12 antibody was a

purified mouse IgG_1 monoclonal antibody from a mouse hybridoma and was specific for human IL-12 p35/p40 heterodimer (R&D Systems; clone 24910). The anti-IL-10 antibody was a purified mouse IgG_{2B} monoclonal antibody from a mouse hybridoma (R&D Systems; clone 23738). The anti-IFN-γ antibody was a purified mouse IgG_{2A} monoclonal antibody from a mouse hybridoma (R&D Systems; clone 25718). Concentrations for each antibody used were based on experiments performed by the manufacturer where the dose used could cause 50% neutralization in activity for each respective cytokine. Control antibodies for each blocking antibody were used, and these were matched by isotype and concentration.

2.1.4 Tissue Processing

For this part of the study, the tissue was processed for the examination of cellular protein expression and mRNA, using the techniques of immunohistochemistry (Section 2.1.13) and *in situ* hybridization(Section 2.1.7), respectively. Proliferation of cells within the tissue was measured using the 5-bromo-2'-deoxyuridine (BrdU) incorporation assay (Section 2.1.16). Co-localization of different proteins was determined by using fluorescent immunohistochemistry (Section 2.1.15). Therefore, after culturing, the tissue was processed according to standard protocols established for the respective techniques.

2.1.5 Fixation

Fixation of the tissues was performed as previously described[513]. Fixation prior to freezing of the tissue has the advantage of maximally retaining the RNA within the tissue and preserving its morphology[514]. Freshly prepared 4% paraformaldehyde (PF) dissolved in phosphate buffered saline (PBS) has proven to be the best compromise for permeability of probes, preservation and maintainenance of cellular morphology.

Before addition of PF, PBS is treated with 0.1% diethylpyrocarbonate (DEPC) for at least 1 hour at 37°C to inactivate RNAses. The DEPC-PBS is autoclaved for at least 15 minutes to inactivate DEPC, converting it to carbon dioxide and ethanol. PF powder is dissolved to a concentration of 4% in PBS at a temperature between 55-66°C in a fume hood. The solution was not allowed to rise above 60°C, as this would reduce the activity of PF. Biopsies were placed in the 4% PF solution for two hours and then washed two times with PBS sucrose (15%) solution 1 hour and overnight in a final wash. The washes removed excess PF, preventing over-fixation, and removed excess water from the tissue. This prevents the tissue from cracking during the freezing process as water would expand as it freezes.

2.1.6 Blocking and Sectioning

Multiple embedding media exists for blocking tissue. For this study, optimal cutting temperature (OCT; Tissue-Tek) media was found suitable for *in situ* hybridization, immunohistochemistry and fluorescent immunohistochemistry. When blocking the nasal biopsy, care must be given in orientating the tissue so that sections of the block contain both the epithelial and submucosal layers. The fixed tissue is placed in a droplet of OCT on a small square of cardboard paper using forceps. The paper is then submerged in isopentane cooled with liquid nitrogen. The OCT immediately freezes over the biopsy.

For *in situ* hybridization, the biopsy blocks are sectioned using a cryostat at a thickness of 5 microns. The glass slides used to collect the section were coated with poly-L-lysine (PLL) solution before use. PLL gives the slides a positive charge causing the adherence of the tissue by ionic bonding[515]. After sectioning, the slides were incubated overnight at 37°C to dehydrate the tissue and maximize adherence. It is

important that the tissue be firmly adhered to the slide due to the rigorousness of the *in situ* hybridization protocol which consists of multiple washes and enzymatic treatments. After incubation, the slides were kept at -80°C to maximally preserve the RNA until use.

For immunohistochemistry and fluorescent immunohistochemistry, biopsy blocks were also cryostat sectioned at a thickness of 5 microns. Slides were immersed in a 60/40 acetone/methanol solution for no longer than 7 minutes to fix the tissue onto the slide. PLL coated slides were not required as immunohistochemistry does not have as many washes as *in situ* hybridization and does not require enzymatic treatment. Fixation in acetone/methanol dehydrates the tissue; therefore, overnight incubation was not necessary. Slides for immunohistochemistry were stored at -20°C to preserve proteins until use.

2.1.7 Hybridization Probe Constructions

In this thesis, antisense RNA probes that were radioactively labelled were prepared to measure the cytokine mRNAs. Probes were derived from cDNA libraries or reverse transcription-PCR (RT-PCR) from native mRNA. The probe DNA were cloned into different vectors, which were replicated in bacteria, extracted with the maxiprep protocols (Qiagen), linearized with different restriction enzymes and transcribed *in vitro*. Antisense probes were used to detect the cytokine mRNAs and sense probes, transcribed from the same plasmids, were used as negative controls for the hybridization. See table 2.1 for plasmid constructs and enzymes used to generate the sense and antisense probes.

Table 2.1 Plasmid constructs and enzymes for *in situ* hybridization

Probe	Cloned fragment size	Vector	Restriction enzyme for antisense probe	Transcription enzyme for antisense probe	Restriction enzyme for sense probe	Transcription enzyme for sense probe	Source
IL-4	318	pGEM-T	Sph1	T7	EcoR1	Sp6	1
IL-5	570	pGEM-4	Xba1	Sp6	BamH1	T7	1
IL-10	880	pGEM-7	Sph1	Sp6	HindIII	T7	2
IL-13	1282	Bluescript	Not1	T7	BamH1	T3	3
IFN-γ	1213	pGEM-1	Xba1	T7	Sma1	Sp6	4
IL-12p35	N/A	pGEM-3	Sph1	T7	HincII	Sp6	5

[1] Glaxo Wellcome Biomedical Research, Geneva, Switzerland
[2] Cloned into pcDSRa from cDNA library and subcloned into pGEM-7 vector [516]
[3] Cloned into pSE1 vector from cDNA library and subcloned into Bluescript vector (ref 177)
[4] Cloned into pBR322 vector and subcloned into pGEM-1 [517]
[5] Cloned into pGEM-3 [518]

For each linearization, plasmid was digested with the restriction enzyme and appropriate salt buffer. Plasmids were digested overnight at 37°C and DNA gel electrophoresis of a sample was performed to determine if the linearization was complete. After linearization, the template DNA was extracted using phenol chloroform, followed by precipitation with 4M sodium acetate solution and ice cold ethanol at -20°C overnight or for 2 hours at -80°C. The DNA was centrifuged and vacuum dried and resuspended in DEPC-treated RNase-free water.

2.1.8 In Vitro Transcription

1.0μg of linearized plasmid was added to 10μl of 5X transcription buffer (200mM Tris-HCl; pH 7.5, 20mM $MgCl_2$, 10mM spermidine, 5mM NaCl), 100mM DTT, 25U/mL of ribonuclease inhibitor (to reduce degradation of probe transcripts), nucleotide mixture (2.5μM of rATP, rCTP, rGTP), 25 mCu of ^{35}S-rUTP and 10 units of specific RNA polymerase (see Table 2.1). The mixture was incubated for 60 minutes in a 37°C water bath. RNase-free DNase was added after incubation to degrade the plasmid. The probe

was extracted from unincorporated nucleotides by addition of 10μg/mL of tRNA, 150μL of 4M NaCl and phenol-chloroform mixture. After centrifugation at 12,000g, the aqueous phase was removed and extracted with an equal volume of chloroform to remove any residual phenol. The aqueous phase was treated with 100μl of 7M ammonium acetate and 750μl of cold absolute ethanol and the probe was precipitated at -20°C overnight. The mixture was centrifuged, the supernatant was removed and was centrifuged and vacuum dried. The pellet was resuspended in DEPC-treated RNase-free water. Incorporation of ^{35}S into the riboprobe was assessed using a β-emission counter by placing 1μl of probe into 5mL of scintillation solution to determine the total counts per minutes (cpm). Probes used for *in situ* hyrbidization were always greater than $1.0X10^6$ cpm.

2.1.9 Pre-hybridization

It is important to prepare the tissue sections for *in situ* hybridization by permeabilizing the cell membranes to allow efficient probe entry and to remove proteins which could bind non-specifically to the probe[519, 520]. Cell surface proteins were removed and membranes permeabilized by immersing the slides for 5 minutes in 0.1M glycine/PBS and then 0.3% Triton-X-100/PBS solution. Intracellular proteins were degraded by applying a solution of 1μg/mL of proteinase K in 1M Tris-HCl (pH 8.0) and 0.5M EDTA and PBS for 20 minutes at 37°C. Further permeabilization and fixation of nucleic acids was performed by immersing the slides in 4% PF-PBS solution for 5 minutes. Non-specific binding was reduced by immersing the slides in 0.25% acetic anhydride and 0.1M triethanolamide for 10 minutes at 37°C followed by immersion in 0.1M N-ethylmalamide and 0.1M iodoacetamide for 20 minutes at 37°C. Tissue was

stabilized by submersion in 50% deionizing formamide in 4X standard saline citrate (SSC) solution for 15 minutes at 37°C. An SSC (1X) solution is composed of 150mM sodium chloride and 15mM sodium citrate. Slides were finally dehydrated by immersion in increasing concentrations of ethanol (70%, 90%, and 100%) for 5 minutes each and were air dried for 2 hours.

2.1.10 Hybridization

Radiolabelled *in situ* hybridization was performed as previously described[519, 520]. Hybridization buffer (90% of total) was composed of 50% deionizing formamide, 20% Denhardt's solution (BSA, Ficoll, and polyvinyl-pyrolidone), 10% dextran sulphate, 0.5% sodium pyrophosphate, 0.5% SDS and 9% of 100mM DTT. To the hybridization buffer, $0.75X10^6$ cpm/section of riboprobe diluted in 10mM DTT (10% of total) was added. Each slide was incubated with 15μL of the complete solution and covered with dimethyldichlorosilane-coated coverslips. Slides were hybridized overnight at 42°C in a humid chamber containing SSC solution. The humid chamber prevents evaporation of the hybridization/probe solution.

2.1.11 Post-hybridization

All washes were performed by immersion the slides in a dish containing wash solutions for 20 minutes in a 42°C water bath. Slides were dipped in 4X SSC to remove the coverslip from the slides. The slides were then washed by three immersions in 4X SSC. The higher temperature and high salt concentration helps remove non-specific binding of the probes to the sample. After the washes, 15μL of 20μg/mL of RNase A dissolved in 4M NaCl, 1M Tris and 0.5M EDTA was added. The RNase A degrades any single stranded RNA, which includes unbound probe and other RNA species, but not

double-stranded probe RNA complexes. After RNase A treatment, the sections were washed with decreasing concentrations of SSC (2X, 1X, 0.5X and 0.1X). After the washes, the slides were dehydrated by immersion in increasing concentrations of ethanol (70%, 90%, 100%) containing 0.3% acetic anhydride for 10 minutes each.

2.1.12 Autoradiography

To visualize cRNA probe binding to mRNA, the slides were dipped in liquid photographic emulsion in the dark and left to dry overnight in complete darkness. Exposure of the emulsion took place in light-proof sealed boxes at 4°C for 10-15 days. Slides were developed under a red light using Kodak D-19 (Eastman Kodak) developing solution for 3 minutes and a half with gentle agitation. The reaction was stopped by placing the slides in water. Fixation of the developing solution was performed by immersing the slides in Rapid Fixer solution for 5 minutes and then rinsing with water for 20 minutes. Using a razor blade, excess emulsion was scraped away. Slides were counterstained with haematoxylin, a blue coloured basic dye that combines with acidic substances such as nucleic acids. This creates a contrast for histological analysis by light microscopy. Slides were dehydrated using increasing concentrations of ethanol (70%, 90%, and 100%) and finally two five minute immersions in xylene. Coverslips were affixed to the slides by using entellan. The slides were then viewed by light microscopy and positive hybridization is observed as a cluster of small black silver grains over counterstained cells. A positive signal was viewed significant accumulation of silver grains over cells compared to the sense control.

2.1.13 Immunohistochemistry

To detect levels of specific inflammatory cells within the tissue, immunohistochemistry was performed on the frozen sections. Sections were hydrated in Tris buffered saline (TBS) and then incubated with a commercial serum-free universal blocking solution (DAKO) for 15 minutes at room temperature. This solution is a mixture of 0.25% casein in PBS, a stabilizing protein and 15mM sodium azide. Casein is a hydrophilic protein which can reduce non-specific binding of antibodies to the tissue, and eliminates the need to use serum for blocking[521, 522]. All antibodies used for immunohistochemistry were diluted in a commercially available antibody diluent (DAKO), composed of 50mM Tris-HCl, 0.1% Tween, 15mM sodium azide and stabilizing proteins that reduce background staining. This antibody diluent is compatible with both polyclonal and monoclonal antibodies.

Table 2.2 Primaries antibodies for immunohistochemistry

Antibody	Supplier	Clone	Isotype	Species	Monoclonal/Polyclonal	Dilution
CD68	DAKO	EBM11	IgG1	mouse	Monoclonal	1/50
MBP	DAKO	BMK13	IgG1	mouse	Monoclonal	1/30
Elastase	DAKO	NP57	IgG1	mouse	Monoclonal	1/30
Tryptase	Cedarlane	AA1	IgG1	mouse	Monoclonal	1/250
CD3	DAKO	T3-4B5	IgG1	mouse	Monoclonal	1/100
CD25	DAKO	ACT-1	IgG1	mouse	Monoclonal	1/5
IL-5	DAKO	14611	IgG	mouse	Monoclonal	1/25
IL-12	Santa Cruz	24910	IgG1	mouse	Monoclonal	1/100
IL-10	DAKO	23738	IgG1	mouse	Monoclonal	1/100
IFN-g	R&D	25718	IgG2a	mouse	Monoclonal	1/50
IL-2	R&D	---	IgG	goat	Polyclonal	1/25
TLR4	Santa Cruz	---	IgG	rabbit	Polyclonal	1/10

The alkaline phosphatase anti-alkaline phosphatase (APAAP) immunohistochemistry technique was used for markers that were detected with primary antibodies derived from mice[523]. Primary antibody characteristics and concentrations

used are in Table 2.2. Sections were incubated with 45 to 60μL of the primary antibody solution and incubated in a humid chamber overnight at 4°C. The following day, slides were washed with two 5 minute washes in TBS. After the washes, 45 to 60μL of rabbit anti-mouse polyclonal IgG (DAKO, 1/10 in diluent) was added for 30 minutes at room temperature. The slides were washed twice again with TBS for 5 minutes. 45 to 60μL of a mixture of mouse monoclonal anti-alkaline phosphatase (DAKO, clone AP1B9) and calf intestinal alkaline phosphatase (DAKO, 1/60 in diluent) were added for 30 minutes at room temperature and washed twice with TBS. To visualize the APAAP complexes, slides were incubated for a maximum of 20 minutes with 0.5mg/mL of fast red chromagen (Sigma) dissolved in a 0.1M Tris solution (pH 8.2) of 0.5mM napthol AS-MX phosphate, 2% dimethylformamide, and 1mM levamisole. Alkaline phosphatase forms red precipitates over the APAAP complexes, which are visible by light microscopy. Slides were counterstained with haematoxylin, a mounting agent was added and coverslips were glued on to the slides using entellan.

IL-2 immunohistochemistry was performed by adding the primary antibody (Table 2.2) overnight at 4°C. The slides were washed and a secondary biotinylated rabbit anti-goat polyclonal antibody (DAKO) was added for 30 minutes at room temperature, and washed twice with TBS. Streptavidin-alkaline phosphatase (DAKO, diluted 1/200) was added to bind to the biotinylated secondary antibodies. After washes, the antibody complexes were stained using fast red similarly to the APAAP technique.

TLR4 immunohistochemistry was performed by adding the primary antibody (Table 2.2) overnight at 4°C. The slides were washed and a secondary biotinylated swine anti-rabbit polyclonal antibody (DAKO, diluted 1/300) was added for 30 minutes at room

temperature, and washed twice with TBS. Streptavidin-alkaline phosphatase (DAKO, diluted 1/200) was added to bind to the biotinylated secondary antibodies. After washes the antibody complexes were stained using fast red similarly to the APAAP technique.

After cover slipping, red positive cells were enumerated using light microscopy. The primary antibody was substituted with an isotype and species matched irrelevant antibody for the negative control of the immunohistochemistry.

2.1.14 Light Microscopy

For *in situ* hybridization and immunohistochemistry, positive cells were counted by two observers unaware of the experimental origin using a light microscope with 400X magnification and an eyepiece with a grid attachment. In either case, it is not possible to quantify the actual number of mRNA copies or protein molecules detected; thus, positive cells were counted, and this technique is referred to as point counting. In the airway epithelium and subepithelium, the number of positive cells was averaged from six to eight random, non-overlapping fields and expressed as the mean number of positive cells per field. Inter-observer variability was determined to be less than 10%.

2.1.15 Fluorescence Immunohistochemistry

Fluorescent immunohistochemistry was used to examine colocalization of different markers. Slides were incubated with serum-free universal blocking solution, washed and different combinations of antibodies were applied. To examine TLR4 positivity on T-cells, slides were incubated with R-phycoerythrin-labelled goat polyclonal anti-CD3 (Cedarlane, undiluted) for 30 minutes at room temperature, washed twice with TBS, and incubated with fluorescein isothiocyanate (FITC)-labelled monoclonal mouse anti-TLR4 (Santa Cruz, clone HTA125, undiluted) for 30 minutes. A similar protocol

was used to examine whether TLR4 positive cells contained IL-10. For this stain instead of anti-CD3 antibody, R-phycoerythrin-labelled mouse monoclonal anti-IL-10 (Cedarlane, clone A5-4, diluted 1/25) antibody was used. The expression of cells positive for CD4 and CD25 was examined by using allophycocyanin labelled mouse monoclonal anti-CD4 (BD Pharmingen, clone L200, diluted 1/60) followed by incubation with FITC-labelled mouse monoclonal anti-CD25 (BD Pharmingen, clone M-A251, diluted 1/50). After addition of the antibodies the slides were washed and covered with PermaFluor (Shandon) mounting media and cover slipped before analysis using a fluorescent microscope (see Section 2.1.17).

2.1.16 BrdU Cell Proliferation Assay

Proliferation of cells in response to LPS within the tissue was studied using BrdU uptake technique[524, 525]. Cell proliferation was studied by addition of BrdU (Roche Diagnostics) to the BCM or BCM with 0.1μg/mL of liposaccharide, and the tissue was cultured for 24 hours. After incubation, tissue was fixed, blocked in OCT, and sectioned as described above. The tissue was incubated with FITC-labelled mouse BrdU specific antibodies (clone BMG 6H8; Roche Diagnostics) for 30 minutes at 37°C to determine cellular incorporation of BrdU. Slides were washed three times with PBS. Slides were covered with PermaFluor mounting medium, coverslipped and evaluated using a fluorescence microscope.

2.1.17 Fluorescence Microscopy

Slides stained for fluorescent immunohistochemistry or for BrdU cell proliferation assay were viewed at 660X magnification using a fluorescence microscope. Fluorescent dyes absorb light at high energy and a certain wavelength. Some of the

energy is converted into heat, but some of the energy is released as a photon at a larger wavelength than the wavelength of the light absorbed. FITC produces green fluorescence, R-phycoerythrin produces red fluorescence and allophycocyanin produces deep red fluorescence. When the green and red fluorescence are overlapped with ImageJ software (National Institutes of Health), of co-localization of antibodies produces a yellow signal. Immunofluorescence for BrdU was semiquantified by use of ImageJ software and expressed in fluorescent cells per field.

2.1.18 Statistics

Analysis was performed using the statistical software package, Systat version 10 (Systat Software Inc.). Immunohistochemistry and *in situ* hybridization results were evaluated similarly. For each marker studied, such as cytokine mRNA or particular cell type, a two-way ANOVA model was performed with treatment (BCM, LPS, allergen and allergen plus LPS) as the factor of interest and with the participant evaluated as the blocking factor. This model shows if a difference exists between the different treatments among the participants. A post-hoc Dunnett's many-to-one test was used to compare the stimulation conditions to the baseline. This was to determine whether the control (BCM) was different from all the stimulations. If a difference was seen using the Dunnett's test, it was be determined where it lies by using a post-hoc Tukey's test. P values less than 0.05 were considered as being significant.

2.2 Cell Line Model Experiments

2.2.1 U-937 Cell Culture Media

Media for cell culture was based on Roswell Park Memorial Institute formulation 1640 (RPMI-1640) basic media used for the culture of peripheral blood leukocytes[526].

RPMI-1640 is bicarbonate-buffered, contains essential amino acids, salts, vitamins, and sugars for growth. RPMI-1640 with 2mM L-glutamine, 1.5g/L sodium bicarbonate was supplemented as per recommend by the ATCC for optimal growth of U-937 cells. The final solution contained 10% fetal calf serum, 10mM hydroxyethylpiperazine ethanesulfonic acid (HEPES) buffer, and 1.0mM sodium pyruvate. 10mL/L of 100X penicillin/streptomycin antibiotic solution was added to prevent bacterial growth during culture. The complete RPMI culture media was filter sterilized using a vacuum. U-937 cell cultures were maintained between $0.25X10^6$ cells/mL and $1.5X10^6$ cells/mL, and all experiments were conducted when the cells were in log phase of growth. Stimulations of the U-937 cells were performed first by seeding the cells into a 12 well plate with a density of $1X10^6$cells/mL. U-937 cell culture and stimulations were performed in a 37°C incubator containing 5% CO_2 and 95% air.

2.2.2 Real-time QPCR

Real-time QPCR was performed to determine TLR4 mRNA expression under various conditions. All experiments used commercially available recombinant human IL-4 (R&D Systems). U-937 cells were seeded for stimulation and incubated with concentrations of IL-4 ranging from 0.1ng/mL to 50ng/mL for 12 hours and with 10ng/mL of IL-4 for time points ranging from 6 hours to 24 hours. After incubations, the cells were centrifuged and washed with 1mL of PBS. After the wash, total RNA was extracted from the cells using the RNeasy mini kit (Qiagen). Cells were incubated in a cell lysis solution containing β-mercaptoethanol and guanidium isothiocyanate. The cells were homogenized using the needle aspiration technique and RNA was extracted using silica membrane columns. RNA on the membrane was washed and eluted using RNase

free water. RNA samples were quantified using a spectrophotometer and only RNA samples with an A_{260}/A_{280} ratio greater than 1.9 were used.

1.0μg of RNA from each sample was used for oligo deoxythymidylate (oligo dT) mediated reverse transcription using the enzyme SuperScriptII reverse transcriptase (Invitrogen). RNA was mixed with 1μL of 0.5μg/μL of oligo dT, 1μL of dNTP mix (10mM of dATP, dCTP, dTTP and dGTP) and water to a final volume of 12μL. This mixture was heated to 65°C for 5 minutes and quickly chilled on ice. The contents were centrifuged and 4μL of reverse transcription buffer (250mM Tris-HCl, pH 8.3; 375mM KCl; 15mM $MgCl_2$), 2μL of 0.1M DTT and 1μL of ribonuclease inhibitor (40units/μL) were added. This mixture was heated at 42°C for 2 minutes and 1μL (200units) of SuperScriptII RT was added. This final solution was incubated at 42°C for 50 minutes. The enzyme was inactivated by heating the solution for 15 minutes at 70°C. The cDNA could then be used as a template for PCR and was stored at -20°C until needed.

Real-time QPCR was performed using the LightCycler real-time system (version 1.2; Roche). This system consists of a thermal chamber that uses air to rapidly heat and cool the PCR samples. Samples are loaded in glass capillaries which have an optimal surface-to-volume ratio to ensure rapid equilibration between the air and the reaction components. The fluorescent dye Sybr Green I was used to quantify the PCR reactions in real-time. The excitation and emission maxima of Sybr Green I are 494 nm and 521 nm, respectively. The LightCycler system uses a blue light emitting diode, which emits light at 470 nm exciting the Sybr Green I and a fluorescence detector, which takes up light at 530nm. The LightCycler software records the data and calculates the threshold cycle (Ct) for each sample.

Commercially available PCR mixes have been developed for the LightCycler system. For this thesis, all experiments were performed using the QuantiTect Sybr Green PCR mix for a two-step RT-PCR (Qiagen). A two-step RT-PCR is performed by doing the reverse transcription reaction separate from the PCR. In a one-step reaction, the reverse transcription reaction and PCR are done in a single reaction tube. One-step reactions have less risk of contamination, but are less efficient reactions. The QuantiTect mix contains HotStarTaq DNA Polymerase, PCR buffer (Tris HCl, KCl, $(NH_4)_2SO_4$, and 5mM $MgCl_2$; pH 8.7), dNTPs, and Sybr Green I. For each reaction, 1μL of cDNA was added to 10μL of mix, 1μL of primers (20μM of each) and 8μL of nuclease-free distilled deionized water. HotStarTaq is a modified Taq DNA polymerase that is in its inactive state at room temperature. This prevents the formation of misprimed products and primer-dimers during reaction setup and the first denaturation step, leading to high PCR specificity and accurate quantification. The enzyme was activated by an incubation step of 15 minutes 95°C before the PCR.

Primer sequences for the TLR4 and for the house-keeping gene aminolevulinate delta-synthase 1 (ALAS1) are found in Table 2.3. ALAS1 is the first enzyme in the heme synthesis pathway, catalyzing the condensation of glycine with succinyl-CoA to form delta-aminolevulinic acid[527]. ALAS1 is expressed constitutively in all tissues of the body. The expression of ALAS1 did not vary significantly between samples of U-937 cDNA, validating its use as a house-keeping gene for normalization of expression of other genes. For both PCR reactions, identical conditions were used. After 15 minutes of incubation at 95°C, 35 cycles of amplification were performed. Each cycle consisted of 15 seconds at 94°C to denature the DNA, 30 seconds at 56°C to cause annealing of the

primers to the DNA, and 20 seconds at 72°C to allow extension of the primers by the polymerase. After extension, the LightCycler activated the blue LED and detected levels of Sybr Green I fluorescence.

DNA standards for the real-time QPCR assay were constructed by first performing a PCR using cDNA from the U-937, and running a DNA gel electrophoresis of the products. The bands were cut out with a razor and purified by using the QIAquick Gel Extraction kit (Qiagen). Extracted bands were incubated at 50°C to melt the agarose in a high salt solution containing guanidine thiocyanate. DNA was bound to the gel membrane, was washed and eluted in 10mM Tris-HCl. After purification, the PCR products were ligated into the plasmid pGEM-T (Promega) by TA-cloning using T4 DNA ligase. 3μL of PCR product was added to 5μL of ligation buffer (60mM Tris-HCl at pH 7.8, 20mM $MgCl_2$, 20mM DTT, 2mM ATP, and 10% polyethylene glycol), 1μL of the plasmid pGEM-T (50ng/μl), and 1μL of T4 DNA ligase (3 Weiss units/μl). The ligation took place overnight at 4°C. After the ligation, 5μL of the plasmid solution was mixed with 25μL of MAX Efficiency DH5α Competent cells (Invitrogen). The bacteria and plasmid were incubated on ice together for 30 minutes. The bacteria were heat-shocked for 45 seconds in a 42°C water bath, and quickly put on ice again for 5 minutes. 200μL of SOC medium (0.5% yeast extract, 2.0% tryptone, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, and 10mM $MgSO_4$) was added and the bacteria were placed in a 37°C shaker incubator at 225rpm for an hour. The bacterial suspension was poured and spread onto a solid agar plate containing LB/ampicillin medium (0.5% yeast extract, 1.0% tryptone, 10mM NaCl, 100μg/mL ampicillin, and 1.5% agar). 30 minutes before plating the bacteria, 100μl of 100mM IPTG and 20μl of 50mg/mL X-Gal was spread over the

LB/ampicillin plate for blue and white selection. The bacteria were cultured overnight and white colonies were selected and placed in 3mLs of LB/ampicillin liquid broth overnight, in a 37°C shaker incubator at 300rpm. Purification of plasmid DNA was performed using the QIAprep Spin Miniprep kit (Qiagen). DNA was washed and eluted in 50μL of 10mM Tris HCl (pH 8.5). 2μL of the plasmid preparations were digested with restriction enzymes and fragments were analyzed by DNA gel electrophoresis to determine if the PCR products were cloned into pGEM-T. 50μL of the miniprep cultures containing positive clones was added to 150mL of LB/ampicillin liquid broth overnight, in a 37°C shaker incubator at 300rpm. Plasmid DNA was extracted using the HiSpeed Plasmid Maxi kit (Qiagen). Plasmid DNA was quantified with a spectrophotometer and copy numbers were calculated using the following formula:

$$\textbf{(Amount in copies/μl)} = \frac{\textbf{(}N_A\textbf{) X (DNA sample concentration in g/μl)}}{\textbf{(Molecular weight in g/mole)}}$$

N_A is Avogadro's number (6.022×10^{23} copies/mole). The molecular weight of double-stranded DNA can be obtained by multiplying the number of base pairs of the DNA by the average molecular weight per base (660g/mole/bp). The plasmids were also sequenced by using high throughput terminal dideoxylnucleotide fluorescent capillary electrophoresis by the McGill University and Genome Québec Innovation Centre using T7 and Sp6 primers and enzymes. The standard curve was produced by making five different log dilutions ($5X10^6$ copies of plasmid to $5X10^2$ copies of plasmid) of purified pGEM-T plasmid with the cloned insert; 1μL of each dilution of plasmid DNA was added instead of cDNA to the Quantitect mix with the primers. Plasmid dilutions for the standard curve and a negative control, containing every reagent except DNA, were run alongside of each set of U-937 cDNA samples in the LightCycler system. Absolute

quantification of both TLR4 and a house-keeping gene ALAS1 was performed by comparing the Ct values of the samples to the standard curve. The copy numbers of TLR4 were normalized with the copy numbers of the ALAS1.

Table 2.3 Primer sequences for real-time QPCR

PCR Target	Primer name	Sequence	Tm	Product size (bp)	Genome Position
TLR4	TLR4 Primer 1	CAACCAAGAACCTGGACCTG	60	151	Exon 2
	TLR4 Primer 2	GAGAGGTGGCTTAGGCTCTG	59		Exon 3
ALAS1	ALAS1 Primer 1	CCCATGAGTTTGGAGCAATC	60	156	Exon 9
	ALAS1 Primer 2	ACCCTCCAACACAACCAAAG	60		Exon 10

2.2.3 Flow Cytometry

Surface expression of TLR4 by U-937 cells was determined by using flow cytometry. After culturing the U-937 cells with and without IL-4 the cells were prepared for flow cytometry. Cells were washed by adding 1mL of staining buffer (PBS and 1% fetal calf serum), centrifuging for 5 minutes at 300g and removing the staining buffer. $1X10^6$ U-937 cells were used per staining condition. All antibodies used were diluted in staining buffer. Cell pellets were resuspended in 100μL of staining buffer containing $2.5\mu g/1X10^6$ cells of mouse IgG_{2A} monoclonal anti-human TLR4 antibody (BD Pharmingen; clone HTA125). The cells were incubated with the primary antibody at 4°C for 30 minutes. The cells were washed twice with staining buffer and the cell pellets were resuspended in 100μL of staining buffer containing $0.25\mu g/1X10^6$ cells of goat polyclonal FITC-labelled anti-mouse IgG_{2A} antibodies (Santa Cruz). Again, cells were incubated at 4°C for 30 minutes. The cells were washed twice and resuspended in 600μL of staining buffer and were ready for flow cytometry analysis, using a FACSCalibur flow cytometry system and CellQuest Software (BD Biosciences). FITC emits green fluorescence and is detected by the fluorescent 1 (FL1) sensor of the FACSCalibur.

Relative TLR4 surface expression on the cells was determined using the mean fluorescent intensity (MFI) and substracting the MFI of the background. Negative controls for the experiment included cells that were not stained with any antibodies and cells that were incubated with the secondary antibody but not the first antibody.

2.2.4 NF-κB luciferase reporter assay

pNF-κB Luc (BD Biosciences) is a reporter plasmid containing four tandem copies of the NF-κB consensus sequence. Large amounts of endotoxin free purified preparations of pNF-κB Luc plasmid were made using EndoFree Plasmid Maxi kit (Qiagen). Endotoxin levels were assessed to be less than 0.125 EU/mL by the limulus amebocyte lysate (LAL) gel clot assay (Biowhittaker). U-937 cells were washed with sterile PBS and $1X10^6$ cells were seeded into a 12 well culture plate. Each transfection mix was prepared by mixing 1.0μg of pNF-κB Luc and 0.03μg of *Renilla* luciferase control vector with serum-free and additive free RPMI-1640, for a total of 75μL. 6μL of Superfect transfection agent (Qiagen) was added and was mixed in by vortexing, and the tubes were centrifuged to collect everything at the bottom of the tube. Superfect transfection agent is a solution of polyamidoamine dendrimers which provides high transfection efficiencies in a broad number of cell lines. The tranfection mix was incubated at 10 minutes at room temperature before addition of 200μL of cell growth medium, containing serum and antibiotics. This solution was pipetted up and down twice to mix and immediately added drop-wise onto the cells in the wells. The cells were incubated for 24 hours with the transfection mixture in a 37°C incubator.

After the transfections, the cells were stimulated with or without 10ng/mL of IL-4 for 24 hours, and then stimulated with 0.25μg/mL of LPS from *E.coli* 0111:B4 for 6

hours. The cells were collected from the 12 well plates and placed in microcentrifuge tubes, pelleted by centrifugation for 5 minutes at 300g, and washed twice with 1mL of PBS. 250μL of lysis buffer (Promega; 25mM Tris-phosphate pH 7.6, 2mM DTT, 2mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100) was added to the cell pellets and was mixed for 15 minutes on a rocking platform. Cells in this lysis solution are ready for the luciferase assay and can be stored at -20°C until use.

The luciferase assay was performed by using the Dual-luciferase reporter assay system (Promega) and a Lumat LB9507 luminometer (Berthold). Reagent formulations for the dual-luciferase reporter assay are proprietary and specially designed for this assay. For the assay 100μL of LARII solution is aliquoted into an optically clear polystyrene tube. 20μL of cell lysate is added to the LARII and mixed by pipetting 2 to 3 times. The tube was placed in the luminometer for a 10 second acquisition of light production by the firefly luciferase reaction. 100μL of Stop & Glo Reagent was then added and the tube was vortexed for 10 seconds to quench the firefly luciferase. The tube was placed again in the luminometer for a 10 second acquisition of light production by the *Renilla* luciferase. Relative light units (RLUs) were calculated by normalizing firefly luciferase activity against *Renilla* luciferase activity. Negative controls for the reaction included cells that contained no plasmids in the transfection mix and cells that were transfected but were not stimulated with IL-4 or LPS.

2.2.5 Luciferase Assay: TLR4 Upstream region

Transcriptional regulation of TLR4 by IL-4 was measured by a luciferase reporter assay. 4.3-kilobases of U-937 genomic DNA containing the promoter and the region upstream of the TLR4 gene was amplified using the TaqPlus Precision PCR System

(Stratagene) similar to previously described method[496]. The TaqPlus Precision PCR System is composed of a specially optimized PCR buffer for long-range PCR reactions and a mixture of Pfu and Taq DNA polymerases, which catalyze highly efficient addition of nucleotides with extremely low error rates. A mixture was prepared of 10μL of 10X TaqPlus Precision buffer, 0.8 μL of dNTPs (25mM of dATP, dCTP, dTTP and dGTP), 1μL of genomic DNA (250 ng/μl), 2.5μL of each primer (Table 2.4; 100 ng/μl) and 1μL of TaqPlus Precision polymerase mixture (5 U/μL). The mixture was vortexed to mix and centrifuged briefly to collect all drops to the bottom of the tube. The PCR mixture was overlayed with mineral oil to avoid evaporation and placed in a thermal cycler. The PCR tubes were heated at 94°C for 1 minute. 35 cycles were performed of the following steps: 94°C for 1 minute, 57°C for 1 minute and 72°C for 5 minutes. PCR products were separated by DNA gel electrophoresis and the appropriate band was excised and purified with the QIAquick Gel Extraction kit.

The TLR4 gene possesses a natural NcoI site and the 3' primer was designed to use this site for cloning. The 5' primer used for the PCR added an SstI at the 5' end of the PCR product for directional cloning in the luciferase reporter plasmid pGL3-Basic (Promega). Both pGL3 and PCR product were digested overnight with SstI and NcoI in water and One-Phor-All proprietary potassium acetate buffer (GE Healthcare). The digestion products were run on a DNA agarose gel and purified with QIAquick Gel Extraction kit. 3μL of digested PCR product was mixed with 1μL of digested pGL3, 4μL of 5X ligase reaction buffer (250mM Tris-HCl, pH 7.6; 50mM $MgCl_2$; 5mM ATP; 5mM DTT; and 25% polyethylene glycol), 1μL of T4 DNA ligase and 11μL of distilled water. This solution was incubated overnight at 4°C. 5μL of the ligation reaction was

transformed into MAX Efficiency DH5α Competent cells by heat shock (section 2.2.2). pGL3 has the ampicillin resistance gene, but it does not contain the β-galactosidase gene; therefore, bacteria transformed with the vector was grown on agar plates containing LB/ampicillin media, but a blue and white selection was not performed. Colonies that grew on the agar plate were cultured for plasmid minipreps (section 2.2.2). Restriction mapping was used to confirm that the DNA sequence cloned in the correct orientation. EndoFree maxipreps (Qiagen) was performed on positive cultures to prepare large amounts of endotoxin free purified plasmids. Endotoxin levels were assessed to be less than 0.125 EU/mL by the Limulus Amebocyte lysate gel clot assay. Samples were sequenced by the McGill University and Genome Québec Innovation Centre using primers used for the PCR and primers designed at 600 base pair intervals, to sequence the entire insert.

Table 2.4 Primer sequences for cloning

PCR Product	Primer name	Sequence	Product size (bp)	Restriction Enzyme
TLR4-N	TLR4-N Primer 1	5'-CTGAGCTCCACATTCTGCAGTA AACTTGGAGGC -3'	4371	SstI
	TLR4-N Primer 2	5'-CACGCAGGAGAGGAAGGCCA TGGCTG-3'		NcoI
TLR4-N2	TLR4-N2 Primer 1	5'-CTGAGCTCTATCAAGCGGTTT CCTCTCC-3'	3450	SstI
	TLR4-N2 Primer 2	5'-CACGCAGGAGAGGAAGGCCA TGGCTG-3'		NcoI
TLR4-N3	TLR4-N3 Primer 1	5'- CTGAGCTCCTAGGCCGTGGGA TATGTTC -3'	3072	SstI
	TLR4-N3 Primer 2	5'- CACGCAGGAGAGGAAGGCCA TGGCTG -3'		NcoI
STAT6	STAT6 Primer 1	5'-GGGGTACCCCGGCACGACGGGA AG-3'	3324	Kpn1
	STAT6 Primer 2	5'-GTTTCTAGAAACCACATGTCA G-3'		Xba1

U-937 cells were seeded into 12 well plates and were transfected with 6μL of Superfect, 1μg of TLR4-N plasmid and 0.03μg of *Renilla* control vector. The cells were transfected for 24 hours and stimulated for different time points with 10ng/mL of IL-4 before performing the luciferase assay. A Dual-luciferase reporter assay was performed similarly to the NF-κB luciferase reporter assay (Section 2.2.4). Negative controls included cells transfected with TLR4-N plasmid but were not stimulated with IL-4, as well as untransfected cells.

2.2.6 Luciferase Assay: STAT6

The source of STAT6 was a pVL1393 baculovirus vector containing the full length human STAT6 (Dr James Ihle, Saint Jude Children's Research Hospital). The STAT6 insert in the vector was amplified using the TaqPlus Precision PCR System in a similar reaction as was used to amplify TLR4-N fragment (section 2.2.5). The primers used added KpnI and XbaI restriction endonuclease sites to the end of the PCR product (Table 2.4). The PCR product was purified from gel and digested overnight with KpnI and XbaI in water and One-Phor-All buffer. The plasmid pcDNA3.1/Hygro (+) (Invitrogen) was also cleaved with KpnI and XbaI. The resulting fragments were extracted from gel and ligated together using T4 DNA ligase (section 2.2.5). Ligated product was transformed into MAX Efficiency DH5α Competent cells and plasmids were extracted using EndoFree maxipreps. Plasmids were sequenced and endotoxin levels were below 0.125 EU/mL by the LAL gel clot assay. The pcDNA-STAT6 plasmid contains a start and stop codon as well as a Kozak sequence necessary for translation[528, 529]. The pcDNA-STAT6 plasmid contains a human cytomegalovirus immediate-early promoter/enhancer sequence that permits high-level expression of STAT6[530]. It also

contains a bovine growth hormone polyadenylation signal to promote increased transcription termination and polyadenylation of the mRNA[531]. There is also β-lactamase gene to select for bacteria that have been transformed with the plasmid.

The ability of the transfected pcDNA-STAT6 plasmid to cause overexpression of STAT6 in U-937 cells was determined by Western blotting technique. $1X10^6$ U-937 cells were transfected with 6μL of Superfect and several concentrations of pcDNA-STAT6 ranging from 0.5μg to 2.0μg for 24 hours. Cells were centrifuged and incubated for 1 hour at 4°C with 1mL of lysis buffer containing 20mM Tris, pH 7.5, 150mM NaCl, 2mM EDTA, and 1% of the non-ionic detergent NP-40. In addition, the lysis buffer contained a complete Protease Inhibitor Cocktail Mini tablet (Roche Applied Sciences) to prevent proteases from digesting the extracts. 30μg of proteins from the cell lysates were separated out by SDS-PAGE with a Laemmli glycine based discontinous buffering system. Lysates were solubilized in hot 2X SDS gel sample buffer and heated in a boiling water bath for 5 minutes before loading into the wells of the vertical polyacrylamide gel apparatus. Proteins were transferred to a PVDF membrane for immunoblotting. The membrane was first incubated for 1 hour with blocking solution containing 3% BSA in TBS (50mM Tris, pH 7.4, 0.5M NaCl and 0.05% Tween 20). The membrane was incubated overnight at 4°C with agitation in TBS containing 1% BSA and 1/2000 dilution of rabbit anti-STAT6 polyclonal antibodies (R&D Systems). The membrane was repeatably washed with TBS and incubated for one hour with TBS containing 1% BSA and a secondary goat anti-rabbit polyclonal antibody linked to horseradish peroxidase (HRP; R&D Systems; dilute 1/2500). The membrane was washed again with TBS and bands were visualized using the proprietary reagent mixture Enhanced

Chemiluminescence (ECL) Plus (Amersham). The high intensity chemiluminescence signal was detected by incubating the membrane with autoradiography photographic film for 15 seconds in an x-ray film cassette. The film was then immediatedly developed with an automatic film developer to produce a picture of the STAT6 bands. Antibodies were stripped off by submerging the PVDF membrane in 100mM 2-mercaptoethanol, 2% SDS and 62.5mM Tris-HCl (pH 6.7) for 30 minutes at 50°C. The membrane was washed two times for 10 minutes in TBS. The membrane was incubated for 1 hour with blocking solution and incubated for 1 hour with mouse monoclonal anti-human β-actin antibodies (R&D clone AC-15; diluted 1/500) in TBS containing 1% BSA. The membranes were washed twice with TBS and then incubated for 1 hour with a secondary rabbit anti-mouse polyclonal antibody linked to horseradish peroxidase (HRP; R&D; diluted 1/10,000). The membrane was washed again and the bands were visualized by using ECL Plus and photographic film. Bands of proteins for both STAT6 and β-actin were measured by densitometry, and the expression of STAT6 was normalized to expression of β-actin. Prestained protein standards were used to determine the size of the protein bands for the sample.

A luciferase assay was performed using an optimized dose of pcDNA-STAT6 and TLR4-N reporter plasmid. The plasmid pcDNA3.1 without any cloned insert was used as a negative control for the experiment and was also transfected with the TLR4-N reporter plasmid. $1X10^6$ U-937 cells were transfected with 6μL of Superfect, 2μg of pcDNA-STAT6 or pcDNA 3.1, 1.0μg of TLR4-N reporter vector and 0.03μg of *Renilla* control vector. The cells were rested for 24 hours and stimulated with or without 10ng/mL of IL-4. Several time points were taken where cells were harvested and lysates were assayed

for firefly and *Renilla* luciferase activity using the Dual Luciferase Reporter Assay System as previously described in section 2.2.4.

2.2.7 Tyrphostin AG 490 stimulation

$1X10^6$ U-937 cells were incubated with 25μM of AG 490 (2-cyano-3-(3,4-dihydroxyphenyl)-N-(benzyl)-2-propenamide; Sigma) dissolved in DMSO (stock 10mM) for 16 hours as previously described[532]. 10ng/mL of IL-4 was then added for 12 hours. After stimulations, the cells were centrifuged and the RNA was extracted for reverse transcription and TLR4 and ALAS1 mRNA levels were quantified by real-time QPCR (section 2.2.2). All samples were compared to baseline expression. Negative controls for the experiments included cells stimulated with the vehicle DMSO and AG 490 without IL-4 stimulation.

2.2.8 Truncations of TLR4-N Reporter Plasmid

The luciferase assay was used to determine where IL-4 responsive elements were positioned in the upstream region of the TLR4 gene. This was performed by cloning progressively shorter inserts of the upstream region of the TLR4 gene into the plasmid vector pGL3-Basic. The upstream region of TLR4 was also analyzed by MatInspector Release professional 6.0 (www.genomatix.de) to determine potential STAT6 binding sites in the upstream region of TLR4.

The TLR4-N2 (3.4 kilobases) and TLR4-N3 (3.0 kilobases) plasmids were made similarly to the TLR4-N plasmid using primers and the TaqPlus Precision PCR System (section 2.2.5). The primers contained restriction endonuclease cleavage sites for cloning into pGL3-Basic(Table 2.4).

Digestion of TLR4-N with HindIII can yields several fragments[496]. One of these contains 1.0 kilobases of the upstream region of TLR4 gene attached to a full pGL3. This fragment was extracted from a DNA gel electrophoresis product and was ligated using T4 DNA polymerase as described in section 2.2.5. The ligation product was transformed into bacteria and the resulting plasmid was named TLR4-H. Similarly, digestion of TLR4-N with PstI and ligation yielded TLR4-P which contains 300 base pairs of the upstream region of TLR4[496].

MAX Efficiency DH5α Competent cells were transformed with each of the constructs and plasmids were extracted using EndoFree maxipreps. Endotoxin levels were assessed to be less than 0.125 EU/mL by the LAL gel clot assay (Biowhittaker) and a sample of all the plasmids were sent for sequencing to confirm the construct sequence. U-937 cells were seeded into 12 well plates and were transfected with 6μL of Superfect, 1.0μg of each plasmid and 0.03μg of *Renilla* control vector. The cells were transfected for 24 hours and stimulated for 12 hours with 10ng/mL of IL-4 before performing the luciferase assay (section 2.2.4). Negative controls included cells transfected with the plasmids but were not stimulated with IL-4, as well as untransfected cells, and cells transfected with a pGL3 plasmid containing no insert (pGL3 Basic).

2.2.9 STAT6 Chromatin Immunoprecipitation assay

STAT6 chromatin immunoprecipitation (ChIP) was performed using the EZ-ChIP kit (Upstate). The ChIP allows the precipitation of DNA complexed to STAT6 bound to it by STAT6-specific antibodies. PCR was used to determine what DNA was precipitated. $20X10^6$ cells were grown with or without IL-4 (10ng/mL) for 6 hours. Cells were centrifuged and resuspended in 10mL of RPMI-1640 in a culture dish. 540μL

of a freshly prepared 18.5% formaldehyde solution (final concentration 1%) was added to the cells and incubated at room temperature for 10 minutes. 1mL of 1.25M glycine was added to dish, swirled to mix, and incubated for 5 minutes to quench the formaldehyde. Cells were centrifuged for 5 minutes at 300g and were resupended in 10mL of cold PBS to wash. The wash was repeated and the cells were resuspended in 1mL of cold PBS containing 5μL of Protease Inhibitor Cocktail II (Upstate). Cells were spun for 5 minutes at 4°C at 700g. 1mL of SDS lysis buffer (1% SDS, 10mM EDTA, 50mM Tris, pH 8.1) was added to the pellet also containing 5μL of Protease Inhibitor Cocktail II.

The samples in the SDS lysis buffer were put on ice and each tube was sonicated for two 15 second pulses with an intensity of 5 using a Fisher model 60 sonic dismembrator. Between pulses, the samples were cooled on ice for 30 seconds. This sonication protocol was optimized to break apart the DNA of the genome into fragments ranging from 200 to 1000 base pairs, which facilitates the immunoprecipitation. The sonicated lysates were centrifuged at 4°C for 10 minutes at 12,000g. 100μL of the supernatant was aliquoted into microfuge tubes.

900μL of Chromatin Immunoprecipitation Dilution buffer (0.01% SDS; 1.1% Triton X-100; 1.2mM EDTA; 16.7mM Tris-HCl, pH 8.1; and 167mM NaCl) containing 5μL of Protease Inhibitor Cocktail II was added to each 100μL aliquot needed for the experiment. 60μL of Protein G Agarose beads (Upstate) was added to each sample to preclear the samples. The bead mixture is composed of 50% gel slurry containing agarose beads, 400μg/mL of sonicated salmon sperm DNA, 1mg/mL of BSA, 3mg/mL of recombinant protein G, 50% TE buffer (10mM Tris-HCl, pH 8.0; and 1mM EDTA) and 0.05% sodium azide. The mixture of Protein G agarose beads and the sonicated lysates

was incubated for 1 hour at 4°C on an agitator. The beads were pelleted by centrifuging the samples for 1 minute at 3000g. Immunoprecipitating antibodies were added to each sample and incubated overnight with agitation at 4°C. 1μg of mouse monoclonal antibodies specific for human RNA polymerase II (Upstate; clone CTD448) was added to samples to serve as a positive control for the experiment. 1μg of normal mouse IgG (Upstate) was used as a negative control for the RNA polymerase II immunoprecipitation. 2.5μg of rabbit polyclonal antibodies specific for human STAT6 (R&D Systems) was added to samples for immunoprecipitation of STAT6-DNA complexes. 2.5μg of normal rabbit serum (R&D Systems) was used as a negative control for the STAT6 immunoprecipitation.

60μL of protein G agarose beads were added to the samples and incubated for 1 hour at 4°C with rotation to make the antibody complexes bind to the Protein G. The complexes were precipitated by centrifuging for 1 minute at 3000g. The supernatant was removed and the pellet was repeatably washed with 1mL of cold buffers to remove non-specific binding. After each buffer was added, the mixture was incubated for 5 minutes on a rotating platform at 4°C followed by a 1 minute centrifugation at 3000g and removal of the supernatant. The first buffer was a low salt immune complex buffer (Upstate) composed of 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl (pH 8.1) and 150mM NaCl. The second buffer was a high salt immune complex buffer (Upstate) composed of 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl (pH 8.1) and 500mM NaCl. The next buffer was a LiCl immune complex buffer (Upstate) composed of 0.25M LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid (sodium salt), 1mM EDTA, and 10mM Tris (pH 8.1). The pellet was washed with TE buffer two times.

The samples were resuspended in 100μL elution buffer (1% SDS and 100mM $NaHCO_3$) and incubated for 15 minutes. The agarose was pelleted by centrifugation for 1 minute at 3000g and the supernatant was collected into a new tube. The pellet was resuspended again in 100μL of elution buffer and incubated for 15 minutes. The supernatant was collected and combined to the first one.

To all tubes containing elution buffer, 8μL of 5M NaCl was added and incubated overnight at 65°C to reverse the DNA-protein crosslinks. 1μL of 10mg/mL of RNase A (in sterile water) was added and incubated for 30 minutes at 37°C. Finally, 4μL of 0.5M of EDTA, 8μL of 1M Tris-HCl (pH 6.5) and 1μL of 10mg/mL of proteinase K were added to the samples which were incubated at 45°C for 2 hours.

DNA was purified from the samples by using silica membrane DNA extraction columns (Upstate) and eluted in 50μL of 10mM Tris-HCl. After purification of the DNA, real-time QPCR was used to quantify the binding of STAT6 and RNA polymerase II to specific regions of DNA by using different primers. The ΔΔCt method was used to quantify the extracted fragments with the reference condition being cells incubated without IL-4 before the ChIP[533]. The reference for the ΔΔCt method was the fragment of the promoter of the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is known to bind RNA polymerase II. Two sets of primers were designed to amplify the regions containing potential STAT6 binding sites determined by MatInspector (Table 2.5). Negative controls for the experiment include ChIP using antibodies that do not bind to STAT6, and cells that were not stimulated with IL-4.

Table 2.5 Primer sequences for STAT6 ChIP assay

PCR Target	Primer name	Sequence	Genome Position
STAT6 site 1	STAT6-1 Primer 1	5'- ACACATGGTCTGCCT TCTGG -3'	STAT 6 site 3,550 base pairs upstream from start codon of TLR4
	STAT6-1 Primer 2	5'- CCAGGGAAGTCCTTT CAACT -3'	
STAT6 site 2	STAT6-2 Primer 1	5'- CTGGCTTGAAATGAC CCACA -3'	STAT6 site 3240 base pairs upstream from start codon of TLR4
	STAT6-1 Primer 2	5'-TGATTCTTCATCCTG CTGTTG -3'	
GAPDH promoter	GAPDH Primer 1	5'- TACTAGCGGTTTTAC GGGCG-3'	RNA Polymerase II site that is about 350 base pairs upstream from the start codon of GAPDH
	GAPDH Primer 2	5'-TCGAACAGGAGGAGC AGAGAGCGA-3'	

2.2.10 Statistics

As the same cell line was used for all experiments, a single factor ANOVA was performed using Systat. A post-hoc Dunnett's many-to-one test was used to compare the stimulation conditions to the baseline. This was to determine whether the control (unstimulated) is different from all the stimulations. If a difference was seen using the Dunnett's test, it was determined where it lies by using a post-hoc Tukey's test. A P value less than 0.05 was considered as being significant.

2.3 Peripheral Blood Experiments

2.3.1 Patient Characteristics

Five children (9.8±3.3 years old) were recruited from the Patient Testing Center (PTC) of the Montreal Children's Hospital. Children were in the hospital for other tests that required blood draw and were recruited on site to participate in this study. Children were screened by using a questionnaire filled out by the child and parent. Informed consent was obtained from the parents. Patients were of both sexes (three female; two male). Individuals were excluded if they had had any viral or bacterial infections during

the month or were taking anti-inflammatory medication regularly or had serious blood disorders. The study was approved by the Research Ethics Board of the Montreal Children's Hospital.

Intravenous blood was drawn by an experienced blood technician or nurse from the PTC. 5mLs of blood were collected into a sterile tube containing sodium heparin to prevent the blood from clotting. Blood was kept at room temperature and was brought to the Meakins Christie Laboratories for analysis.

2.3.2 Ficoll Gradient Centrifugation and Culture

Blood was taken out of the heparin tubes and room temperature PBS (pH 7.2) was added to a volume of 30mL. 15mL of Ficoll-Paque PLUS (Amersham) was aliquoted to 50mL tubes. The diluted blood was carefully layered over the Ficoll. The samples were centrifuged for 30 minutes at room temperature at 1000g. The upper layer containing plasma and platelets was drawn off using a sterile pipette but leaving the PBMC layer undisturbed at the interface of the Ficoll layer. The PBMC layer was carefully collected without removing excess Ficoll. The PBMC layer was washed twice with 50mL of PBS, centrifuging 10 minutes at 300g each time. The cell pellet was resuspended in 1mL of RPMI-1640 with added factors (section 2.2.1) and a cell count was performed. $1X10^6$cells/mL were dispensed in a 6 well culture dish and stimulated with or without 10ng/mL of IL-4.

2.3.3 Flow Cytometry on PBMCs

After 24 hour culture the PBMCs were collected from the culture dish and washed with cold PBS. PBMCs were resuspended in PBS at a concentration of $0.5X10^6$cells/mL and 1mL aliquots were used for each condition of the flow cytometry. The cells were

centrifuged again and resuspended with 1mL of PBS containing 20μg of human IgGs (Bayer Inc.; stock 50mg/mL of human IgG and 10% maltose at a pH 4.2) to block non-specific binding antibodies to cells. The cells were incubated with the IgGs for 30 minutes at 4°C. Cells were centrifuged for 5 minutes at 300g and the supernatant was removed. Cell pellets were resuspended in 95μL of cold staining buffer with 5μL (0.05 mg/mL) of the first antibody and were incubated for 30 minutes at 4°C. Antibodies used included: mouse monoclonal antibody specific for human TLR4 (Serotec; clone HTA125) conjugated to the fluorescent dye Alexa Fluor 488, purified mouse IgG_{2A} (Serotec; clone MRC OX-34) conjugated with Alexa Fluor 488 was used as an isotype control, mouse monoclonal antibody specific for human CD3ε (R&D Systems; clone UCHT1) which was conjugated to FITC and purified mouse IgG1 (R&D Systems; clone MOPC-21) conjugated with FITC used as an isotype control. The cells were washed twice with cold staining buffer by centrifugation for 5 minutes at 300g. The cells were then incubated for 30 minutes at 4°C with 5μL of the second antibody diluted with 95μL of staining buffer. The second antibody used was a mouse monoclonal antibody specific for human CD4 (R&D Systems; clone RPA-T4) conjugated to the fluorescent dye allophycocyanin (APC) and its isotype control was a mouse IgG1 (R&D Systems; clone MOPC-21). The cells were washed twice with 1mL of cold staining buffer and the pellet was resuspended in 500μL of staining buffer before proceeding to analysis using the FACSCalibur flow cytometry system and CellQuest Software (BD Biosciences). Both the Alexa Fluor 488 and FITC emit green light when excited by an argon laser and their fluorescence is captured by the FL1 sensor of the flow cytometry system. APC emits red light when excited by a red diode laser and the fluorescence is captured by the FL4 sensor

of the flow cytometry system. Fluorescence in both the FL1 and FL4 channels was detected simultaneously for cells stained with both the first and second antibodies. Negative controls for the experiment included cells that were not stained with any antibodies and cells that were incubated with the isotypes of both the first and second antibody used.

The forward scatter (FSC) and side scatter (SSC) parameters were also determined during the acquisition of fluorescence and were used to define the monocyte and lymphocyte fractions. The monocyte fraction was gated and the MFI was used to compare TLR4 expression of monocytes stimulated with and without IL-4. CD4 positive cells that were also positive for TLR4 were determined by simultaneous analysis of the FL1 and FL4 data. This data was plotted on a scatter plot and different quadrants were assigned for negative cells, cells positive for TLR4, cells positive for CD4 and double positive cells. The percentage of double positive cells from the total CD4 population was determined for each patient in the study. CD3 expression of CD4 positive cells was also determined in a similar fashion.

2.3.4 Statistics

The average MFI for TLR4 in the monocytic cell fraction that was stimulated with IL-4 was compared to unstimulated monocytes using the paired two sample Student's T-test. The average percentage of TLR4 positive CD4 positive cells of total CD4 positive cells stimulated with IL-4 was compared to unstimulated TLR4 positive CD4 positive cells using the paired two sample Student's T-test. P values less than 0.05 were considered as being significant.

Chapter 3: Results

3.1 Immune Responses to LPS in Nasal Mucosa

3.1.1 Inflammatory Cells in Nasal Mucosa after Ex Vivo Challenges

Immunohistochemistry was performed to determine the presence of inflammatory cells in the nasal explants of atopic children (n=8) cultured *ex vivo* with LPS, allergen and LPS and allergen stimulations. In tissues cultured in media (BCM) without challenge (baseline), monocyte/macrophages (CD68; mean immunopositive cells per field ± stand deviation; 6.4±2.7), eosinophils (MBP; 2.1±1.8), neutrophils (elastase; 3.1±2.5), mast cells (tryptase; 7.9±3.4) and T cells (CD3; 25.8±9.5) were detected (Figure 3.1). Exposure of the atopic mucosa to LPS alone caused a significant increases ($P<0.05$) in monocyte/macrophages (15±4.7), neutrophils (7.3±3.4), mast cells (12.6±5.6) and T cells (121.4±24.7) but not eosinophils (1.4±1.2). Monocyte/macrophages, neutrophils, mast cells increased about 2-5 fold from baseline, but the cells present in the greatest number after LPS exposure, the T cells, increased about 4-5 five times compared to baseline.

Exposure of the nasal explants to allergens, depending on the atopy of the patient, caused a significant increase in mast cells (17.6±9.7), eosinophils (9.1±2.9) and T cells (41.5±16.3) compared to baseline. A smaller, although significant, increase in monocyte/macrophages (9.9±2.2) and neutrophils (6.1±2.7) was detected. When the nasal explants were challenged with specific allergens and LPS at the same time, differences were seen compared to either challenge alone. LPS inhibited the allergen-induced increase in monocyte/macrophage (7.3±2.5), eosinophils (3.8±1.5) and mast cells (9.1±4.5) making the levels not significantly different from baseline. Neutrophils

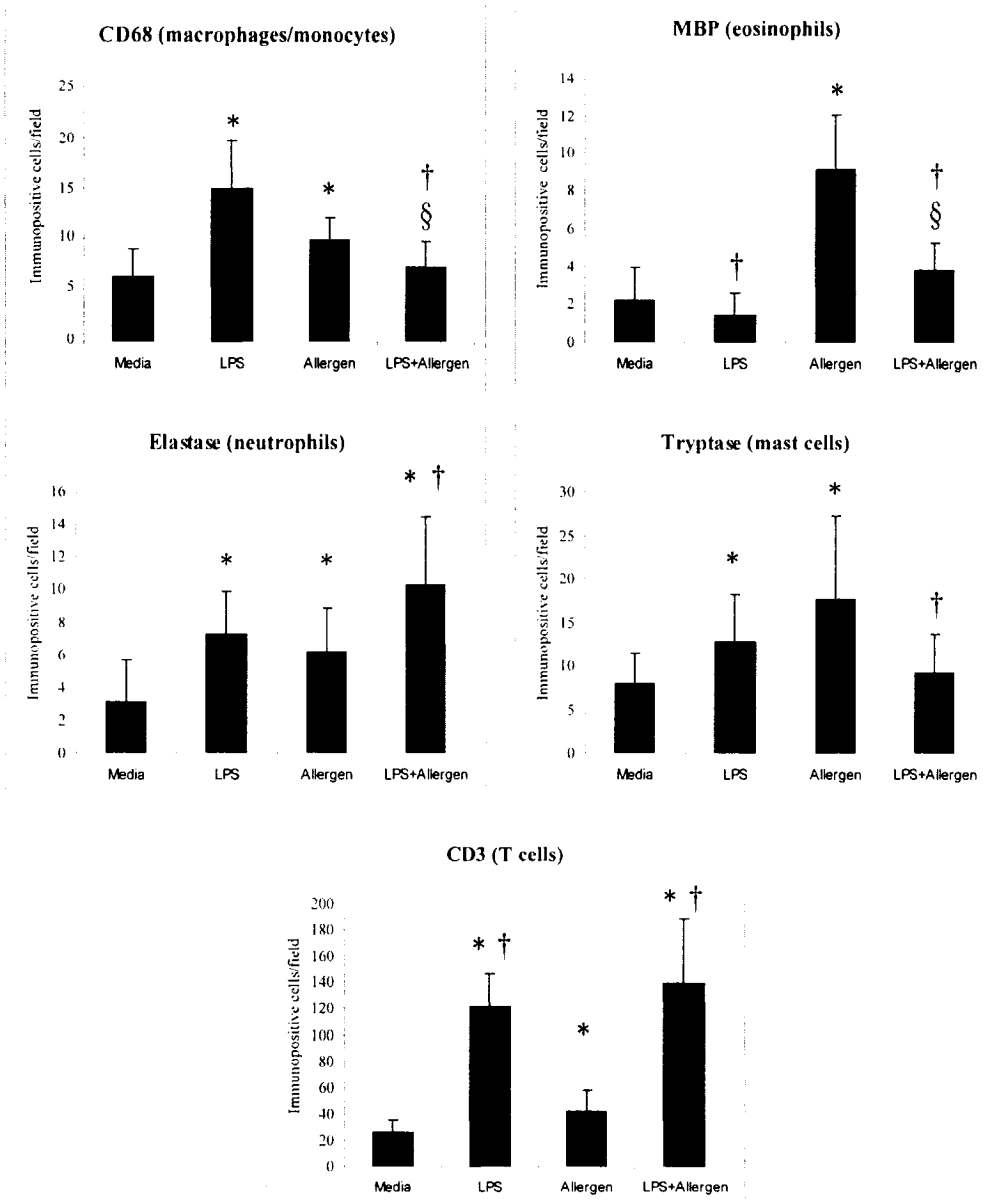

Figure 3.1. Levels of inflammatory cells in nasal mucosa of explants from atopic children after LPS, allergen or allergen and LPS challenge as measured by immunohistochemistry. CD68 is a marker for macrophages and monocytes, MBP is a marker for eosinophils, elastase is a marker for neutrophils, tryptase is a marker for mast cells and CD3 is a marker for T cells. All data is expressed in numbers of immunopositive cells/field. MBP = major basic protein; CD = Cluster of differentiation. *P<0.05 versus baseline. † P<0.05 versus allergen challenge. § P<0.05 versus LPS challenge.

(10.3±4.2) were significantly higher than baseline but simultaneous challenge did not increase levels more than single challenges. LPS and allergen challenges increased the levels of T cells (138.9±48.8). This level was significantly higher than in biopsies challenged with allergen alone but not different from biopsies challenged with LPS alone.

3.1.2 CD25 Expression and IL-2 Expression in the Nasal Mucosa

IL-2 is a potent growth factor for T cells and increases in CD3 positive cells after LPS stimulation may be due to induction of this cytokine. A baseline (20.3±8.1) level of IL-2 immunopositive cells was found to be present within the nasal mucosa of explants (n=8) cultured with BCM (Figure 3.2). Significant increases in IL-2 immunopositive cells were seen after incubation of the nasal explants with both LPS (83.1±17.7) and with allergen (59.4±14.2). Simultaneous challenge also led to a significant increase in IL-2 (80±11.5) compared to baseline and compared to allergen challenges, but was not different from LPS challenge alone.

CD25 is part of the receptor of IL-2, and LPS and allergen may increase number of cells responsive to IL-2 by inducing this receptor. A baseline level of CD25 (15.4±5.3) immunopositive cells was found in the mucosa of the nasal explants (n=8). A significant increase in CD25 immunopositive cells was seen upon LPS challenge (69.8±8.8) and allergen challenge (52.4±6.1). Simultaneous challenge of LPS and allergen also significantly increased CD25 immunopositive cells (73.3±12.5) compared to baseline and allergen challenge alone, but this effect was not significantly different from LPS challenge alone. With fluorescent immunohistochemistry it was determined that about 60% of the T cells were positive for CD25 (data not shown).

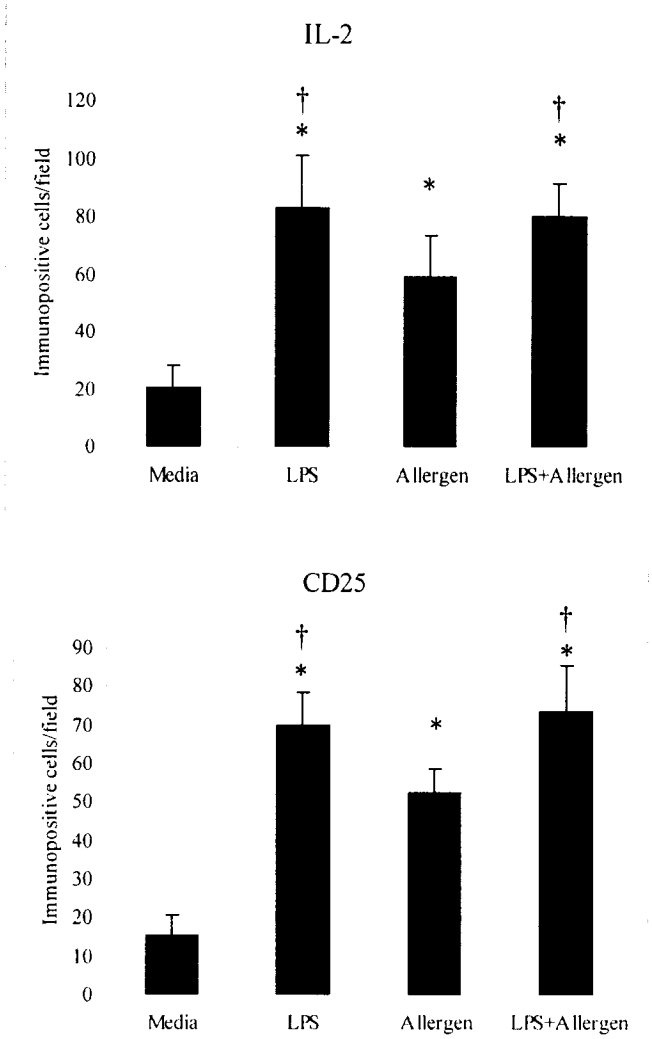

Figure 3.2. Levels of IL-2 and CD25 in nasal mucosa of explants from atopic children after LPS, allergen or allergen and LPS challenge as measured by immunohistochemistry. All data is expressed in numbers of inflammatory cells/field. *P<0.05 versus baseline. † P<0.05 versus allergen challenge.

3.1.3 Cytokine mRNA Expression in the Nasal Mucosa

The expression of cytokine mRNAs in the explanted nasal mucosa (n=8) of atopic children was measured by radioactive *in situ* hybridization (Figure 3.3). Tissue cultured in BCM without challenge expressed baseline levels of IL-4 (mean mRNA positive cells per field ± stand deviation; 2.1±2.1), IL-5 (3.3±2.4), IL-13 (4.5±2.6), IFN-γ (1.4±1.3), IL-12 (1.1±1.1) and IL-10 (6.4±3.7) mRNA positive cells. LPS did not cause significant increases of mRNA positive cells for the Th2 cytokines IL-4 (2.5±3.0), IL-5 (2.6±2.5) and IL-13 (3.3±2.2), but did cause significant increases in mRNA positive cells for the Th1 cytokines IFN-γ (17.4±10.1) and IL-12 (6.8±3.9). LPS also caused a significantly large increase, almost sevenfold baseline levels, in the immunoregulatory cytokine IL-10 (41.8±15.5) mRNA positive cells compared to baseline. Allergen challenge caused significant increases in IL-4 (6.7±4.1), IL-5 (16.0±10.8), IL-13 (10.0±6.7) and IL-10 (14.4±7.7) mRNA positive cells, but not in IFN-γ (1.9±1.4) and IL-12 (1.3±1.5) mRNA positive cells. LPS was added to allergen to determine whether it could inhibit induction of Th2 cytokine mRNA. LPS abrogated the allergen induced increase in the number of cells positive for mRNA of the Th2 cytokines IL-4 (2.4±2.6), IL-5 (5.1±2.2) and IL-13 (5.1±3.3) making levels of positive cells not significantly different from biopsies cultured in BCM without challenge. Simultaneous challenge of the biopsies with allergen and LPS caused significant increases in IL-10 (62.8±24.8), IFN-γ (24.3±9.4) and IL-12 (16.3±6.6) mRNA positive cells.

3.1.4 Neutralizing Antibodies for IL-10, IL-12 and IFN-γ: Th2 Cytokines

Neutralizing antibodies for IL-10, IL-12 and IFN-γ were used to determine which of these cytokines could mediate the ability of LPS in reducing allergen induced Th2

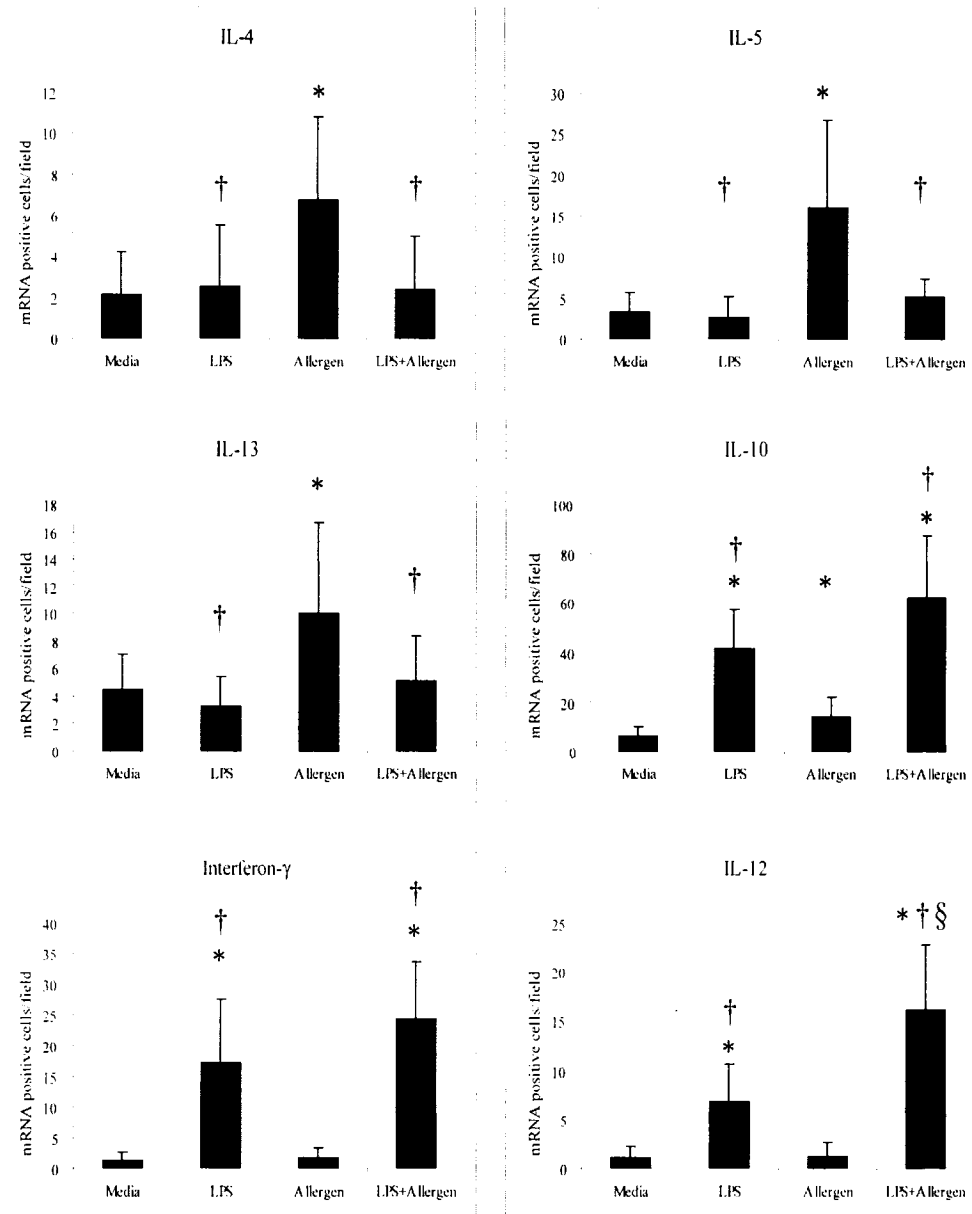

Figure 3.3. Levels of cytokine mRNAs in nasal mucosa of explants from atopic children after LPS, allergen or allergen and LPS challenge as measured by *in situ* hybridization. IL-4, IL-5 and IL-13 are known as Th2 cytokines, while IFN-γ and IL-12 are known as Th1 cytokines. IL-10 is known as a regulatory cytokine. All data is expressed in numbers of mRNA cells/field. IL = interleukin; IFN = interferon. *P<0.05 versus baseline. † P<0.05 versus allergen challenge. § P<0.05 versus LPS challenge

cytokine production (n=4). Allergen induced increases in the mRNA positive cells for the Th2 cytokines IL-4 (6.8±1.5), IL-5 (16.0±4.1), and IL-13 (10.0±2.5) (Figure 3.4). LPS significantly abrogated the allergen induced increase in these cytokine mRNAs when the biopsies were simultaneously challenged with LPS and allergen: IL-4 (3.8±0.7), IL-5 (4.5±0.5) and IL-13 (5.8±0.5). Neutralization of IL-10 significantly blocked the effect of LPS on IL-4 (7.8±0.9), IL-5 (14.1±1.3) and IL-13 (12.4±0.5). Neutralization of IL-12 also inhibited the effect of LPS on IL-4 (6.6±1.0), IL-5 (13.0±0.8) and IL-13 (11.5±1.0). Neutralization of IFN-γ did not inhibit the effect of LPS on IL-4(5.1±0.5) but did inhibit the effect on IL-13 (8.2±1.0) and IL-5 (10.1±0.9). The IL-12 and IFN-γ neutralizing antibodies were combined to determine if inhibiting both major Th1 associated cytokines would have a greater effect than either neutralizing antibody alone. Neutralization of IFN-γ and IL-12 brought the level of IL-4, IL-5 and IL-13 similar to levels cells after allergen challenge. When isotype controls for each neutralizing antibody were used, levels of IL-4(4.2±0.7), IL-5(4.2±0.5) and IL-13(5±0.5) were not different to the effects of challenging the tissue with allergen and LPS (data not shown).

Immunohistochemistry for IL-5 was used to determine if levels of protein positive cells were affected by the neutralizing antibodies (Figure 3.5). Levels of IL-5 immunopositive cells were increased in the mucosa by culturing the tissue with allergen (12.4±3.0), and this increase was blocked by the addition of LPS (3.1±0.5). Neutralizing IL-10 (13±4.3), IL-12 (12.2±2.8), IFN-γ (15.1±4.9) and both IL-12 and IFN-γ (12.9±2.1) all inhibited the effects of LPS on allergen-induced increases in IL-5 immunopositive cells.

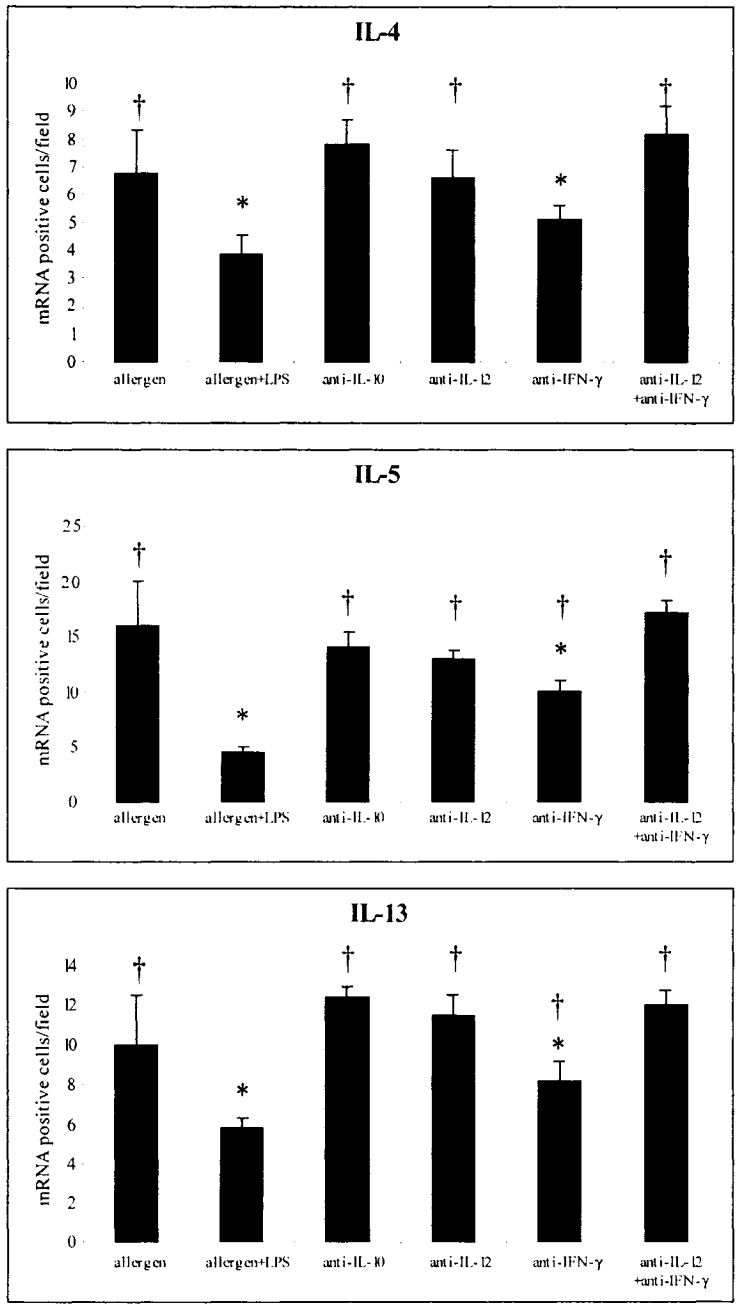

Figure 3.4. Levels of Th2 cytokine mRNAs in nasal mucosa of explants from atopic children after allergen and LPS challenge as measured by *in situ* hybridization. Neutralizing antibodies were used to block the effects of IL-10, IL-12, IFN-γ, and both IL-12 and IFN-γ. All data is expressed in numbers of mRNA cells/field. IL = interleukin; anti-IL-10 = IL-10 neutralizing antibody; anti-IL-12 = IL-12 neutralizing antibody; anti-IFN-γ = IFN-γ neutralizing antibody. *$P<0.05$ versus allergen. †$P<0.05$ versus allergen and LPS challenge.

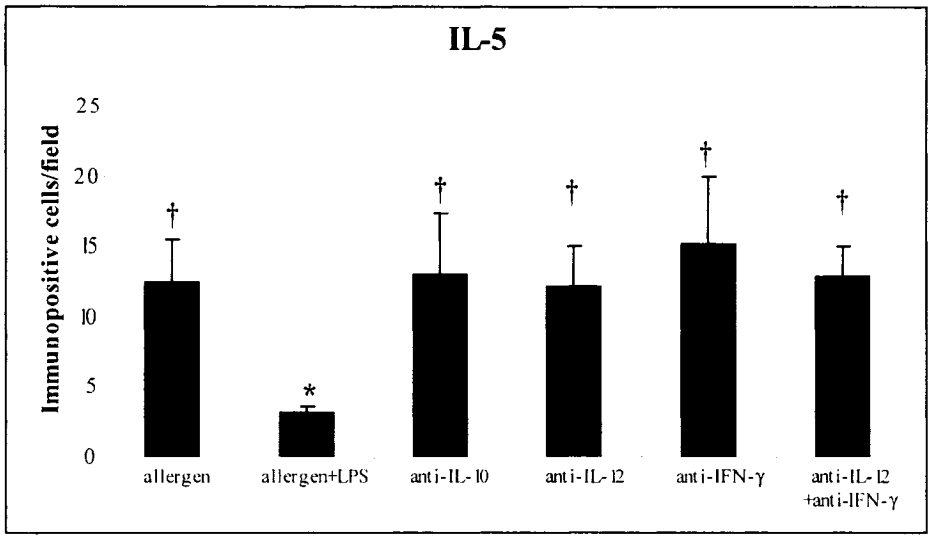

Figure 3.5. Levels of IL-5 immunopositive cells in nasal mucosa of explants from atopic children after allergen and LPS challenge as measured by immunohistochemistry. Neutralizing antibodies were used to block the effects of IL-10, IL-12, IFN-γ, and both IL-12 and IFN-γ. All data is expressed in numbers of protein positive cells/field. *P<0.05 versus allergen. †P<0.05 versus allergen and LPS challenge.

3.1.5 Neutralizing Antibodies for IL-10, IL-12 and IFN-γ: Th1 Cytokines and IL-10

In situ hybridization was also used to measure the impact of the neutralizing antibodies on levels of IL-10, IL-12 and IFN-γ mRNA positive cells (Figure 3.6). This was performed to determine if these cytokines could influence the expression of each other. Low levels of IFN-γ (1.9±0.6), IL-12 (1.3±0.5) and IL-10 (14.4±2.9) mRNA positive cells were seen when the tissue was incubated with allergen alone. LPS challenge in the presence of allergen induced the levels of all three cytokines: IFN-γ (20.3±1.2), IL-12 (17.8±1.9) and IL-10 (55.3±4.2). Neutralization of IL-10 (13.7±0.5) and of IL-12 (10.2±0.5) blocked the induction of IFN-γ, but blocking IFN-γ (17.2±0.9) had no effect. Combination of neutralizing antibodies against IL-12 and IFN-γ (9.3±0.8) significantly blocked the effect of LPS on levels of IFN-γ positive cells, but the effect was not different from using neutralizing antibodies against IL-12 alone. Neutralization of IL-10 (5.9±0.5) and neutralization of IFN-γ (9.4±2.3) reduced the induction of IL-12 mRNA positive cells by LPS. Blocking IL-12 (16.5±2.3) had no effect on LPS induced IL-12 mRNA positive cells, but combining neutralizing antibodies for both IL-12 and IFN-γ (3.5±0.4) produced a greater effect than using either neutralizing antibodies alone. Neutralization of IL-10 had no significant effect on IL-10 (50.3±4.8) levels of mRNA positive cells induced by LPS, but neutralization of IL-12 (22.1±0.9) significantly lowered levels of IL-10 mRNA positive cells, and was not different from allergen challenge alone. Neutralization of IFN-γ (40±2.2) also significantly lowered levels of IL-10 mRNA positive cells, but not as much as IL-12 neutralization. Combining IL-12 and IFN-γ (18.1±1.2) neutralizing antibodies significantly decrease IL-10 mRNA positive

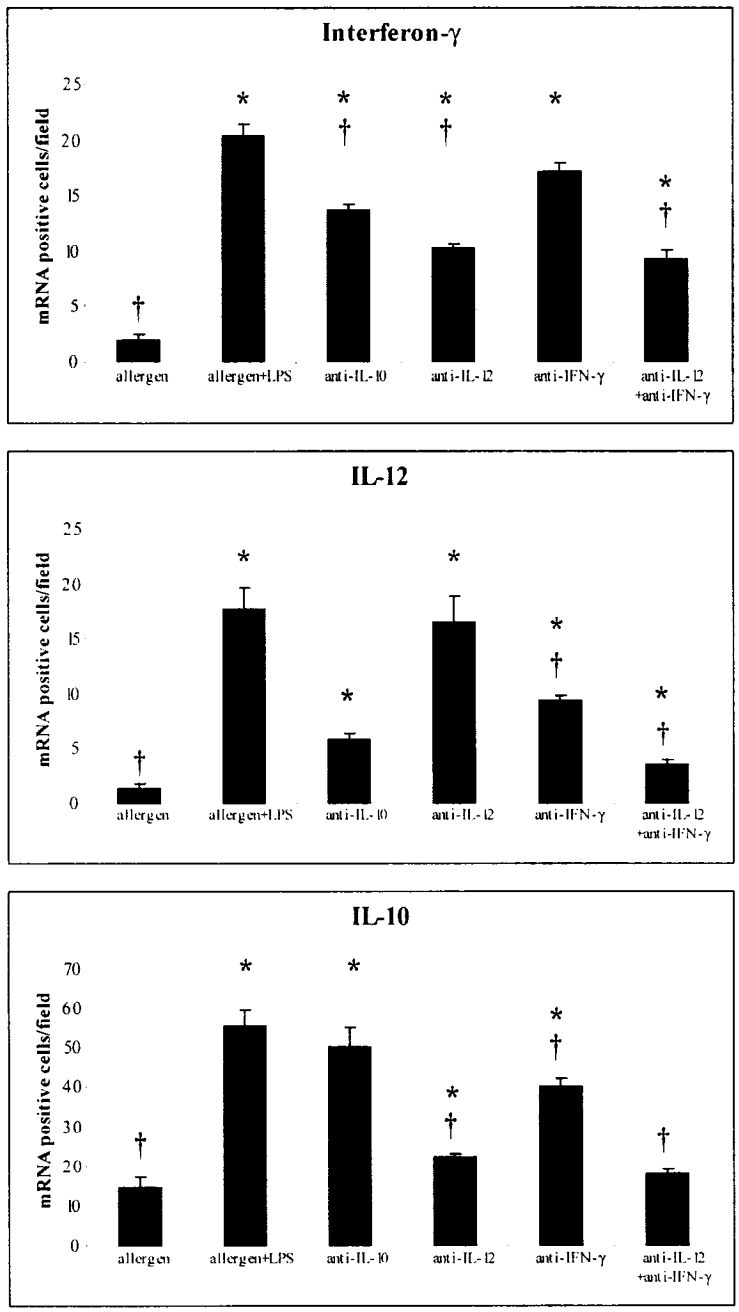

Figure 3.6. Levels of Th1 cytokine and IL-10 mRNAs in nasal mucosa of explants from atopic children after allergen and LPS challenge as measured by *in situ* hybridization. Neutralizing antibodies were used to block the effects of IL-10, IL-12, IFN-γ, and both IL-12 and IFN-γ. All data is expressed in numbers of mRNA cells/field. *P<0.05 versus allergen. †P<0.05 versus allergen and LPS challenge.

cells induced by LPS, but the effect was not significantly greater than the use of IL-12 neutralizing antibodies alone.

Immunohistochemistry for IFN-γ, IL-12 and IL-10 was used to determine if neutralizing antibodies affected the levels of cytokine protein in the positive cells (Figure 3.7). Levels of IFN-γ, IL-12 and IL-10 immunopositive cells were increased by LPS challenge with allergen (17.3±1.1, 11.7±0.9 and 52.9±6.7 respectively) compared to allergen stimulation alone (3.7±1.1, 3.6±0.8 and 16.5±2.7 respectively). Neutralization of IL-10 or of IL-12 with antibodies significantly reduced the LPS induced levels of IFN-γ (11.2±0.9) immunopositive cells. IFN-γ neutralization had no significant effect on IFN-γ (14.1±2.2) immunopositive cells, while neutralization of both IFN-γ and IL-12 reduced levels of IFN-γ (8.4±0.3) to levels similar tissue incubated with IL-12 neutralizing antibodies alone. Blocking IL-10 and IFN-γ (6.2±0.2) reduced levels of IL-12 (8.5±0.6) immunopositive cells induced by LPS. Blocking of IL-12 (13.5±3.3) had no significant effect on levels of IL-12 immunopositive cells, while addition of IFN-γ neutralizing antibodies to IL-12 (4.2±1.5) neutralizing antibodies caused a significant reduction in IL-12 immunopositive cells that was significantly greater than using IFN-γ neutralization alone. IL-10 neutralization (49.7±6.6) had no significant effect on IL-10 immunopositive cells. Both neutralization of IFN-γ (39.9±1.2) or neutralization of IL-12 (30.0±3.9) significantly reduced the LPS induction of IL-10 immunopositive cells. IL-12 had a greater effect than IFN-γ on IL-10 immunopositive cell reduction. Combination of the IFN-γ and IL-12 (19.6±4.1) neutralizing antibodies reduced levels of IL-10 immunopositive cells, but the combination was not significantly greater than use of IL-12 alone.

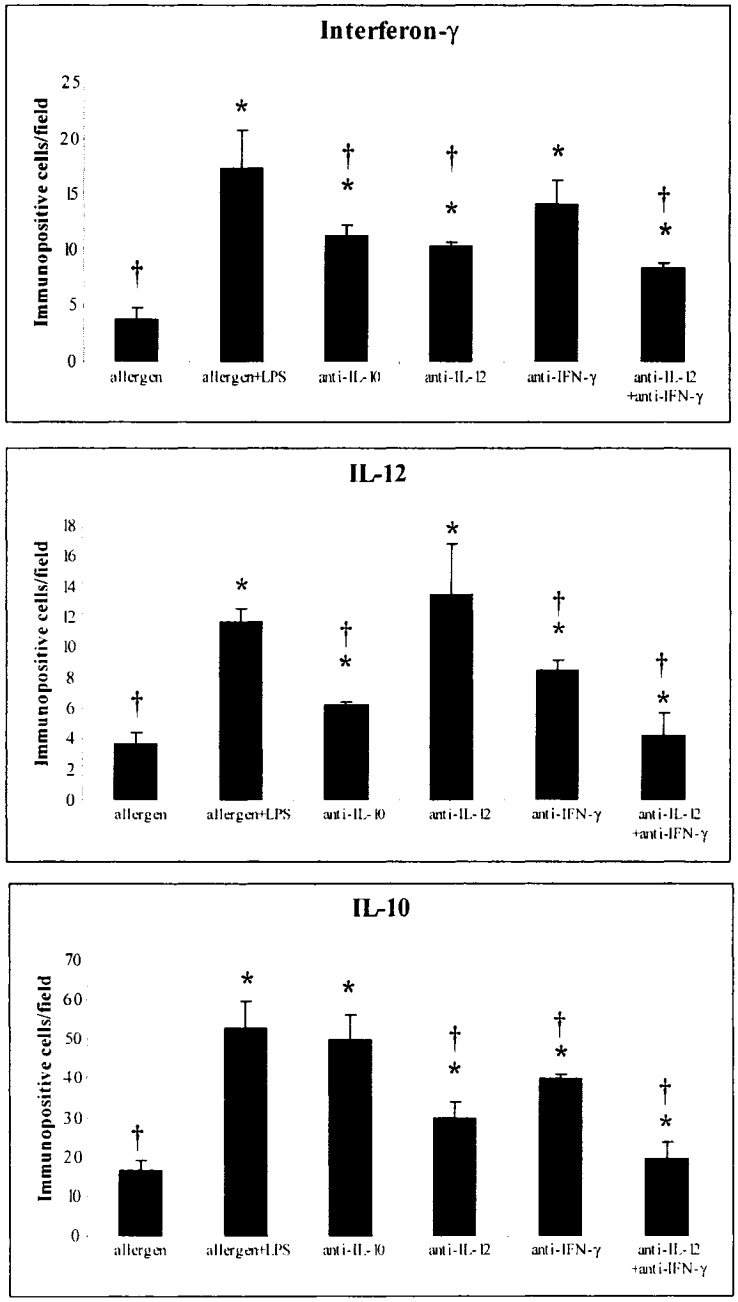

Figure 3.7. Levels of Th1 cytokine and IL-10 immunopositive cells in nasal mucosa of explants from atopic children after allergen and LPS challenge as measured by immunohistochemistry. Neutralizing antibodies were used to block the effects of IL-10, IL-12, IFN-γ, and both IL-12 and IFN-γ. All data is expressed in numbers of immunopositive cells/field. *P<0.05 versus allergen. †P<0.05 versus allergen and LPS challenge.

3.1.6 TLR4 Immunopositivity in Children and Cell Proliferation with LPS

In an earlier set of experiments, immune responses in the nasal mucosa of non-atopic children to LPS were measured but not of atopic children[293]. Immunohistochemistry for TLR4 was performed on the nasal mucosa of non-atopic children (n=7) and also atopic children (n=8) to determine whether a difference occurred between the patient groups at baseline and upon stimulation with different doses of LPS for 24 hours (Figure 3.8). A dose-dependent increase in TLR4 immunopositive cells was detected in the non-atopic group when comparing biopsies incubated in BCM (11.7±1.3) to stimulations using 0.001μg/mL (15.2±2.2), 0.01μg/mL (20.7±2.5), 0.1μg/mL (35.7±3.2) and 1μg/mL (41.5±4.3) of LPS. Similarly, biopsies from atopic children had a dose dependent increase in TLR4 (0.001μg/mL, 13.8±1.8; 0.01μg/mL, 22.5±2.2; 0.1μg/mL, 34.0±3.5; and 1μg/mL, 43.5±3.0) compared to baseline (10.2±1.0). No significant difference was determined between levels of TLR4 between atopic and non-atopic children at baseline or at any dose of LPS used.

Proliferation of cells, as measured by BrdU incorporation, was measured in explants from atopic children (n=3) to determine if LPS caused proliferation of inflammatory cells (Figure 3.9). Incubation of the biopsies for 24 hours with 0.1μg/mL of LPS (47.0±14.6) caused a significant increase in BrdU incorporation compared to biopsies cultured in BCM (7.0±1.1). Incubation of the tissues with allergen (9±1.6) did not cause a significant increase in BrdU incorporation, but when LPS was added to allergen (54±8.9), a significant increase in BrdU immunopositive cells was seen. Comparison of LPS to LPS and allergen yielded no significant difference, suggesting that

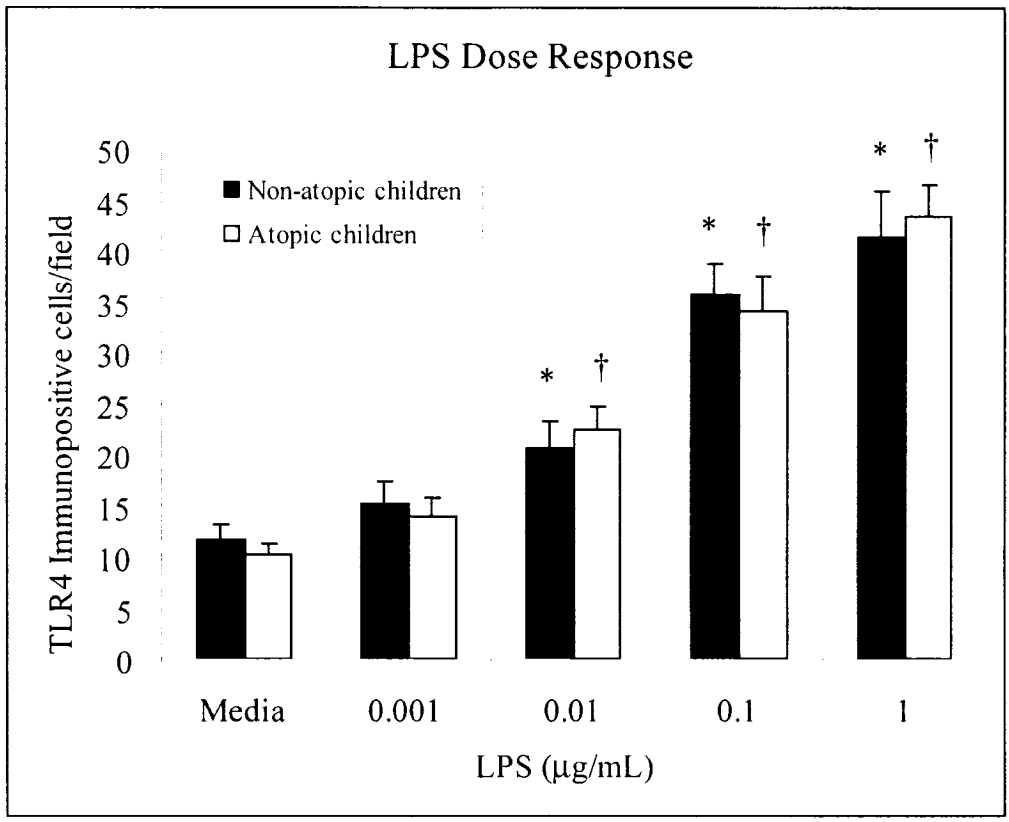

Figure 3.8. Levels of TLR4 positive cells in nasal mucosa of explants from non-atopic and atopic children after LPS challenge as measured by immunohistochemistry. Increasing doses of LPS were added to media ranging from 0.001 μg/mL to 1 μg/mL. All data is expressed in numbers of immunopositive cells/field. *P<0.05 versus non-atopic explants cultured in media alone. †P<0.05 versus atopic explants cultured in media alone.

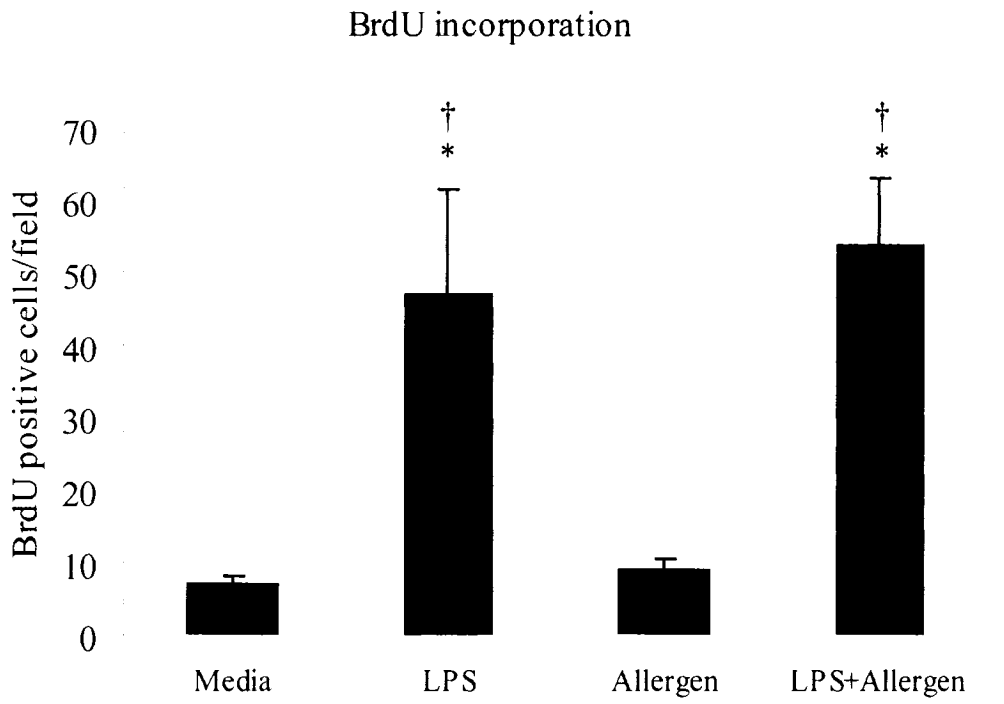

Figure 3.9. Levels of BrdU incorporation in nasal mucosa of explants from atopic children after LPS, allergen or LPS and allergen challenges as measured by fluorescent immunohistochemistry. All data is expressed in numbers of BrdU immunopositive cells/field and was semi-quantified using ImageJ software. BrdU = 5-bromo-2'-deoxy-uridine. *$P<0.05$ versus explants cultured in media alone. †$P<0.05$ versus explants cultured with allergen.

allergen did not cause a significant proliferation of cells as measured by BrdU incorporation.

3.1.7 CD3 and IL-10 Expression in Atopic Children and Atopic Adults

Atopic children (n=8; Figure 3.10) were then compared to atopic adults (n=7) for the response of the nasal mucosa to LPS. Atopic children and adults both expressed similar baseline levels of T cells (CD3 immunopositive cells) in the mucosa of the nasal tissue (26.5±4.3 and 29.1±3.9 respectively). Allergen stimulation of the tissue led to a significant increase of T cells in both children (42.2±7.0) and adults (51.7±8.7), but levels of T cells were not different between adults and children. LPS caused a great increase in T cells in the nasal mucosa of atopic children (123.5±10.4) but not in atopic adults (33.5±4.8). Simultaneous challenge of the biopsies with allergen and LPS caused an increase of T cells in adults (141.3±20.0) not different from allergen alone. The increase was significantly greater in children (54.3± 7.8) and not different from LPS alone.

The expression of IL-10 mRNA positive cells, as measured by *in situ* hybridization, was determined in atopic adults and was compared to the levels of atopic children (Figure 3.11). Baseline levels of IL-10 mRNA positive cells were detected in both children (7.0±1.7) and adult (8.2±1.4) atopic nasal mucosa. Significantly, increased levels of IL-10 mRNA positive cells were seen in both children (14.9±3.4) and adults (18.3±2.3) after allergen challenge, but were not different from each other. LPS caused a dramatic increase in IL-10 mRNA positive cells in the nasal mucosa of children (42.8±7.0) but not in adults (18.3±2.3). Simultaneous challenge with allergen and LPS led to strong increase in IL-10 mRNA positive cells in the nasal mucosa from children (64.8±9.6) and a smaller but significant increase in the nasal mucosa from adults

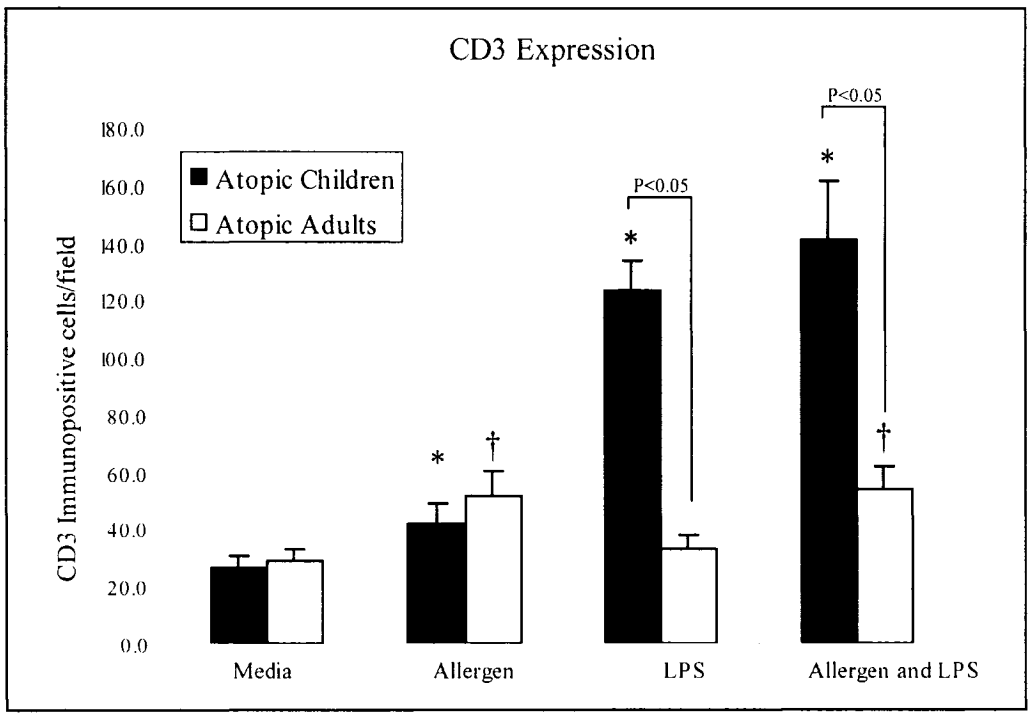

Figure 3.10. Levels of CD3 positive cells in nasal mucosa of explants from atopic children and atopic adults after LPS, allergen or allergen and LPS challenge as measured by immunohistochemistry. *P<0.05 versus biopsies from atopic children incubated in media. † P<0.05 versus biopsies from atopic adults incubated in media. Other comparisons P<0.05 are labeled on the graph.

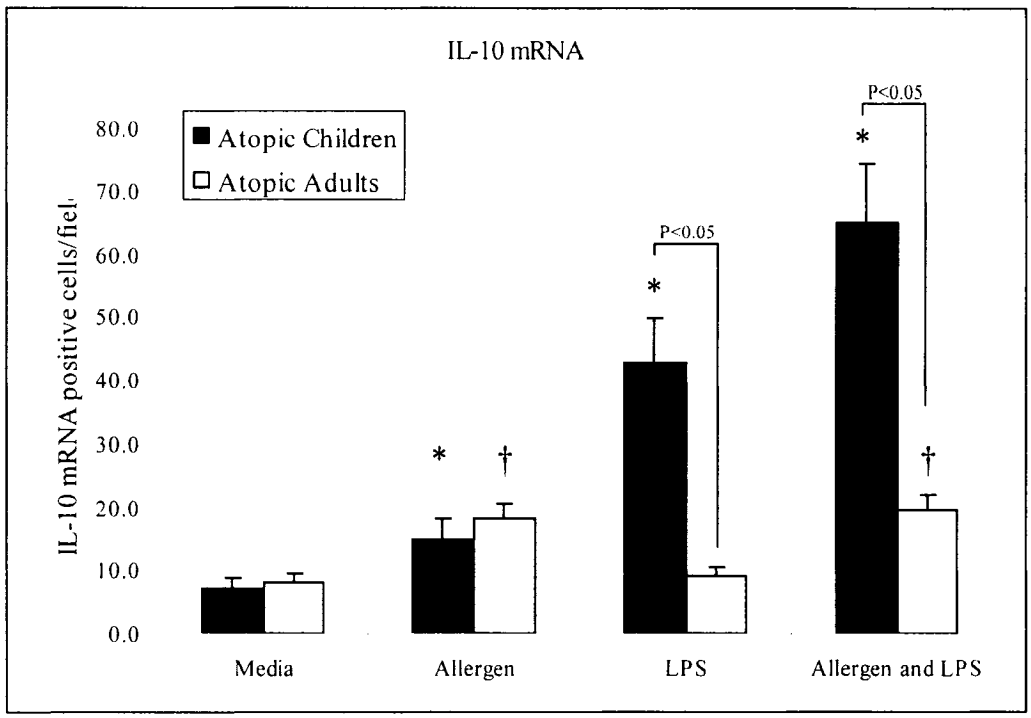

Figure 3.11. Levels of IL-10 mRNA in nasal mucosa of explants from atopic children and atopic adults after LPS, allergen or allergen and LPS challenge as measured by *in situ* hybridization. *P<0.05 versus biopsies from atopic children incubated in media. † P<0.05 versus biopsies from atopic adults incubated in media. Other comparisons P<0.05 are labeled on the graph.

(19.4±2.5). The increase of T cells and IL-10 mRNA positive cells in adults after the simultaneous challenge was not different from challenges using allergen alone, suggesting that the cells of the nasal mucosa from atopic adults is unresponsive to LPS. Some commercial LPS preparations can be potentially contaminated with other bacterial components such as lipoproteins, which can activate TLR2. A purified version of LPS (>99% purity), that activates only TLR4 LPS receptor, was used to stimulate explants from atopic children (n=3) and atopic adults (n=3). The effects of the purified version of LPS were not significantly different from the less pure formulations in terms of T cells and IL-10 mRNA positive cell levels (data not shown). This clearly supports the involvement of TLR4 in mediating LPS effects in the explant model.

3.1.8 TLR4 Expression in Adults and Children

LPS caused no effects in the nasal mucosa of atopic adults compared to children and this may be due to decreased LPS receptor components. Therefore, levels of TLR4 positive cells where measured by immunohistochemistry in atopic children (n=8), non-atopic children (n=7), atopic adults (n=8) and non-atopic adults (n=7; Figure 3.12). Biopsies were either cultured for 24 hours in BCM or in BCM with 0.1μg/mL of LPS. Baseline levels of TLR4 were detected in non-atopic children (11.3±1.5), atopic children (10.0±1.0) and non-atopic adults (4.3±1.0) and were almost undetectable in atopic adults (1.0±0.3). After LPS stimulation, TLR4 positive cells increased significantly in non-atopic children (34.9±3.0), atopic children (33.0±3.4) and non-atopic adults (13.6±1.7), but no observable increase was detected in atopic adults (1.0±0.5). Levels of TLR4 positive cells in the non-atopic adults were significantly lower compared to children, but

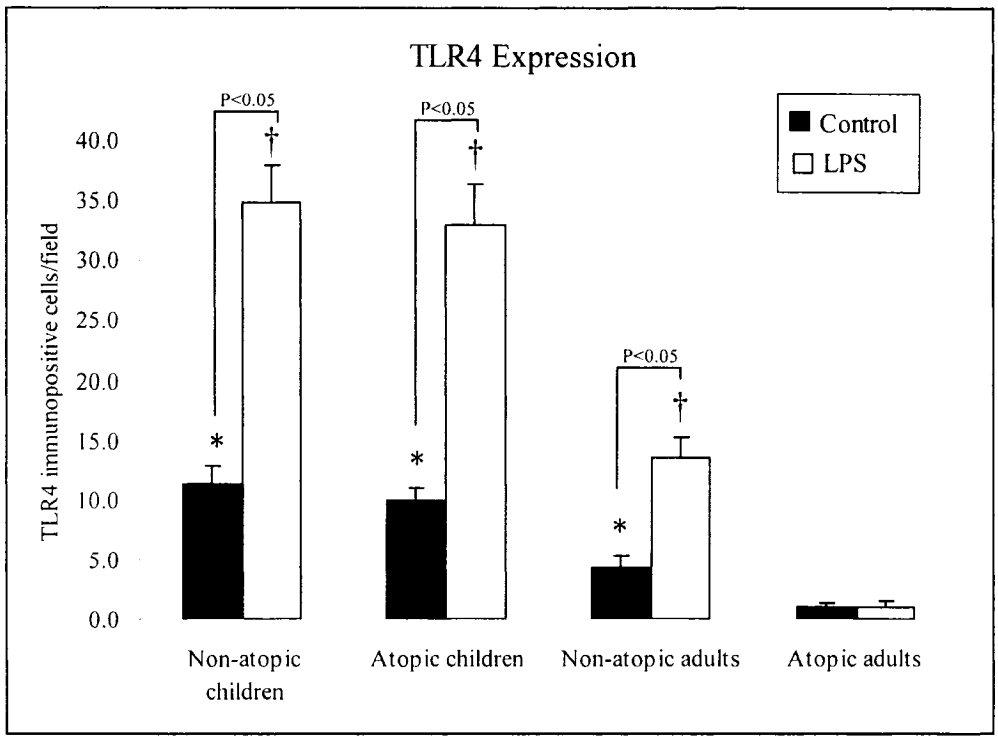

Figure 3.12. Levels of TLR4 immunopositive cells in nasal mucosa of explants from atopic and non-atopic children and adults after LPS as measured by immunohistochemistry. Biopsies were stimulated for 24 hours with 0.1 μg/mL of LPS. *P<0.05 versus biopsies from atopic adults incubated in media. † P<0.05 versus biopsies from atopic adults incubated with LPS. Other comparisons with P<0.05 are labeled on the graph.

levels of TLR4 positive cells in atopic adults were significantly lower to all other groups of patients.

3.1.9 Coexpression of Markers in Nasal Mucosa of Children

As T cells were the cell type that responded the greatest to LPS stimulation, it is possible that LPS had direct effects on these cells. Expression of TLR4 was determined on T cells (CD3 positive cells) using flurescent immunohistochemistry (Figure 3.13). Many T cells were also positive for TLR4 in the atopic nasal musosa and stimulation of the tissue with LPS led to a strong increase in the number of T cells and TLR4 positive cells (Figure 3.14). IL-10 was colocalized to TLR4 positive cells using fluorescent immunohistochemistry in tissue challenged with allergen, and levels of double positive cells increased greatly when the biopsies were challenged with LPS and allergen (Figure 3.15). CD4 and CD25 double positive cells were found in allergen stimulated tissue, but when the tissue was simultaneously challenged with allergen and LPS, a strong induction in the amount of these cells was seen (Figure 3.16).

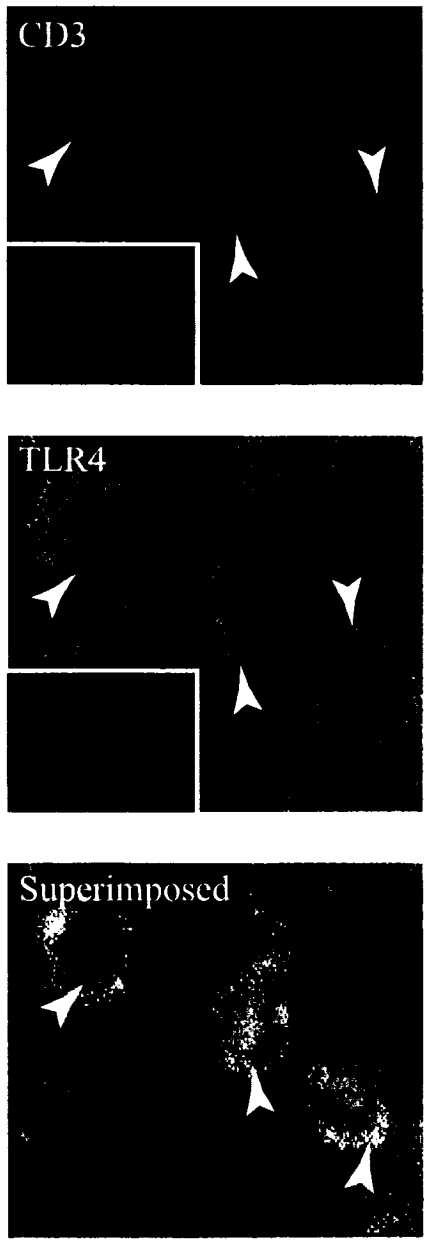

Figure 3.13. Detection of CD3 positive and TLR4 positive cells by colocalization using fluorescent immunohistochemistry. Atopic nasal mucosa from children was stained with R-phycoerythrin-labelled anti-CD3 antibodies and FITC-labelled anti-TLR4 antibodies. When stimulated with a laser, phycoerythrin fluoresces red (CD3 staining) and FITC fluoresces green (TLR4 staining). When the two signals are superimposed, any colocalization of the signals is seen as yellow. Images of isotype control antibodies labeled with the same fluoresecent dye as their respective antibody are found in the insets. Arrows denote positive staining or colocalization.

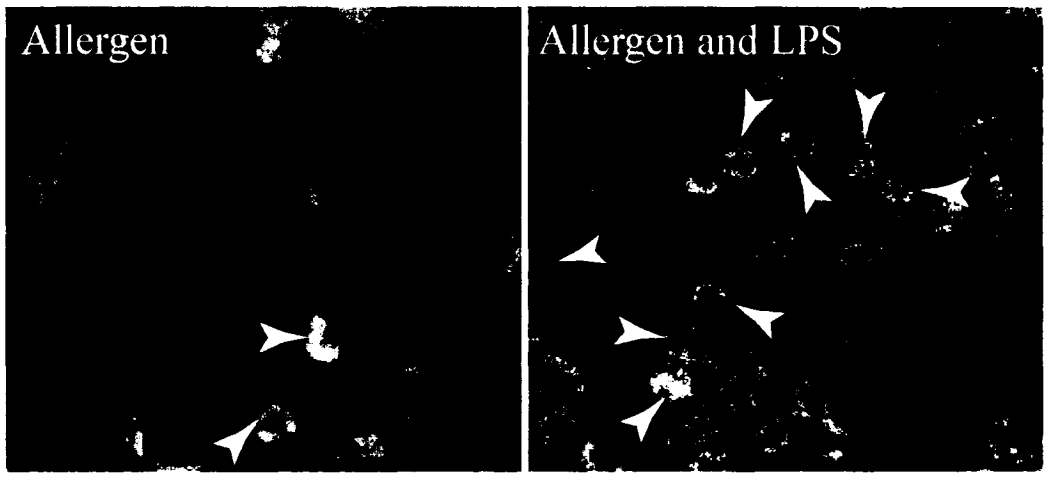

Figure 3.14. Detection of CD3 positive and TLR4 positive cells by colocalization using fluorescent immunohistochemistry at baseline and with LPS stimulation. Atopic nasal mucosa was stained with R-phycoerythrin-labelled anti-CD3 antibodies and FITC-labelled anti-TLR4 antibodies. When stimulated with a laser phycoerythrin fluoresces red (CD3 staining) and FITC fluoresces green (TLR4 staining). When the two signals are superimposed, any colocalization of the signals is seen as yellow. Arrows denote positive colocalization.

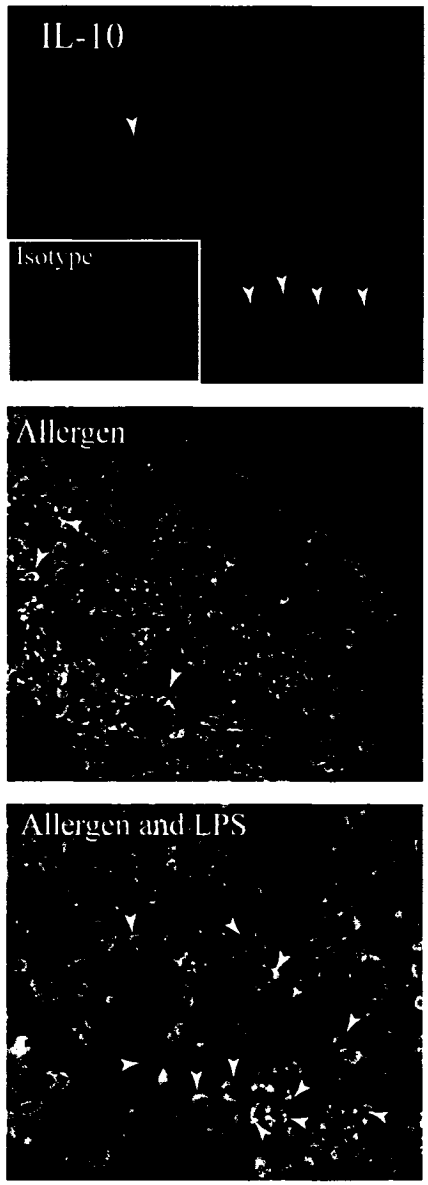

Figure 3.15. Detection of IL-10 positive and TLR4 positive cells by colocalization using fluorescent immunohistochemistry. Atopic nasal mucosa was incubated with allergen (middle) and allergen and LPS(bottom panel). Atopic nasal mucosa was stained with R-phycoerythrin-labelled anti-IL-10 antibodies and FITC-labelled anti-TLR4 antibodies. When stimulated with a laser phycoerythrin fluoresces red (IL-10 staining) and FITC fluoresces green (TLR4 staining). When the two signals are superimposed, any co-localization of the signals is seen as yellow. The top panel shows staining for IL-10 alone in allergen stimulated tissue. Phycoerythrin labelled isotype control for the anti-IL-10 antibody is found in the top panel inset. Arrows denote positive staining or colocalization.

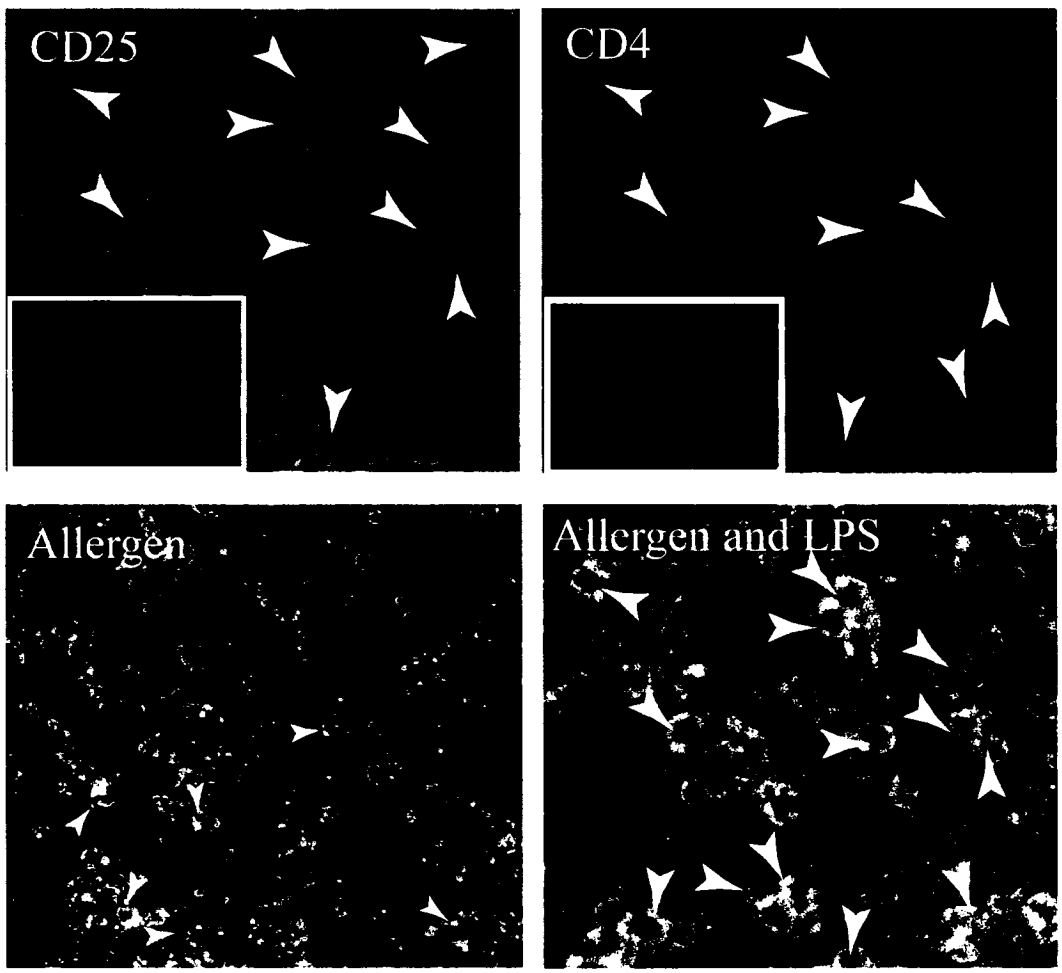

Figure 3.16. Detection of CD4 positive and CD25 positive cells by colocalization using fluorescent immunohistochemistry. Mucosa was cultured with allergen (bottom left panel) and allergen and LPS (bottom right panel). Atopic nasal mucosa was stained with allophycocyanin (APC) anti-CD4 and fluorescein isothiocyanate (FITC) anti-CD25 antibodies. When stimulated with a laser, FITC fluoresces green (CD25 staining) and APC fluoresces red (CD4 staining). When the two signals are superimposed, any colocalization of the signals is seen as yellow. Images of isotype control antibodies labeled with the same fluorescent dye as their respective antibody are found in the insets. Arrows denote positive staining or colocalization.

3.2 TLR4 Expression and Regulation in U-937 Cells

3.2.1 NF-κB Luciferase Assay

To determine the effect of IL-4 on the LPS responsiveness of U-937 cells, the activation of the NF-κB signal transduction was measured using the NF-κB luciferase assay (n = 3; Figure 3.17). Baseline levels of NF-κB activation were detected, and incubation of the cells with 10ng/ml IL-4 for 24 hours (mean fold upregulation compared to baseline level ± standard deviation; 1.1±0.1) did not cause any significant effect. Incubation of cells for 6 hours with 0.25μg/ml of LPS (3.0±0.3) caused a significant increase in the NF-κB reporter plasmid. When cells were incubated for 24 hours with IL-4 and then stimulated with LPS (IL-4 preincubation), a significant increase in NF-κB reporter activity was measured (1.8±0.1), but this increase was significantly decreased compared to cells stimulated with LPS without IL-4 preincubation. Conversely, if cells were only given IL-4 at the time of LPS challenge (IL-4 coincubation) NF-κB reporter activity was significantly induced (2.8±0.5) but was not different to cells stimulated with LPS alone.

3.2.2 TLR4 mRNA and Protein Surface Expression

As the inhibitory effects of IL-4 required a preincubation period, this suggests that LPS receptor components were downregulated and TLR4 expression in U-937 cells was determined after IL-4 stimulation. U-937 cells showed a high basal expression of TLR4 mRNA as measured by real-time QPCR (n=4; Figure 3.18). Cells were incubated with 0.1ng/ml to 50ng/ml of IL-4 for 12 hours and the lowest dose required to reach maximum suppression of TLR4 mRNA was found to be 10ng/ml. This is the dose used in all other experiments. When the cells were incubated with the optimal dose of IL-4, there was a

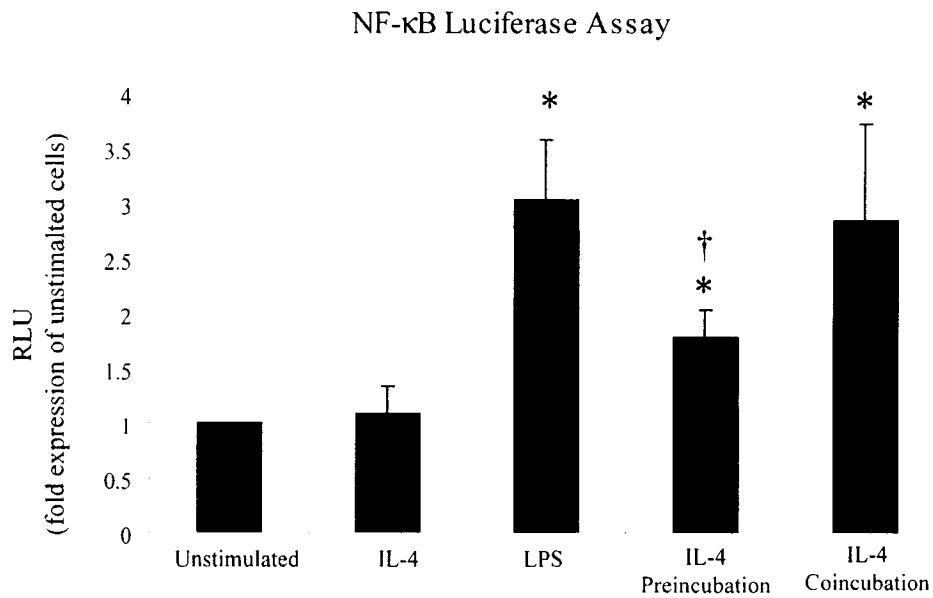

Figure 3.17. Activation of NF-κB in U-937 cells by IL-4 and LPS as measured by the NF-κB luciferase reporter assay. U-937 cells were transiently transfected with luciferase reporter plasmid activated by NF-κB. A *Renilla* luciferase vector was used to correct for transfection efficiency. Transfected cells were either left unstimulated, stimulated with 10ng/ml of IL-4 for 24 hours, stimulated with 0.25μg/ml of LPS for 6 hours, incubated with IL-4 for 24 hours (IL-4 preincubation) followed by LPS stimulation for 6 hours or stimulated with LPS and IL-4 (IL-4 coincubation). Relative light units (RLUs) were obtained by performing the dual-luciferase assay. Results are expressed as the RLU fold expression compared to the RLU of unstimulated cells. NF-κB = nuclear factor κB; *$P<0.05$ versus baseline, †$P<0.05$ versus LPS treatment.

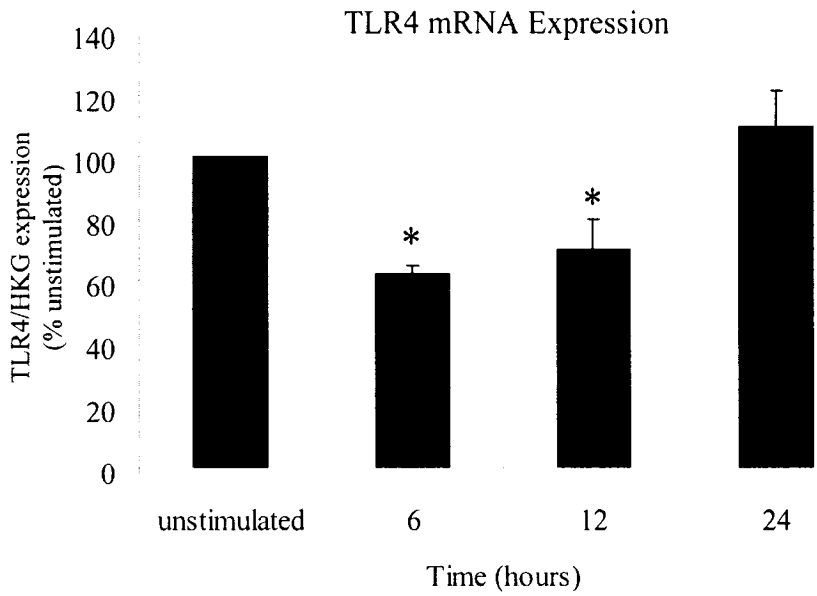

Figure 3.18. Expression of TLR4 mRNA in U-937 cells as measured by real-time QPCR. U-937 cells were incubated with 10ng/ml of IL-4 for 0 to 24 hours. RNA was extracted at different time points and TLR4 and ALAS1 house-keeping gene RNAs were quantified using plasmid standards. Results are expressed as copies of TLR4 versus copies of the house-keeping gene ALAS1 compared to unstimulated controls. *$P<0.05$ versus unstimulated cells (n=4).

rapid and significant reduction in the expression of TLR4 mRNA as early as 6 hours (percent of mRNA expression of unstimulated cells expression ± standard deviation; 62.3±4.7%). Downregulation of TLR4 mRNA was maintained at 12 hours (69.9±17.1) but was returned to baseline by 24 hours (109.6±20.8).

Surface expression of TLR4 of U-937 cells was evaluated using flow cytometry. 94.8±5% of the U-937 cells were positive for surface staining of TLR4. IL-4 did not reduce the number of TLR4 positive cells, but reduced the individual cell surface TLR4 intensity measured by using the MFI, a measure of the amount receptor expression on the cells based on the amount of fluorescence detected (Figure 3.19). TLR4 MFI was significantly reduced at 12 hours (percent of unstimulated cells ± standard deviation; 91.9±0.9%) and 24 hours (86.9±1.4%) but returned to basal levels by 48 hours (99.4±4.2%).

3.2.3 TLR4 Promoter Transcriptional Activity

To determine if IL-4 had an effect on the regulation of transcription, the proximal promoter of the human TLR4 gene, ranging from 4.3 kb of the ATG start codon, was cloned into a luciferase reporter plasmid pGL3-Basic. This construct was named TLR4-N as an Nco1 restriction endonuclease site was used to clone the fragment. U-937 cells were transiently transfected with TLR4-N and the luciferase assay was performed (n= 3). As a negative control, U-937 cells were also transfected separately with pGL3-Basic, the luciferase reporter plasmid that does not contain a promoter upstream of the luciferase gene. U-937 cells transfected with TLR4-N had a significantly greater level of luciferase activity than the control pGL3-Basic (Figure 3.20; mean fold upregulation compared to pGL3-Basic ± standard deviation; 5.9±0.9). This demonstrates a high baseline level of

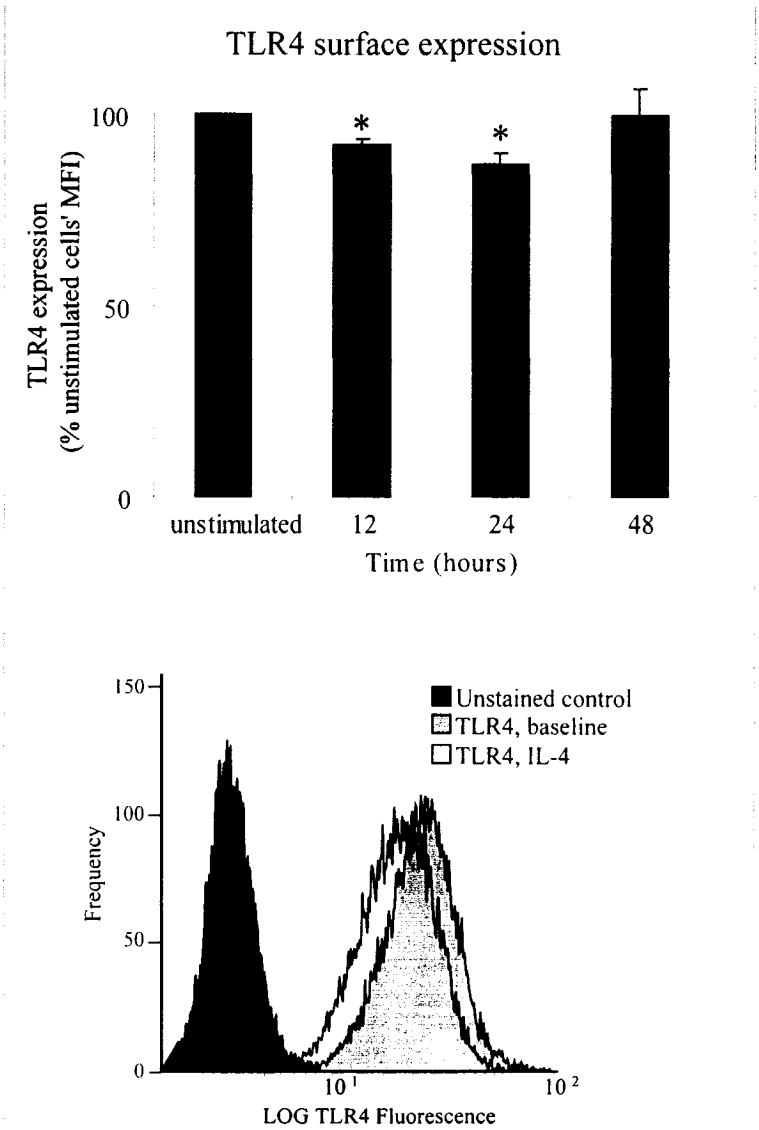

Figure 3.19. Expression of TLR4 protein cell surface expression in U-937 cells as measured by flow cytometry. U-937 cells were incubated with 10ng/ml of IL-4 for 0 to 48 hours. Cells were stained with a primary mouse anti-TLR4 antibody and a secondary FITC-labelled anti-mouse antibody(top panel). The flow cytometry histogram (bottom panel) shows a representative example of 24 hour stimulation with IL-4 (□) compared to unstimulated control (□). Background fluorescence was determined by incubation of the cells with FITC-conjugated secondary antibody alone (■), and was subtracted from the sample MFI values. Results are expressed as a percent of unstimulated cells for each time point. *P<0.05 versus unstimulated cells.

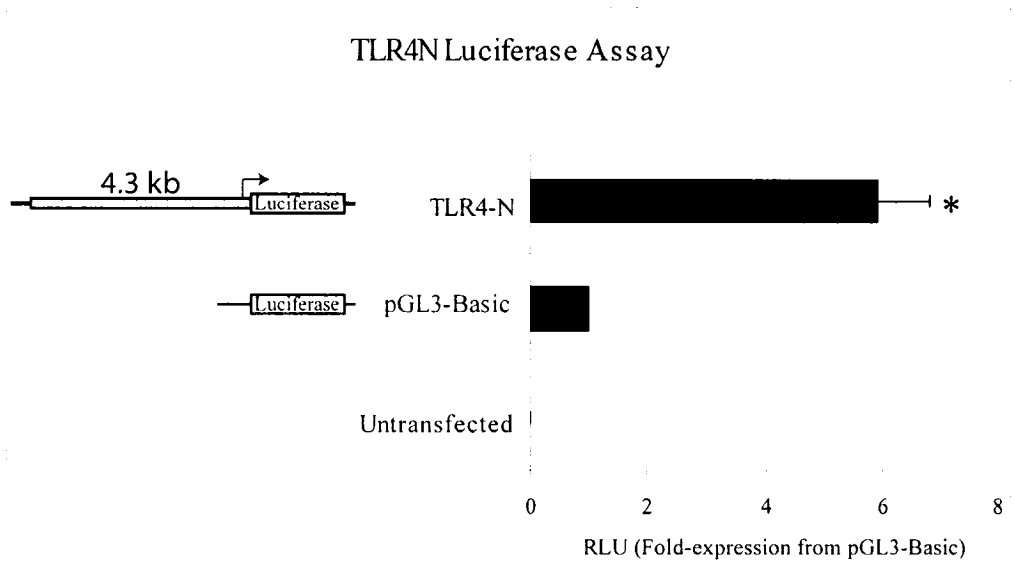

Figure 3.20. Activation of upstream proximal promoter of TLR4 in U-937 cells as measured by the luciferase reporter assay. U-937 cells were transiently transfected with a luciferase reporter plasmid containing 4.3 kb of the upstream region of TLR4 (TLR4-N). pGL3-Basic is the reporter plasmid without any cloned insert and was transfected into the U-937 cells to serve as a negative control. Untransfected cells were used as another negative control. A *Renilla* luciferase vector was used to correct for transfection efficiency. Relative light units (RLUs) were obtained by performing the dual-luciferase assay. Results are expressed as the RLU fold expression compared to the RLU cells transfected with pGL3-Basic. *$P<0.05$ versus pGL3-Basic.

transcription in unstimulated cells. TLR4-N transfected U-937 cells were stimulated with IL-4 for different lengths of times before performing the luciferase assay (Figure 3.21). Decreased reporter activity occurred at 6 hours (percent of activity of TLR4-N in unstimulated cells ± standard deviation; 67.0±13.7%), and was maintained at 12 hours (71.7±15.4%) but was lost at 24 hours (87.6±12.7%).

3.2.4 STAT6 transfection and TLR4 promoter transcriptional activity

IL-4, through the IL-4 receptor activates STAT6 signal transduction [534]. To determine if STAT6 plays a role in inhibiting TLR4 transcription, a plasmid containing a full length human STAT6 plasmid (pSTAT6) was constructed and transfected into U-937 cells. Different concentrations of pSTAT6 were transfected into cells ranging from 0.5μg to 2.0μg (n=3). STAT6 levels were evaluated by immunoblotting and 2.0μg was the optimal dose causing a 2.5-fold increase in STAT6 expression compared to baseline levels (Figure 3.22). Transfection of pcDNA, which is the expression plasmid used to clone and overexpress STAT6, did not have any effect on endogenous STAT6 levels compared to untransfected cells.

U-937 cells were transfected with the plasmid TLR4-N and pSTAT6 to determine if STAT6 overexpression could affect transcription of TLR4. pcDNA was also transfected with TLR4-N into separate U-937 as a negative control. After transfection, the cells were stimulated with IL-4 for different lengths of time. IL-4 caused a decrease in TLR4-N activity in cells transfected with pcDNA at 6 hours (percent of activity of TLR4-N in unstimulated cells ± standard deviation; 64.9±11.9%), 12 hours (74.9±14.2%) but not at 24 hours (94.5±10.9%). The effects of pcDNA were comparable to the results seen when cells received TLR4-N alone (Figure 3.23). When cells transfected with

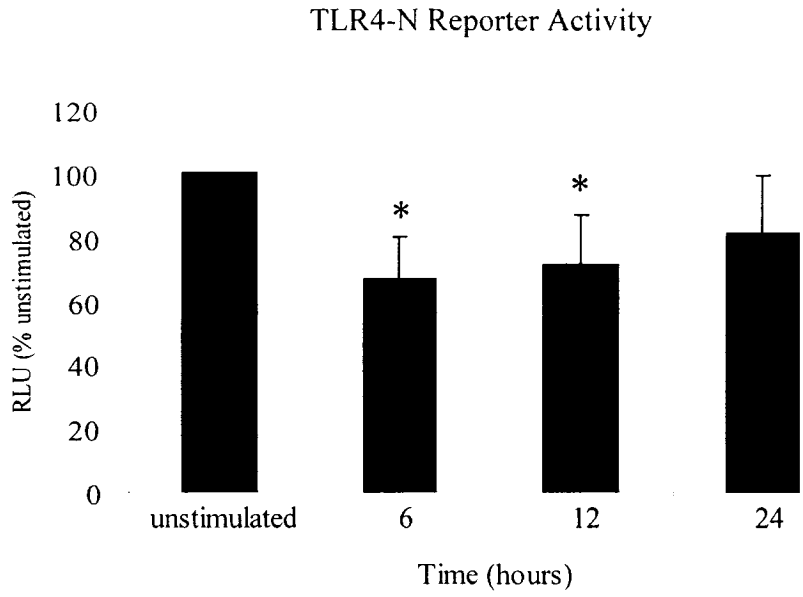

Figure 3.21. Activation of upstream proximal promoter of TLR4 in U-937 cells as measured by the luciferase reporter assay. U-937 cells were transiently transfected with a luciferase reporter plasmid containing 4.3 kb of the upstream region of TLR4 (TLR4-N). A *Renilla* luciferase vector was used to correct for transfection efficiency. Transfected cells were stimulated with 10ng/ml of IL-4 for 0 to 24 hours. Relative light units (RLUs) were obtained by performing the dual-luciferase assay. Results are expressed as a percent of the RLU of unstimulated cells. *$P<0.05$ versus baseline.

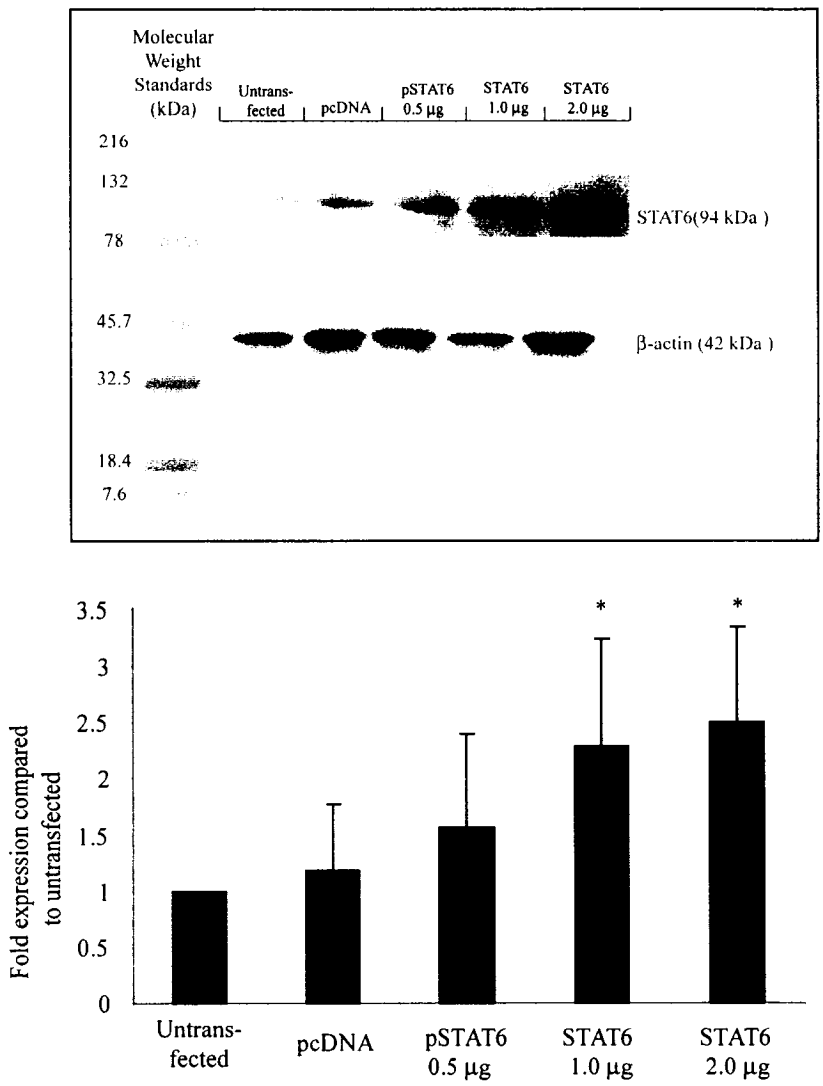

Figure 3.22. Expression of STAT6 by U-937 cells transfected with a constitutive STAT6 expression plasmid (pSTAT6), as measured by immunoblotting. U-937 cells were transiently transfected wtih different concentrations of pSTAT6 from 0.5µg/mL to 2.0µg/mL for 24 hours. An SDS-PAGE was performed on the lysates, and after transfer to a PVDF membrane, STAT6 and β-actin were probed using antibody and chemiluminescence. A representative example of a developed immunoblot is shown in the top panel, next to an image of the molecular weight protein standards used. Amounts of protein were semi-quantified using densitometry (bottom panel). pcDNA is the expression plasmid without STAT6 and was used as a negative control. Results are expressed as a density units of STAT6 bands (expected size 94 kDa) versus the house-keeping gene β-actin (expected size 42 kDa) for each condition compared to untransfected cells. *$P<0.05$ versus baseline.

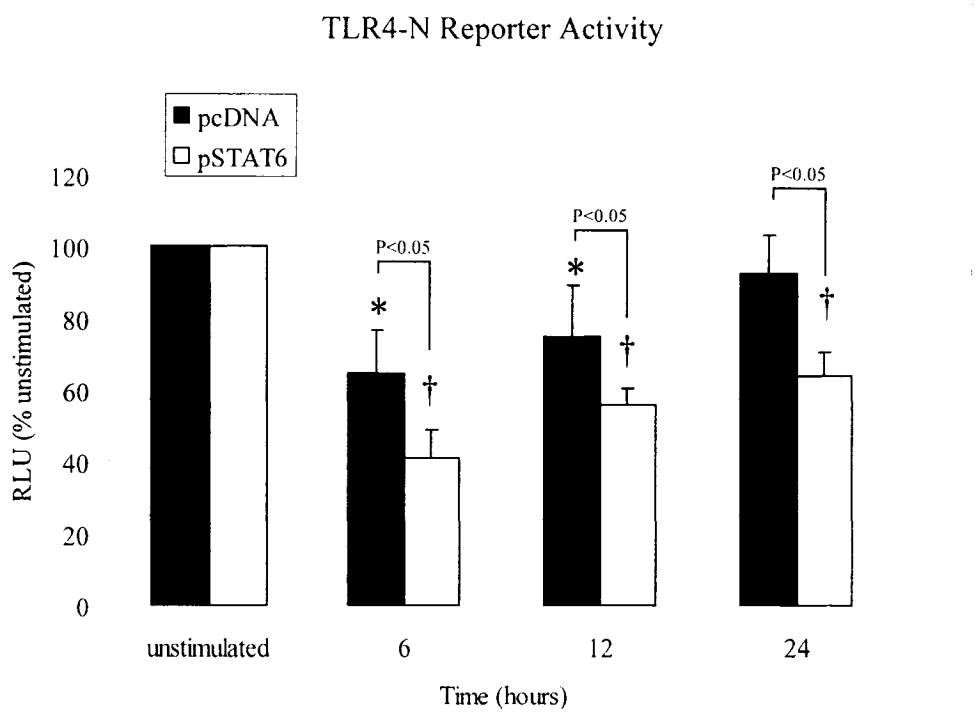

Figure 3.23. Activation of upstream proximal promoter of TLR4 in U-937 cells as measured by the luciferase reporter assay. U-937 cells were transfected with TLR4-N luciferase reporter plasmid and pSTAT6. As a negative control, pcDNA, the plasmid used to clone STAT6, was transfected in separate cells. A *Renilla* luciferase vector was used to correct for transfection efficiency. Transfected cells were stimulated with 10ng/ml of IL-4 for 0 to 24 hours. Relative light units (RLUs) were obtained by performing the dual-luciferase assay. Results are expressed as a percent of the RLU of unstimulated cells. *P<0.05 versus unstimulated cells transfected with pcDNA. †P<0.05 versus unstimulated cells transfected with pSTAT6. Other comparisons with P<0.05 are labeled on the graph.

pSTAT6 were stimulated with IL-4, TLR4-N reporter activity was significantly decreased at 6 hours (41.0±8.1%), 12 hours (55.7±4.5%) and 24 hours (64.3±6.6%). The decrease was significantly greater than the decrease seen in cells transfected with pcDNA at 6 hours and 12 hours. Unlike cells transfected with pcDNA, pSTAT6 transfected cells maintained a significantly lower activity in TLR4-N reporter activity at 24 hours. It is possible that stimulation of the cells with IL-4 caused an increased transcription of STAT6 from pSTAT6, but this is unlikely as analysis of the plasmid showed no STAT6 binding sites in its sequence.

3.2.5 Tyrphostin AG 490 and TLR4 mRNA Expression

Tyrphostin AG 490 is a potent inhibitor of the Janus Kinase-2 (Jak2) protein tyrosine kinase[535-537]. It can also inhibit Jak1 and Jak 3, but AG 490 does not significantly inhibit other kinases such as Lck, Lyn, Btk, Syk and Src. It is the most common agent used to block tyrosine phosphorylation of STAT6 phosphorylation by Jak tyrosine kinases; therefore, AG 490 was used to block IL-4 induced tyrosine phosphorylation of STAT6, and TLR4 mRNA levels were measured to determine the impact of this agent. Spiekermann et al. have shown that AG 490 can be toxic for U-937 cells[538], but with the dose of 25μM that was used, the cell viability was 95±5% as measured by Trypan blue exclusion. Treatment of U-937 cells with DMSO (diluted 1/400) alone, the vehicle used for AG 490, had no significant effect (Figure 3.24; 103.9±10.4%). 25μM of AG 490 alone caused no significant effect on levels of TLR4 mRNA expression (107.7±9.5%). IL-4 caused a significant decrease in TLR4 mRNA expression (79.7±3.4%) compared to unstimulated cells (100%). Pre-incubation of U-937

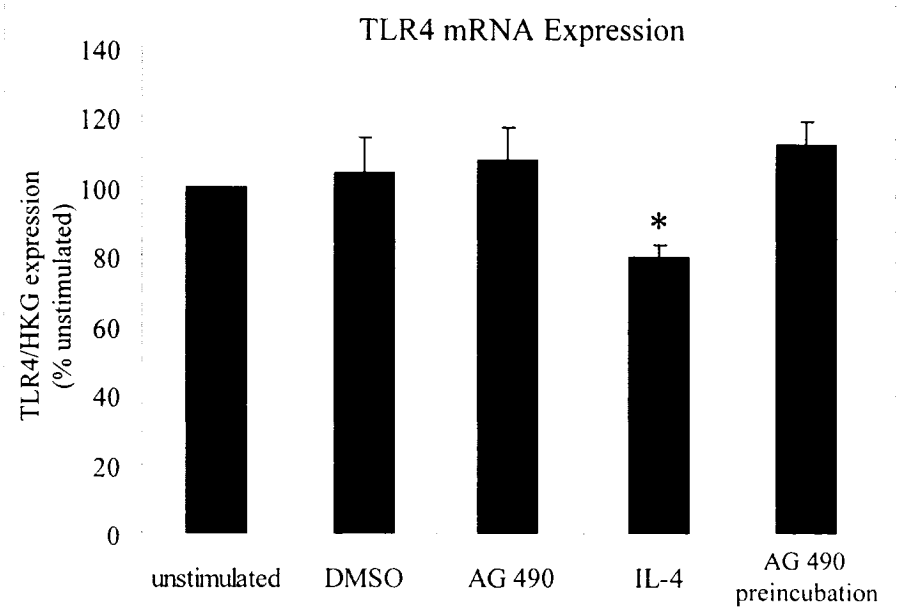

Figure 3.24. Expression of TLR4 mRNA in U-937 cells as measured by real-time QPCR. U-937 cells were incubated with 10ng/ml of IL-4, with DMSO (0.025%), and 25μM of tyrphostin AG 490 for 12 hours. Some cells were incubated with AG 490 for 16 hours before incubation for 12 hours with IL-4 (AG 490 preincubation). RNA was extracted at different time points and TLR4 RNA and ALAS1 house-keeping gene were quantified using plasmid standards. Results are expressed as copies of TLR4 versus copies of the house-keeping gene ALAS1. *$P<0.05$ versus unstimulated cells (n=4).

cells with AG 490 before challenge with IL-4, abrogated the decrease in TLR4 mRNA levels caused by IL-4 (111.9±6.9%) as it was not significantly different from baseline.

3.2.6 TLR4 Promoter Transcriptional Activity of Different Plasmid Constructs

The MatInspector software was used to map out potential STAT6 binding sites in the upstream region of proximal promoter of the TLR4 gene, cloned in the TLR4-N plasmid. MatInspector is computer software that uses sophisticated algorithms to compare DNA sequences of interest and determine potential transcription factor binding sites based on consensus sequences and a probability weight matrix[539]. Results of MatInspector are shown as possible transcription factors binding the sequence of interest, the core similarity value (maximum value of 1.0), and the matrix similarity value (maximum value of 1.0). It is recommended that values for the core be as high as possible if not 1.0, and that for a good match the matrix usually has a similarity of >0.80[539]. Six potential sites were determined having a core similarity (similarity to most highly conserved sequences) of 1.0 and matrix similarity (similarity to all residues in the sequence to known sequences that can bind STAT6) of ranging from 0.92 to 0.96 (Figure 3.25).

Different luciferase reporter plasmids containing different lengths of the upstream region of TLR4 were constructed to determine the region containing the IL-4 responsive region (Figure 3.26). U-937 cells transfected with the plasmid TLR4-N had a significant reduction in luciferase reporter activity when incubated with IL-4 for 12 hours (mean fold upregulation compared to pGL3-Basic ± standard deviation; 3.3±0.9) versus luciferase reporter activity at baseline (4.8±1.3). IL-4 incubation did not result in a significant reduction in luciferase reporter activity of TLR4-N2 (5.1±1.0), TLR4-N3 (6.0±1.5),

Number	Sequence (consensus sequence: nnnnn**TTCC**nnn**GAA**nnnn)	Core similarity	Matrix similarity	Position upstream from start codon (nucleotides)
1	ttcat**TTCC**cag**GAA**aagg	1.0	0.960	3346
2	tccac**TTCC**taa**GAA**tgag	1.0	0.954	3036
3	tacag**TTCC**taa**GAA**gcgg	1.0	0.937	2616
4	agccc**TTCC**tct**GAA**ccac	1.0	0.921	2325
5	taact**TTCC**agg**GAA**ctaa	1.0	0.938	2079
6	gtttc**TTCC**caa**GAA**gggg	1.0	0.916	746

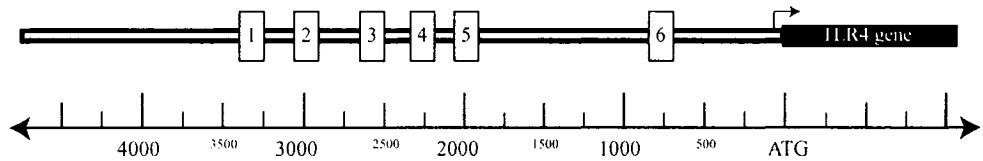

Figure 3.25. Potential STAT6 binding sites determined using MatInspector Professional transcription factor analysis software. Six potential sites were determined with a high core similarity and matrix similarity. The core nucleotides are labelled in bold text and in capital letters. The position and sequence of the potential binding sites with respect to the ATG start codon of the TLR4 gene is shown above.

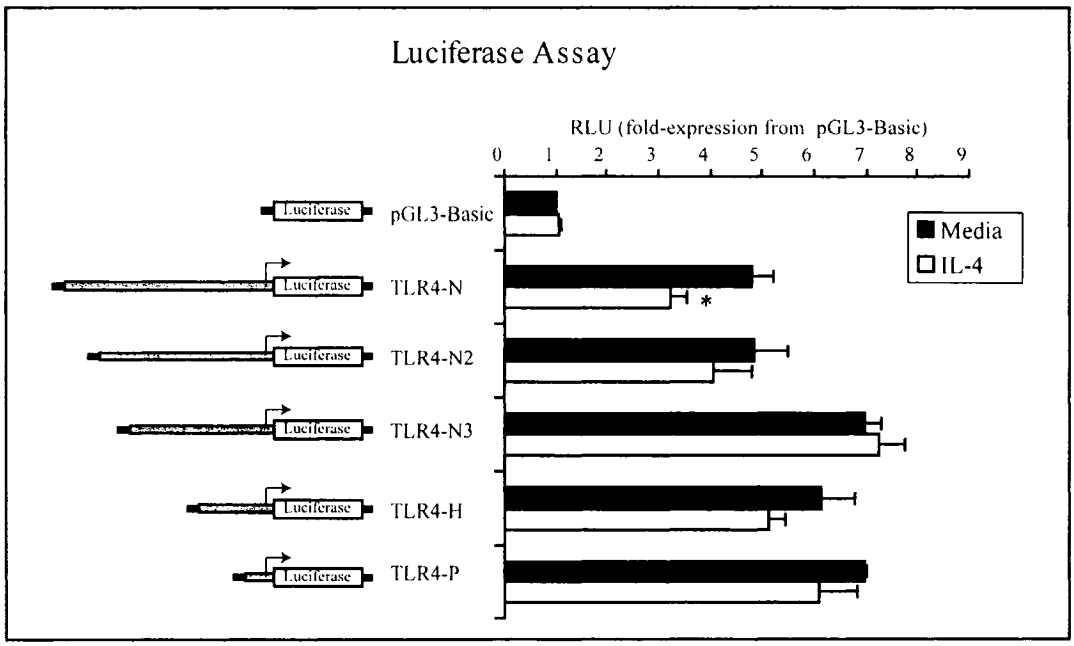

Figure 3.26. Activation of upstream proximal promoter of TLR4 in U-937 cells as measured by the luciferase reporter assay. U-937 cells were transiently transfected with luciferase reporter plasmids containing different lengths of the upstream region of TLR4. After transfections cells were incubated with and without 10ng/mL of IL-4 for 12 hours. pGL3-Basic is the reporter plasmid without any cloned insert and was transfected into the U-937 cells to serve as a negative control. A *Renilla* luciferase vector was used to correct for transfection efficiency. Relative light units (RLUs) were obtained by performing the dual-luciferase assay. Results are expressed as the RLU fold expression compared to the RLU cells transfected with pGL3-Basic. *$P<0.05$ versus baseline.

TLR4-H (4.1±2.2) and TLR4-P (7.3±0.5) compared to levels of unstimulated cells (6.1±1.9, 6.9±0.2, 4.8±1.9 and 7.0±0.3 respectively). These results suggest that only TLR4-N contains an IL-4 responsive region (shown in Figure 3.27).

3.2.7 STAT6 Chromatin Immunoprecipitation

STAT6 ChIP was used in combination with real-time QPCR to determine if the potential STAT6 binding sites can bind to STAT6 protein upon stimulation with IL-4 (n=3). Only one of the potential STAT6 binding sites determined using MatInspector software was found in the IL-4 responsive region determined using the luciferase assay. The potential STAT6 binding site in the IL-4 responsive region (site 1) and the potential STAT6 binding site closest to the IL-4 responsive region (site 2) were investigated (Figure 3.27). Upon IL-4 stimulation, a significant increase in STAT6 binding to site 1 (mean fold upregulation compared to baseline level ± standard deviation; 3.7±0.9) was determined compared to unstimulated cells. There was no significant increase in binding of STAT6 to site 2 upon IL-4 stimulation (0.5±1.2). The isotype control was not significantly different to the unstimulated cells for both site 1 (0.5±1.2) and site 2 (0.3±1.4). Therefore, it was concluded that STAT6 can bind to the potential STAT6 binding site in the IL-4 responsive region.

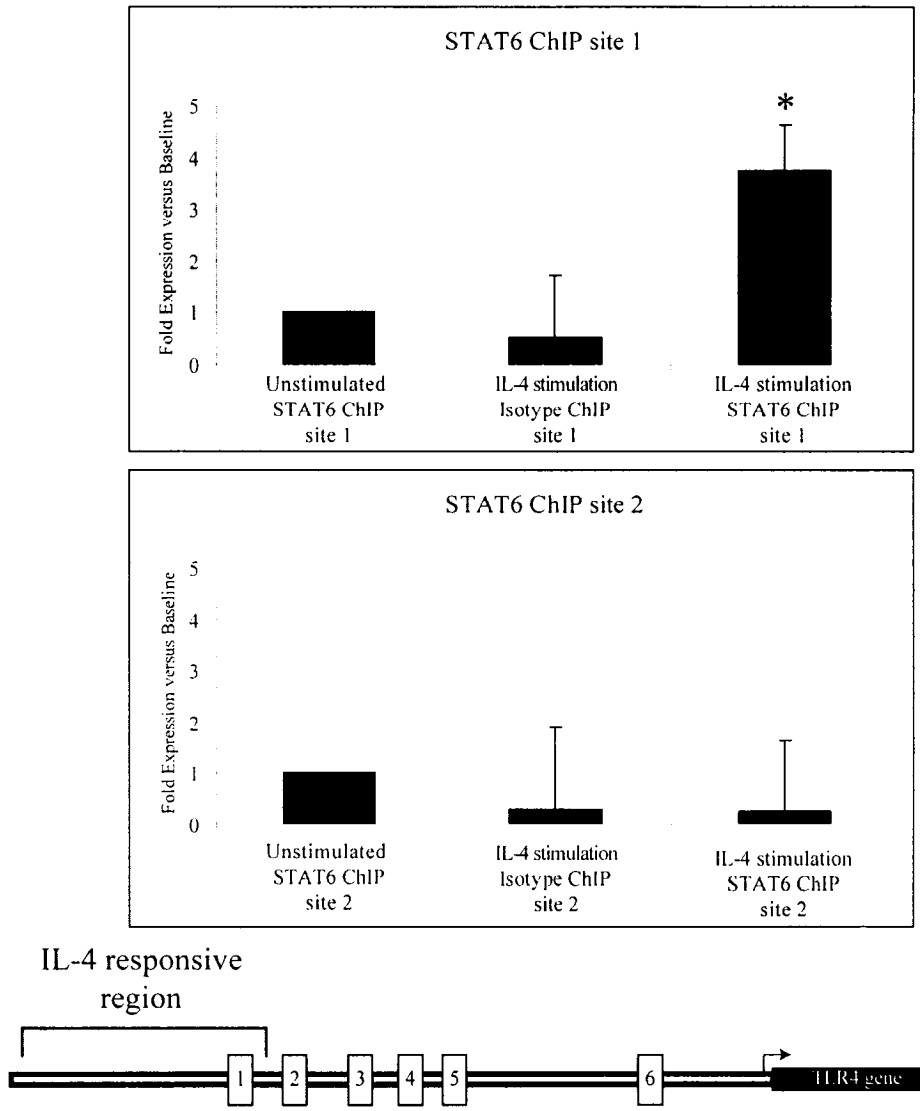

Figure 3.27. Measurement of STAT6 binding to DNA upstream of the TLR4 gene measured by STAT6 chromatin immunoprecipitation (ChIP). U-937 cells were incubated with 10ng/ml of IL-4 for 6 hours and DNA bound to activated STAT6 was purified by ChIP. Real-time QPCR was used to determine whether STAT6, after challenge with IL-4, could bind to the potential STAT6 binding site (site 1) in the IL-4 responsive region. An isotype control was used in the ChIP as a negative control for background staining. Primers were also designed for the potential STAT6 binding site (site 2) outside of the IL-4 responsive region but closest to site 1. Quantification was performed using ΔΔCt method using a ChIP for RNA polymerase II binding to the GAPDH promoter as a reference. Results are expressed as fold expression of IL-4 stimulated cells compared to unstimulated cells. *$P<0.05$ versus isotype (n=4).

3.3 TLR4 Expression in Peripheral Blood Mononuclear Cells from Children

3.3.1 Flow Cytometry Monocytes and $CD4^+$ Cells

IL-4 could inhibit TLR4 expression in the U-937 cell line, and blood from children was investigated to confirm that it was not a cell line specific effect. The FSC and SSC parameters were used to gate the monocyte and lymphocyte fractions(Figure 3.28; n=5), similar to a procedure by Mita et al.[489]. Monocyte fractions made up 15-20% of the PBMCs isolated from children's blood. TLR4 expression of the monocyte population cultured in media had a high baseline level of TLR4 positive cells with a mean MFI of 155±8.5 (Figure 3.29). When incubated with IL-4 for 24 hours, the absolute number of monocytes expressing TLR4 did not differ significantly, but the MFI decreased significantly (126±15.5).

PBMCs were stained with anti-CD4 and anti-TLR4 antibodies (Figure 3.30). A population of the PBMCs were positive for both TLR4 and CD4 protein surface expression (Figure 3.31; mean percent of total CD4 population±standard deviation; 16.0±7.1%). In contrast to the monocytes, when the PBMCs were stimulated with IL-4, the population of CD4 positive and TLR4 positive cells was significantly decreased (8.1±5.6%). To confirm that the CD4 positive cells were T cells, the PBMCs were stained with both anti-CD4 and anti-CD3 antibodies (Figure 3.32). It was determined that greater than 99.5% of the cells that express high levels of CD4 are T cells (CD3 positive). Incubation with IL-4 did not significantly alter the levels of expression of CD4 demonstrating that reduction of the $CD4^+/TLR4^+$ is due to decreased surface expression of TLR4.

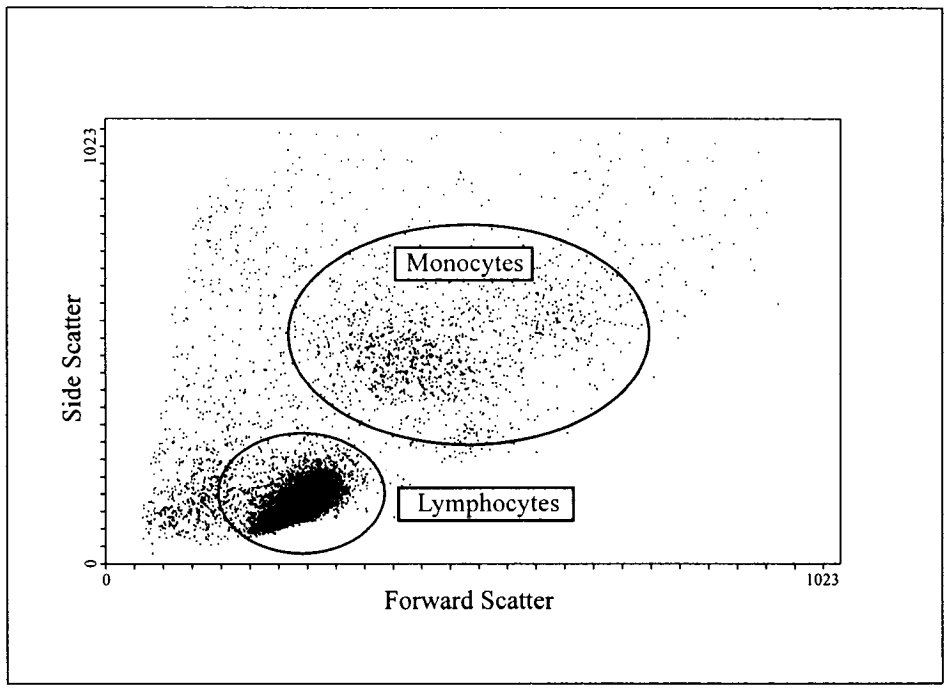

Figure 3.28. Forward and side scatters acquired by flow cytometry from PBMCs isolated by Ficoll gradient centrifugation. Two clear populations of cells were seen. Cells with low forward scatter and low side scatter are the lymphocyte population while cells that have a higher forward scatter (larger cells) and a higher side scatter (more granular cells) are the monocyte population.

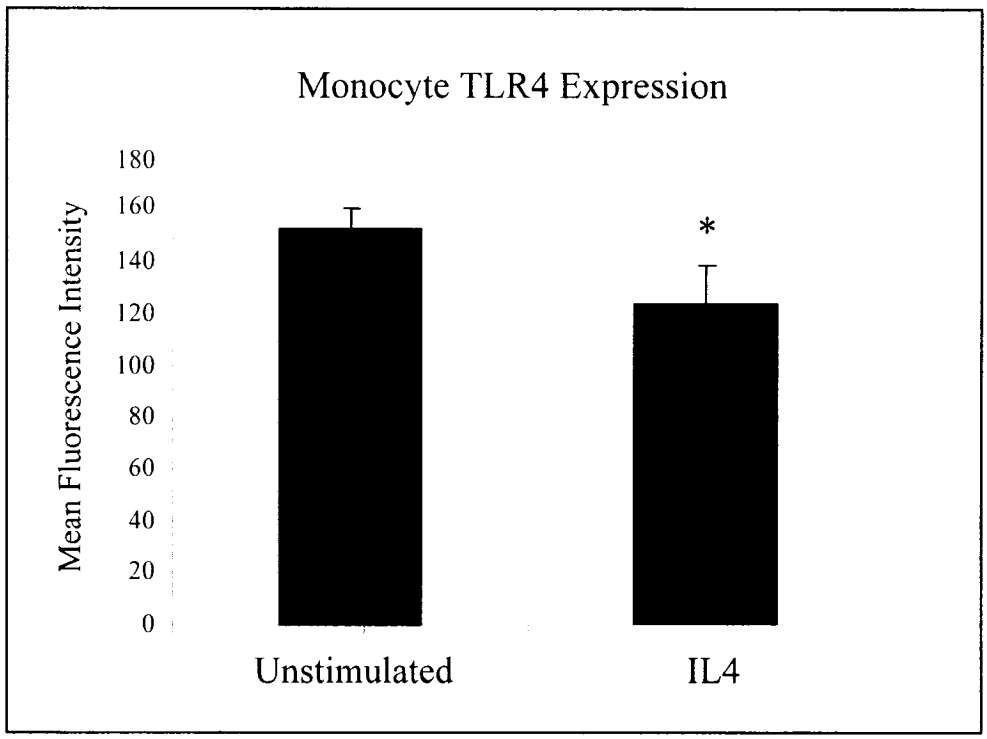

Figure 3.29. Surface expression of TLR4 on monocyte population from PBMCs as measured by flow cytometry. PBMCs were purified from whole blood by Ficoll gradient centrifugation and cultured for 24 hours with and without 10ng/mL of IL-4. Cells were stained with an Alexa Fluor 488 labelled monoclonal antibody for TLR4 and fluorescence was acquired on the monocyte population by using the foward scatter and side scatter parameters. An isotype control, labelled with Alexa Fluor 488, was used to determine background fluorecscence. Results are expressed as the raw value of the mean fluorescence intensity minus the background fluorecescence.*$P<0.05$ versus unstimulated cells.

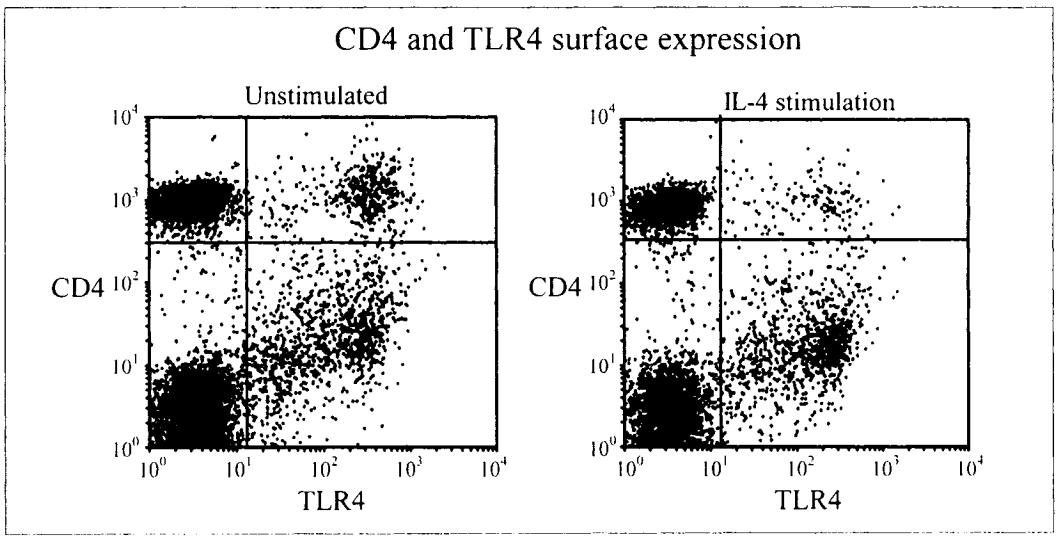

Figure 3.30. Dot plots for the surface expression of CD3 and TLR4 from PBMCs as measured by flow cytometry (representative example). PBMCs were purified from whole blood by Ficoll gradient centrifugation. Cells were cultured with 10ng/mL of IL-4 (right) or cultured unstimulated (left) for 24 hours. Cells were stained with Alexa Fluor 488 labelled monoclonal anti-TLR4 and APC labelled monoclonal anti-CD4 antibodies. Isotype controls, labelled with FITC or APC respectively, were used to determine background fluorescence.

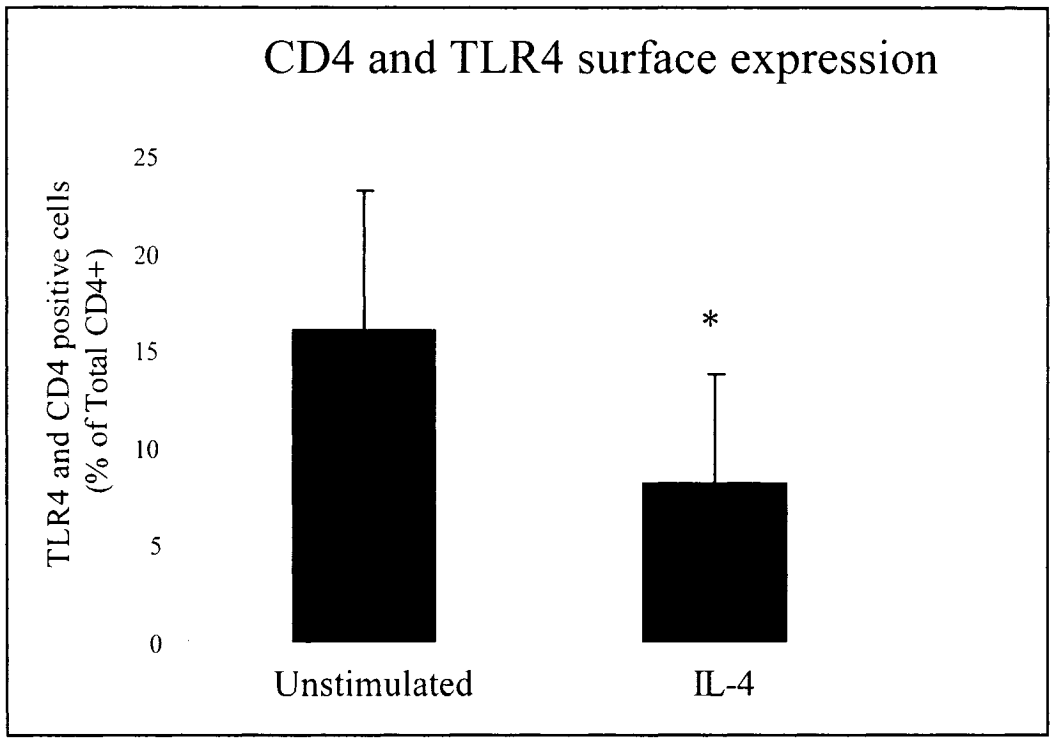

Figure 3.31. Surface expression of TLR4 and CD4 on PBMCs as measured by flow cytometry. PBMCs were purified from whole blood by Ficoll gradient centrifugation and cultured for 24 hours with and without 10ng/mL of IL-4. Cells were cultured with or without 10ng/mL for 24 hours. Cells were stained with Alexa Fluor 488 labelled monoclonal anti-TLR4 and APC labelled monoclonal anti-CD4 antibodies. Isotype controls, labelled with FITC or APC respectively, were used to determine background fluorescence. An isotype control, labelled with Alexa Fluor 488, was used to determine background fluorecscence. Results are expressed as the percent of TLR4 and CD4 positive cells from the total population of CD4 positive cells. *$P<0.05$ versus unstimulated cells.

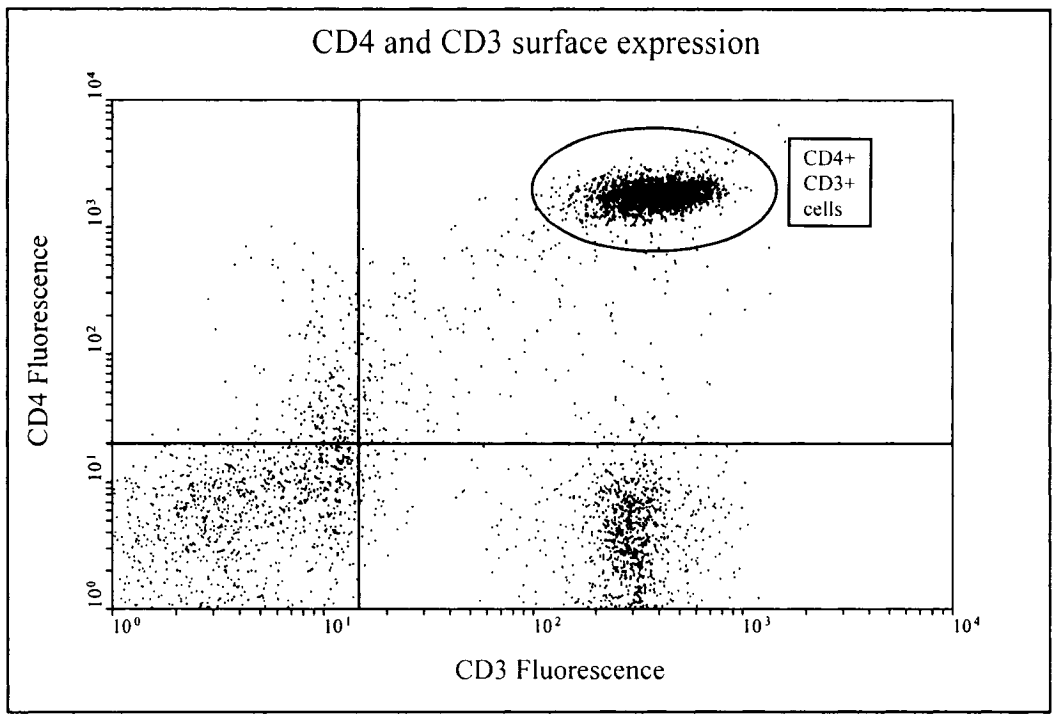

Figure 3.32. Dot plot for the surface expression of CD3 and CD4 from PBMCs as measured by flow cytometry (representative example). PBMCs were purified from whole blood by Ficoll gradient centrifugation. Cells were stained with a FITC labelled monoclonal antibody for CD3 and a APC labelled monoclonal antibody for CD4. Isotype controls, labelled with FITC or APC respectively, were used to determine background fluorescence. Circled cells are the cells that express high levels of both CD4 and CD3.

Chapter 4: Discussion

4.1 Introductory Remarks

Infectious diseases have been a longstanding and still existing cause of morbidity and mortality. It is only recently in history that humans have been able to defend themselves with better sanitation, urbanization, vaccination and antibiotics. These measures have decreased the load of exposure to pathogens and may be associated with a rise in other inflammatory diseases. The hygiene hypothesis suggests that to develop proper immunoregulatory mechanisms to prevent irregular damaging responses, as in the case of allergic diseases, the immune system must be exposed to a certain amount of low-grade pathogens or at least components from pathogens[247]. In other words, bacterial components would induce immunoregulatory mechanisms against the development of atopy and Th2 inflammation.

The complex regulatory components of the immune system are slowly being elucidated. These components are necessary to prevent overactive immune responses. One immunoregulatory mechanism is the ability of the cytokines of the two arms of the immune system, the cell-mediated response and the humoral response, to counter-regulate each other. Another mechanism is the induction of anti-inflammatory cytokines, such as IL-10, which has general anti-inflammatory effects. In allergic responses, Th2 cytokines predominate to produce an IgE-mediated humoral response; therefore, promoting Th1 cytokine production and IL-10 production with bacterial components such as LPS could reduce Th2 inflammation in allergic disease.

Th2 cytokines have been shown to inhibit responses to LPS. This may be a mechanism for development of persistent allergic inflammation; LPS may be present in

the environment, but it can not provide its benefits because of overwhelming Th2 responses. The Th2 cytokine IL-4 has been shown to downregulate expression of the main LPS receptor component TLR4[489], but the mechanism by which this occurs has not been elucidated and also if Th2 cytokines has similar effects on T regulatory cells.

The above premises form the basis of this thesis. It was determined that LPS can benefit allergic inflammation in children, by promoting Th1 cytokines and IL-10. The results discussed here were acquired by using nasal mucosal tissue resected from children and adult subjects who were sensitive or insensitive to allergens. Atopic nasal mucosa from children reacted to allergen with a typical Th2 cytokine inflammatory profile, but allergen could reduce these effects by inducing IFN-γ, IL-12 and IL-10. The shift in cytokine profiles was associated with increased production of immunoregulatory IL-10 and proliferation of $CD3^+$ T lymphocytes. T cells also expressed TLR4 and the markers CD4 and CD25. The effects of LPS in children were mediated by TLR4, and similar responses were not seen in nasal tissue from allergic adults. While TLR4 is generally thought to be expressed by dendritic cells, macrophages and monocytes, it has been recently reported to be expressed on murine $CD4^+CD25^+$ regulatory T cells[433]. Results in this thesis provide evidence that TLR4 is highly expressed on $CD3^+$ T lymphocytes in humans.

The difference seen between children and adults and between atopic adults and non-atopic adults in TLR4 expression suggests downregulatory mechanisms at play during the development of long-standing atopy. In a cell model present in this thesis, IL-4 rapidly decreased the transcriptional activity of the upstream region of TLR4, TLR4 mRNA, TLR4 protein surface expression as well as LPS responsiveness. This suggests a

form of transcriptional regulation. STAT6 is a transcription factor and its activation is the result of one of the major pathways of IL-4 signal transduction. IL-4's inhibitory effect on TLR4 was enhanced and prolonged if STAT6 was overexpressed and inhibition of tyrosine phosphorylation abolished the effect of IL-4 on TLR4 expression. This evidence points to the role of STAT6 in downregulating TLR4 expression at the level of transcription. STAT6 mediates its functions by binding to specific sequences of DNA, and computer analysis of the upstream region of the TLR4 gene revealed six potential STAT6 binding sites. Truncations of the upstream region of TLR4 gene revealed an IL-4 responsive region and STAT6 was shown to bind to this region. In addition, results from this thesis demonstrate for the first time that $TLR4^{+}CD4^{+}$ T cells are found in the peripheral blood of children. These cells are also highly responsive to IL-4, which can downregulate TLR4 expression after 24 hours of incubation with the cells. Taken together, these findings point to a role of LPS in inhibiting allergic responses via TLR4 and that Th2 cytokines can limit these responses by reducing TLR4 expression.

4.2 Discussion of Results

The first part of this thesis largely focused on the nasal explant model, where small sections from the inferior turbinate are cultured outside the body with various stimuli. The advantage of this technique is that it allows the study of the effects of various inflammatory challenges without being confounded with the influence of cellular recruitment from the systemic circulation; all responses to challenges are solely derived from tissue constituents. At the same time, it affords a view into local immune responses in a tissue that is regularly exposed to environmental challenges and which participates in the symptoms of allergic rhinitis. The explanted nasal mucosa model has been used

successfully in the past as part of numerous studies in allergic rhinitis. This model has been used to describe local eosinophilic differentiation [43], local upregulation of Th2 transcription factor GATA3[62] and production of Th2 cytokines and IgE [90, 91]. Despite the advantages of this model, there are limitations worth mentioning. Firstly, the small size of these biopsies limits the ability for the number of stimulations that could be used for each patient. Secondly, again due to small amounts of tissue, an accurate quantification of mRNA or protein production following LPS stimulation is limited even with powerful techniques such as laser capture microdissection and real-time QPCR. For this reason, histological techniques, such as *in situ* hybridization and immunohistochemistry, were well suited for use in the explant model. The techniques offer information about cell numbers and cellular expression of both mRNA and protein levels, but not absolute values for these markers. Despite this, these techniques are sufficiently robust to measure numbers of cells expressing a specific mRNA and protein species giving valuable information about local reactions to LPS or allergen. Thirdly, the culture conditions may limit the explant model. The tissue is incubated on filter disks that float over the culture media from which the tissue is nourished. This is to mimic the environment the biopsy had within the body, where the local blood vessels and surrounding tissue would nourish it. In this system, allergen and/or LPS are added to the media and absorbed into the tissue from the submucosa; epithelial cells would not be the first cells to be in contact with the challenge, as would be the case *in vivo*. Although the tissue is not submerged in media, studies where the tissue was incubated with modified nucleotides (BrdU), fluorescently tagged oligonucleotides or nuclear DNA binding dyes (Hoechst 33342) have shown that the elements from the wells of media can adequately

diffuse throughout the tissue[91, 293]; therefore, allergen, which are small lipophilic proteins, or LPS would permeate the tissue as well. Regardless of the limitations, the explant model has been sufficiently characterized, has been successfully utilized in past reports and offers valuable insight into local immune responses using human tissue.

The ability of LPS to prevent allergen-induced inflammation has been described in animal models, but not in humans. Epidemiological studies point to a potential role of LPS inhibiting atopic diseases in children[264, 266, 267], but LPS has been associated as a risk factor for occupational asthma[272] [273, 276] and even in the clinical severity of asthma in adults[275, 278, 279, 540]. LPS challenge of the tissue from atopic children, in this thesis provides evidence supporting the epidemiological studies and points to the possibility that responses to LPS in nasal tissue from children are different from adults. In the explant model, LPS induced a strong induction of Th1 cytokine mRNA (IL-2, IFN-γ and IL-12) positive cells and was associated with increased numbers of cells positive for CD68(macrophages), elastase (neutrophils), tryptase (mast cells) and large increases in CD3 (T cells) positive cells. Allergen challenge produced characteristic increases in Th2 cytokine mRNA production such as IL-4, IL-5 and IL-13 and a slight increase in IL-10. MBP positive (eosinophils) cells and mast cells, prominent cells in allergic inflammation, were also increased by allergen, and this increase was probably related to increases in Th2 cytokines. It is possible that allergen also increased other growth factors for these cell types such as IL-3, stem cell factor (SCF) and GM-CSF involved in mast cell growth, but these were not measured. T cells were also increased by allergen and this may be due to large increases in IL-2 positive cells. IL-2 is a powerful growth inducing cytokine of lymphocytes and is produced upon antigenic or mitogenic activation

of T cells[541]. The receptor for IL-2 is composed of an α-subunit (CD25), a β-subunit (CD122), and a γ-subunit (CD132). On resting T cells, the β and γ chains are expressed constitutively and form a low affinity IL-2 receptor, but upon activation, the CD25 subunit is expressed forming a high affinity receptor[542]. Increased expression of CD25 upon LPS and allergen challenge demonstrates an increase of cells with high affinity for IL-2. CD25 is also constitutively expressed on T regulatory cells in high levels[543], and increased numbers of CD25 positive cells suggests their proliferation. Furthermore, LPS could diminish allergen-induced Th2 inflammation and skew it toward Th1 inflammation in the nasal mucosa of atopic children; in tissues stimulated with both allergen and LPS, the Th2 cytokine mRNA positive cells were all reduced to baseline and IFN-γ, IL-12 and IL-10 mRNA levels were elevated. LPS also decreased the allergen induced increases in mast cells and eosinophils. Similarly to LPS challenges alone, T cell levels increased upon LPS challenge with allergen and was associated with increases in IL-2 and CD25.

Although IgE forms the basis of sensitization, levels of expression with allergen and/or LPS challenge were not measured in this work; this study was restricted to cytokine and inflammatory cell numbers. The primary reason for this was the limited amounts of tissue that were available for the study. A secondary reason is that levels of IgE are much lower in children than in adults and only reach adult values at the age of 10[544]; all the children from which explants were obtained for this study were younger than 4 years old. The production of IgE has been measured in the nasal mucosa of adults in the explant model[90-92]. Not only is IgE production induced by allergen in the tissue, but also IgE isotype switching. These events were shown to depend on local production of IL-4 and IL-13, as downregulating IL-4 mRNA with antisense oligonucleotides or

neutralizing antibodies blocks IgE mRNA production and IgE isotype switching[91, 92]. LPS can inhibit IgE production in animal sensitization models[285, 286]. The mechanism is not clear but may be through induction of Th1 cytokine and IL-10 production. IFN-γ, IL-12 and IL-10 have all been shown in separate studies to directly or indirectly inhibit IgE production[545, 546]. IFN-γ can also downregulate IεCε RNA and CD40L expression, both necessary for IgE isotype switching[547]. Based on these studies, LPS could play a role in inhibiting IgE production in the nasal mucosa of children by downregulating Th2 cytokines and upregulating Th1 cytokines and IL-10.

Neutralizing antibodies against IFN-γ, IL-10 and IL-12 were used to determine which of these cytokines induced by LPS had the most prominent role in downregulating Th2 inflammation. IFN-γ is one of the most important cytokines for the cell-mediated inflammatory response, primarily produced by Th1 cells and has large impacts on macrophage functions[548]. IFN-γ can inhibit both IgE isotype switching and Th2 cell proliferation[549, 550]. Neutralization of IFN-γ, in allergen and LPS challenges, did not cause a significant increase in IL-4, IL-5 or IL-13 mRNA positive cells compared to allergen stimulation alone. However, neutralization of IFN-γ did impact on IL-5 protein positive cells, demonstrating its inhibitory role in Th2 cytokine production and suggesting possible post-transcriptional regulatory mechanisms controlling IL-5 protein synthesis.

IL-10 is produced by many cell types such as monocytes and macrophages, but it is also produced by T cells, including Th1, Th2 and regulatory T cells[551-553]. IL-10 has numerous anti-inflammatory effects. IL-10 can inhibit Th1 and Th2 cytokine production[551]. IL-10 can also inhibit MHC class II molecule and B7 expression

reducing the ability of antigen-presenting cells to present antigen and activate T cells [548, 554, 555]. IL-10 can also inhibit IgE synthesis and eosinophil survival[556, 557]. When IL-10 was neutralized in the nasal mucosa, the inhibitory effects of LPS on Th2 cytokine mRNA production were abrogated. Similarly, neutralization of IL-10 augmented the number of IL-5 protein producing cells. These results show IL-10's potent role in downregulating Th2 cytokine production. Surprisingly, neutralization of IL-10 caused a reproducible reduction of IL-12 and IFN-γ after LPS stimulation. IL-10 has been demonstrated to reduce IFN-γ[516] and IL-12[558], and its neutralization should have caused an increase in their production. This effect needs further investigation to determine if this is an explant-specific phenomenon or an unknown regulatory process found in children. Immunohistochemistry for Th1 and Th2 cytokines after IL-10 neutralization were similar to the *in situ* hybridization results, suggesting that IL-10's effects may be at the level of transcription and not translation.

IL-12 is a heterodimeric cytokine produced by many cell types but mostly antigen-presenting cells in response to bacteria or bacterial products[559, 560]. IL-12 induces cytokine production, proliferation and enhanced cytotoxic ability of NK cells and T cells. IL-12 is particularly a potent inducer of IFN-γ of both NK cells and T cells, and inhibits IL-4 production[561, 562]. IL-12 was greatly induced by LPS challenge of the mucosa, similarly to previous results[293]. Neutralization of IL-12 upon simultaneous LPS and allergen challenge caused increases in IL-4, IL-5 and IL-13 mRNA positive cells, to levels comparable of allergen challenges. At the same time, neutralization of IL-12 reduced IFN-γ mRNA positive and protein positive cells, further demonstrating the functions of IL-12 at inducing Th1 cytokine production and downregulating Th2 cytokine

production. IL-12 is known to induce IL-10 production from T cells, possibly to limit its pro-inflammatory effects[563, 564]. In explants, IL-12 neutralization significantly reduced IL-10 production, supporting the concept that IL-12 plays a role in inducing IL-10, and may act together to prevent allergen-driven Th2 allergic response. Furthermore, it is important to note that the relative number of IL-10-immunoreactive cells following LPS stimulation was almost five times higher than the number of IL-12-immunoreactive cells, suggesting that IL-10 may be the dominant cytokine in LPS-induced inhibition of allergic inflammation. The effects of IL-12 neutralization were seen at the level of mRNA and protein, suggesting that IL-12 inhibits transcription of Th2 cytokines. When both IL-12 and IFN-γ were neutralized together, no additive effect in inducing Th2 cytokines was observed, and the effect was comparable to neutralization of IL-12 alone. This demonstrates that IL-12 has a greater effect than IFN-γ in diminishing allergen induced inflammation. Dual neutralization of IL-12 and IFN-γ caused a similar effect to single neutralization of IL-12 on levels of IL-10, supporting the greater role IL-12 plays in IL-10 induction. Interestingly, neutralization of IL-12 and IFN-γ caused an additive decrease in IL-12 mRNA and protein positive cells, compared to single neutralizations. This suggests that IFN-γ can have a positive feedback on itself by promoting IL-12 production in the tissue. This is supported by findings showing that IFN-γ can induce production of IL-12, and in many cases, IFN-γ is necessary for IL-12 to be produced [565-567].

The nasal mucosa from young children, irrespective of their atopic state, is primed to respond quickly to bacterial products. Similarly to a previous report[293], the cells that seemed to respond the most to LPS stimulation in the mucosa were $CD3^+$ cells. In

only 24 hours, LPS caused a dose dependent increase in $CD3^+$ cells in both the nasal mucosa of atopic and non-atopic children, and for each dose, no difference was seen between the patient groups. Increase in these populations in the nasal mucosa of atopic children was shown to be due to local proliferation of these cells by the BrdU uptake assay. It is also possible that stimulation may increase marker expression and experiments would need to be performed to determine the effects of LPS on total cellularity of the tissue. 60% of the $CD3^+$ cells were positive for CD25, and many of these cells were also positive for CD4. At the same dose of LPS, $CD3^+$ cells and IL-10 mRNA positive cells both rose about 6-fold in the tissue, suggesting that $CD3^+$ were activated to produce IL-10. In addition, numbers of $CD25^+$ cells were similar to IL-10 mRNA positive cells. Taken together, this provides evidence that naturally occurring $CD4^+CD25^+$ immunoregulatory T cells in the nasal explants from children are likely to be the major source of IL-10, which is involved in controlling Th2 responses to allergen upon LPS stimulation. Many cells were positive for TLR4 that were not $CD4^+$, and these may also be involved in production of IL-10. Further experiments would be needed to characterize these cells and their involvment in LPS-mediated downregulation of allergic inflammation.

To activate T cells, usually antigen presentation and accessory costimulatory molecules, such as CD80 and CD86, are necessary. *In vitro* experiments have shown that $CD4^+CD25^+$ T regulatory cells seem to mediate fully competent suppression in the absence of CD80 or CD86[568]. This suggests that, while costimulation is necessary for the development of T-regulatory cells, their suppressor functions may be activated independently of antigen presentation[569]. These concepts support the results obtained

from LPS challenge of the nasal explants; LPS without antigen caused increases in $CD3^+$ cells in both atopic and non-atopic mucosa. A recent report showed that murine $CD4^+CD25^+$ T regulatory cells selectively express TLR4, TLR5, TLR7 and TLR8[433]. In addition, LPS stimulation of murine $CD4^+CD25^+$ T regulatory cells with LPS enhanced suppressor function, survival and proliferation, all of which was independent of antigen-presenting cells. Expression of TLR4 on CD3 positive cells was investigated to determine if this population of cells existed in children. Indeed, many $CD3^+$ cells were positive for TLR4. In addition, allergen did not increase the $TLR4^+CD3^+$, but when the tissue was simultaneously challenged with LPS and allergen, a large increase was seen. TLR4 expression was also co-localized with IL-10 and an increase in double positive cells was seen upon 24 hour incubation with LPS. Overall, the results obtained suggest that $CD4^+CD25^+$ T regulatory cells in the atopic mucosa of children express TLR4, and LPS directly causes this population to proliferate and produce IL-10.

The LPS-induced increase in CD3 immunoreactivity and associated increase in IL-10 mRNA expression in atopic mucosa from children was not observed in atopic adults. LPS unresponsiveness of atopic adults was most likely attributable to the extremely low basal expression of TLR4 when compared to both atopic and non-atopic children. As in other reports, LPS sensitivity seemed dependent upon TLR4 expression[484]. Non-atopic adults had a significantly higher level of TLR4 positive cells and TLR4 positive cells increased with LPS stimulation. Studies have shown that several mutations in TLR4 may account for decreased LPS responsiveness[498, 570], but besides results in this thesis, variability in responsiveness to LPS was never associated with differences in TLR4 expression between atopic and non-atopic individuals.

The levels of TLR4 positive cells in the nasal mucosa of non-atopic adults were significantly lower than both atopic and non-atopic children. Decreased expression of TLR4 in non-atopic adults may be related to the ability of the immune system to decline with age. Recent studies by Renshaw and colleagues have shown that in mice, TLR activation by their ligands and even the abundance of TLRs decrease with age[571]. In other studies, levels of TLR4 on monocytes did not differ with age in both mice and humans[572, 573]. Therefore, differences in numbers of TLR4 positive cells between children and adults may be cell-type dependent. This is supported by the report that T cells in peripheral blood of healthy adults were shown to have low levels of TLR4 mRNA, but this was never compared to levels in children[432]. Levels of TLR4 expression may also depend on PAMP exposure during life, and if an individual is expressed to a lesser load of PAMPs, levels of TLR4 may decrease. To clarify this issue, a larger population of adults would have to be studied and correlations would have to be performed with age and LPS exposure levels.

The protective effect of LPS in atopic children against allergen-induced inflammation is most likely driven directly by TLR4 and not other TLR receptors. This is supported by results obtained using purified LPS preparations, which selectively activates TLR4 only and not TLR2 or other TLRs. Differences in LPS sensitivity between adults and children may not only be limited to TLR4 expression. TLR4 is physically associated with MD-2 in its extracellular domain, and MD-2 is essential for binding of LPS and activation of TLR4[445-447], and its levels have not been investigated in this work. In addition, LPS is transferred to TLR4 via the protein CD14[452, 453]. Recent studies have shown that neonate monocytes have reduced CD14

expression on their surface as well as reduced intracellular Myd88 expression[572, 574]. It is speculated that reduced neonate responsiveness to LPS are to reduce the risk of inflammatory reactions *in utero* that could lead to premature delivery[572]. Thus, there are important differences in the capacity of adults, children and even neonates to respond to bacterial LPS, which may play a vital role in maturation of the immune system.

Atopic nasal mucosa from adults is associated with high expression of Th2 cytokines and Th2-associated transcription factors[37, 61, 90, 91, 575], which may play a role in reducing TLR4 and LPS responsiveness. The immortalized cell line, U-937, was used as a model to determine the regulation of TLR4 by the Th2 cytokine IL-4 and by the transcription factor STAT6. The U-937 monocytic cell line was obtained from the American Type Culture Collection (ATCC), and the original stock was derived by Sundstrom and Nilsson in 1974 from malignant cells of monocytic origin, obtained from the pleural effusion of a patient with histiocytic lymphoma [576]. This cell line was chosen for numerous reasons. U-937 cells are well characterized, used often in research, are easy to culture *in vitro*, express high levels of baseline TLR4 and are sensitive to LPS [577]. They also express functional IL-4 receptor and STAT6 and respond to IL-4 similarly to peripheral blood monocytes [538, 578]. The experiments are also simplified by use of a homogenous cell line and are therefore more reproducible. Many of the experiments required transfection of plasmids into cells and primary cells from patients are very sensitive and vary significantly in the conditions needed to achieve efficient transfection results. Although cell lines give insight into underlying mechanisms, results obtained should be confirmed with primary cells from human subjects.

Several reports have shown that U-937 cells produce TNF-alpha, IL-1β, and IL-10 upon LPS stimulation[306, 579-581]. In these reports, IL-4 could reduce LPS-induced production of TNF-α, IL-10 and IL-1β, but the mechanism has not been determined. Two major pathways of signal transduction are activated by LPS, the early MyD88-dependent pathway and the late MyD88-independent pathway[454-456, 467]. Activation of the early Myd88-dependent pathway leads to rapid activation of NF-κB[341, 456, 467]. The MyD88-independent pathway also involved activation of NF-κB, but with slower kinetics than the MyD88-dependent pathway[467]. In this work, NF-κB activation by LPS was measured with or without IL-4 stimulation. LPS stimulation caused a robust activation of NF-κB which was significantly reduced when U-937 cells were preincubated with IL-4 for 24 hours. If IL-4 was added at the same time as LPS, IL-4 had no significant effect, suggesting that preincubation activates regulatory mechanisms which require time to have an effect. In studies on sepsis, for IL-4 to have protective effects it needed to be administered before LPS exposure [307, 311, 582]. The mechanism by which this is occurs was not determined, but it can be either through downregulation of LPS receptor components, inhibition of signal transduction components or cross talk between signal transduction pathways. One possibility is that IL-4 induces the suppressor of cytokine signaling 1 (SOCS1)[583], which can downregulate IRAK1 activation, necessary for the MyD88-dependent pathway [357, 584].

This work also shows that the mechanism by which IL-4 mediates its inhibitory effects may be related to regulation of TLR4. IL-4 inhibited TLR4 mRNA and transcriptional activity as early as 6 hours in U-937 cells, but it was only after 12 hours that surface expression of TLR4 was significantly decreased. This suggests that time is

required for IL-4 to inhibit LPS signaling machinery, and this may be due to the time it takes for existing TLR4 to be degraded and replaced by nascent TLR4. The time point where a significant reduction in surface expression of TLR4 was seen coincides with the incubation time that caused decreased LPS responsiveness as measured by the NF-κB assay. It is possible that IL-4 can inhibit other components of the LPS receptor, such as MD-2 and CD14, but this was not investigated in this work. IL-4 has been shown to be able to reduce CD14 surface expression in monocytes, but this mechanism has not been investigated for its effects on LPS responsiveness[585].

Downregulation of IL-4 mRNA and transcriptional activity of TLR4 suggests that IL-4 activates transcriptional repression of the gene. IL-4 functions by binding the IL-4 receptor, which consists of an IL-4Rα subunit and the γ common (γ_c) chain subunit[173, 174], or by binding to the IL-13 receptor, which consists of the IL-4Rα subunit and the IL13Rα_1 subunit[185]. The main signal transduction pathway activated by IL-4 binding to its receptor is the Jak-STAT pathway involving Jak1[175] and Jak3 [175, 176] and both can phosphorylate STAT6. Phosphorylated STAT6 homodimerizes, migrates to the nucleus, binds to STAT6-response elements and once there it regulates transcription. Many genes activated by STAT6 are important for allergic inflammation, such as IgE[88, 89] or the chemokine eotaxin[168-170] and STAT6-deficient animals have defective IL-4 responses[58]. STAT6 has been shown to play an important role in LPS-mediated sepsis[314]. STAT6-deficient mice have uncontrolled production of cytokine and chemokine production after challenge with LPS, and the mice also succumb to sub-lethal doses of LPS compared to their wild type counterparts. This suggests an essential role for STAT6 in controlling LPS sensitivity.

As well as an activator of transcription, STAT6 has been shown to inhibit transcription of many genes[586]. The N-terminal domain of STAT proteins mediates DNA binding[587, 588]. The C-terminal part of STAT6 protein is a proline-rich domain involved in activation of transcription, called the transactivation domain[589-591]. Transcription factors function by binding other proteins known as cofactors of transcription that recruit additional cofactors or the basal transcription machinery to the promoter site and/or function as chromatin modifying enzymes. STAT6 has been shown to associate with CREB-binding protein (CBP) while another region associates with nuclear receptor coactivator 1 (NCoA-1)[591, 592]. Both these regions of STAT6 significantly increase transcription, potentially through induction of histone acetylase activity, which opens up chromatin for transcription. STAT6 can inhibit transcription by competing for binding sites of other transcription factors such as STAT1 and NF-κB[593, 594]. STAT6 can also inhibit transcription by competing with other transcription factors for co-activators of transcription[595]. NF-κB has been shown to associate with both CBP and NCoA-1[596, 597], but competition between STAT6 and NF-κB for these factors has yet to be described. In U-937 cells, Jak kinase activity was essential for inhibition of TLR4 mRNA by IL-4; if Jak kinases were inhibited using tyrphostin AG 490, IL-4 had no effect on levels of TLR4 mRNA. Effects of the inhibitor on TLR4 surface expression and transcriptional activity were not measured. Transfection of U-937 cells with STAT6, to cause constitutive overexpression of this protein, allowed for investigation of transcriptional activity of the upstream region of the TLR4 gene. When IL-4 was added to the STAT6 transfected cells, a great reduction in transcriptional activity of the reporter plasmid was observed, which was greater than the reduction and

of longer duration than that provided by endogenous STAT6. As STAT6 inhibited the transcriptional activity of the upstream region of the TLR4 in the reporter plasmid, this suggests that STAT6 plays a direct role in regulating transcription of TLR4.

STAT6-binding sites have been identified in many promoter regions of IL-4 responsive genes. The STAT6 binding site consensus sequence is 5'-TTC$(N)_4$GAA-3', where N_4 is any series of four nucleotides[598]. Sequence analysis of the cloned upstream region of TLR4, which was determined to be sensitive to IL-4, revealed six potential STAT6 binding sites. Truncation of the upstream region cloned into TLR4 revealed that the IL-4 responsive region was present about 3400 base pairs upstream of the start codon of TLR4. This region contained only one potential STAT6 binding site. A STAT6 chromatin immunoprecipitation confirmed that STAT6 does indeed bind to this region of DNA. Although these results show that IL-4 activates STAT6 to bind to a site in the upstream region of the TLR4 gene, the mechanism by which STAT6 functions remains to be determined. Potentially, at this site, STAT6 can inhibit basal transcription of TLR4. In the first analysis of the upstream region of TLR4, Rehli and colleagues determined that an inhibitory region of DNA was present upstream of the TLR4 gene[496]. When this region was removed, transcriptional activity increased more than double, but the nature of this region has not been determined. The STAT6 binding site that was determined in this work was found to be present in this region of the upstream region of TLR4, and it is possible that STAT6 may promote the effects of this inhibitory sequence of DNA. To determine the mechanism by which STAT6 inhibits TLR4 more experiments would be required. For example, mutation of the DNA binding site of STAT6 in the overexpression plasmid could help determine if STAT6 inhibits TLR4 by

competing with other transcription factors for the binding of the DNA. Removal of the transactivation domain of STAT6 would help determine if this region of the STAT6 protein is necessary to mediate its inhibitory effects.

IL-13, another Th2 cytokine, sharing the IL-4 receptor components, can activate STAT6 and be protective against sepsis. IL-13 has been shown to reduce LPS-induced IL-6, IL-8, IL-1β and TNFα[178, 312, 313]. Although this study was largely focused on IL-4, preliminary results showed that IL-13 was capable in decreasing U-937 cell expression of TLR4 mRNA in a time and dose dependent manner (data not shown). These effects are most likely mediated by STAT6 as well. Other cytokines, such as GM-CSF and TGFβ, may have a role in the regulation of TLR4 with IL-4 and IL-13. When used in combination with IL-4, GM-CSF and TGFβ decrease TLR4 expression and LPS responsiveness in monocytes[599].

IL-4 rapidly decreased but did not abolish TLR4 expression. Rapid decrease suggests direct inhibition by STAT6 rather than transcription of indirect inhibitors. Moreover, incomplete inhibition as well as a transient effect seen suggests that IL-4 and STAT6 fine tune responses rather than completely suppress TLR4. The effect on TLR4 surface expression was modest but significant and was smaller than what was observed in primary monocytes[489]. This effect may be related to cell type differences; U-937 cells may be refractory to IL-4 treatment over time. Indeed, it has been reported that there is less STAT6 phosphorylation and less γ_c chain expression in monocytes after incubation with IL-4[306]. This may explain why overexpressing STAT6 in U-937 prolonged IL-4's inhibition of the upstream region of TLR4. Perhaps in other cell types that possess more STAT6 or are more responsive to IL-4, downregulation of TLR4 would be stronger

and of longer duration. Alternate mechanisms to downregulation of signal transduction include activation of endogenous inhibitors of STAT6 such as Src homology-containing phosphatase-1 (SHP-1)[600], protein phosphatase 2A (PP2A)[601] and SOCS-1[602]. Other mechanisms have been proposed for the regulation of STAT6 activity. Removal of a methyl group on Arg^{27} of STAT6 decreases phosphorylation of STAT6 and its nuclear translocation[603]. Regulation of STAT6 has shown to be more complex than once believed, and whether these play a role in the transient nature of IL-4 effects in the U-937 cell model was not determined.

For this work, the role of IL-4 in downregulating TLR4 in U-937 was confirmed in peripheral blood mononuclear cells from children. Peripheral blood is a quick and easy source of leukocytes including lymphocytes, granulocytes, monocytes and macrophages. Peripheral blood was taken from children rather than adults as the studies in the explant model revealed a higher expression of TLR4 in children. In addition, Mita et al. demonstrated that TLR4 expression in monocytes from peripheral blood of adults was downregulated by IL-4[489], but this effect was not determined in monocytes and T cells from children. Monocytes from children expressed high levels of TLR4, and after incubation with IL-4 for 24 hours, the absolute number of monocytes expressing TLR4 did not differ significantly, but the expression level was significantly reduced. Reduction of TLR4 by IL-4 was not as great as that reported by Mita et al., but this may be due to differences in the dose of IL-4 used or even an age specific effect. In addition, use of FSC and SSC parameters to gate for monocytes and lymphocytes provides only a rough estimate of the monocyte population. It is also possible that large lymphocytes such as natural killer cells were present in the monocyte gate, although these cells are found in

lesser numbers in the blood and their expression of TLR4 is still undetermined. Staining for specific cell markers using antibodies is a better strategy to identify the cell populations than FSC and SSC parameters. CD3 staining is specific for T cells as it is a component of the T cell receptor, and all the cells that expressed high levels of CD4 were positive for CD3. Monocytes also express CD4 but have a much lower expression than T cells making it easy to discriminate these two populations. Cells expressing TLR4 and high levels of CD4 were thus T cells, and these cells responded strongly to IL-4; the population of $CD4^{+}TLR4^{+}$ was significantly reduced after incubation of PBMCs with IL-4 for 24 hours. Therefore, $TLR4^{+}$ T cells in the nasal mucosa may be similarly affected by IL-4 and would have a reduced responsiveness to LPS, by loss of expression of the receptor. This may be the mechanism by which atopic adults have reduced TLR4 protein positive cells in their nasal mucosa.

4.3 Implications of these Results

These results demonstrate the ability of the bacterial product LPS to prevent local allergen-induced allergic inflammation in the nasal mucosa of children. Moreover, the results suggest a possible mechanism for the hygiene hypothesis, where exposure of young children to LPS can promote Th1 cytokine production and anti-inflammatory cytokine production in the nasal mucosa by Th1 cells and $TLR4^{+}$ T-regulatory cells. Responses of children were different from atopic adults, suggesting that long-standing allergic rhinitis can impair responsiveness to the benefits of LPS. This was dependent on TLR4 which could be inhibited by IL-4, a mediator of atopy, in a STAT6 dependent mechanism. In addition, IL-4 could inhibit T cell expression of TLR4, suggesting that

allergic responses could downplay the protective role of LPS in inducing the activity of this cell population.

The protective effects of LPS in reducing atopy seem to depend on the age of the patient. This has been observed in epidemiological studies and in LPS airway challenges in adults. In young children, exposure to LPS reduces the risk of atopy, while exposure in adults will aggravate airway inflammation. This may be due to complex interplay between allergens and host cytokine production. Bacterial components may also only have a short and temporary therapeutic window to act. While this work shows that T cells and monocytes from children lose expression of TLR4 upon incubation with IL-4, peripheral blood B cells from healthy adults can have an increased TLR4 expression with IL-4[489]. This denotes both age and cell-specific regulation of TLR4. Activation of mast cells by LPS would promote allergic inflammation instead of downregulating it. LPS stimulated mast cells can produce Th2 cytokines and chemokines to recruit eosinophils[604]. In addition, activation of TLR4 with LPS and FcεRI with antigen leads to a synergistically increased production of TNF-α, IL-6 and IL-13 by activation of MAP kinases[605]. Although not significant, IL-4 seems to increase eosinophil expression of TLR4 mRNA[606]. In addition, activation of eosinophils with LPS led to increased adhesion molecule expression, survival and superoxide production. The cell and age specific effects on TLR4 expression may be due to different levels of expression and activity of transcription factors or accessibility of the TLR4 gene for transcription, but these mechanisms need to be determined. Therefore, LPS may be protective in the initial steps of sensitization to allergen or when IgE levels in the subject are not at their peak.

More research is required to determine the individual role of the different cell types, especially in children, in LPS responses and sensitization.

Evidence, in murine models shows that TLRs may have a role in the development of Th2 immune responses[290, 291]. While high doses promote Th1 cytokine production, low doses of LPS can promote Th2 immune response, primarily through the activation of dendritic cells. In addition, TLR4 deficient mice have defective Th2 cytokine production and IgE-mediated sensitivity to antigen[292]. Although comparable doses of LPS were used in this study, several possibilities may explain the differences obtained. Differences seen may be due to tissue, age and species as immune responses seen in mice may not necessarily translate to immune responses in humans. Deletion of TLR4 gene may also have effects on development of the immune system. In *Drosophila*, Toll protein is not only involved in innate immune responses but is also plays an important functional role in embryogenesis[333-335]. One cannot discount the fact that systemic recruitment plays a role in TLR4 generation of Th2 reactions, and the explant model separates the tissue from the systemic circulation. Despite this, the explant model provides the advantage that human tissue, rather than an animal model is used. Nevertheless, the influence of the systemic immune system in allergic rhinitis needs to be determined to further develop the conclusion with respect to LPS on allergic inflammation.

The nasal passages are in constant exposure to the environment and are the site of inflammation in allergic rhinitis, but can also be colonized by bacteria. According to the results from this study, allergic rhinitis would be a predisposing factor for nasal bacterial infections. In allergic rhinitis patients, damage caused by inflammation and frequent clearance of protective mucus combined with decreased TLR4 levels would lead to

greater colonization of the upper airways by bacteria. The number of studies exploring bacterial sinusitis and allergic rhinitis are limited, and a large variability is seen between studies; patients with both diseases range from 25% to 70% [607]. Nonetheless, patients with allergic rhinitis have a greater incidence of sinusitis than controls[608-611]. In one study, persistent allergic rhinitis was associated with more than two-fold increased risk for sinusitis than non-allergic controls, but it was unclear if it was of bacterial origin[612]. In other studies, the presence of bacteria was clearer; patients with persistent allergic rhinitis are associated with a two fold increase in *Staphylococcus aureus*[613], and in another study, 90% of subjects with a positive skin test to allergens had presence of potential pathogenic bacteria in their nasal cavity compared to 36% of patients with a negative skin test[614]. Despite the link, the immunopathological reasons for this predisposition are unclear and more studies are required. It is possible that decreases in innate resistance in allergic rhinitis may play a role in the predisposition. This work has focused on TLR4 expression in the nasal mucosa and it would be important to determine whether other PRRs are also reduced in allergic rhinitis, leading to an even greater predisposition to infection.

Few reports have looked in detail at the transcriptional regulation of TLR4. Studies include investigation of basal transcription factors in monocytes[496] and transcription under conditions of hypoxia[497]. This work has added to the current knowledge by demonstrating a role of STAT6 in downregulating TLR4 in monocytes and potentially T-regulatory cells. Other transcription factors involved in allergic inflammation may also regulate TLR4. The transcription factor GATA3 has a large role to play in the development of Th2 reactions. Naïve, freshly activated T cells and Th2

cells express high levels of GATA3 but not Th1 cells [59, 615]. The role of GATA3 in the development of Th2 reactions cannot be shown by simple deletion as GATA3 knockout mice die 11-12 days after conception displaying massive internal bleeding, marked growth retardation, severe deformities of the brain and spinal cord, and gross aberrations in fetal liver hematopoiesis[616]. Inhibition of GATA3 with antisense oligonucleotides showed a crucial function for this transcription factor in activation of Th2 cytokines[59]. GATA3 is critical for the production of IL-5 and IL-13 but not for IL-4[617-619]. Like STAT6, GATA3 has been associated with chromatin remodeling of the IL-4, IL-5 and IL-13 genes[620, 621]. The chromatin of these genes is maintained in an open state, by modification of DNA and proteins such as histones, to promote transcription of these genes. In addition, GATA3 has been shown to be important in maintenance of chromatin in its remodeled state[60]. GATA3 has been shown to be increased in allergic asthma in humans and in allergen sensitization models in mice[62, 63, 622]. The current consensus is that GATA3 is a master regulator of Th2 cell generation. The DNA binding sequence of GATA3 is known and preliminary analysis of the upstream region of TLR4 by computer analysis demonstrates nine potential GATA3 binding sites (data not shown). This suggests a potential role of GATA3 in regulating TLR4 expression in T cells. It is known that TLR2 expression can be controlled by chromatin remodeling[623, 624], and it is possible that TLR4 is as well. IL-4 activation of GATA3 may potentially inhibit TLR4 expression by modifying chromatin to shut down gene expression, and this would provide longstanding inhibition of gene expression. This may explain the low expression of TLR4 in adults with allergic rhinitis.

It is still not clear by what mechanism allergens are seen as a threat to the host, as they cause little damage. Similar responses to allergens denote possible structural similarities or even a common receptor. Inhaled allergens are stable proteins, are often proteases of low molecular weight, and have a high proportion of hydrophobic residues[625]. It is possible that a conserved molecular pattern on allergens causes them to react with a PRR. It is even possible that TLR4 may function as a receptor for allergens, as the extracellular domain of this receptor has a broad hydrophobic surface for binding of ligands. Low dose stimulations of LPS induce the production of Th2 cytokines from dendritic cells and TLR4 is essential for the development of Th2 immune responses[290-292]. It is possible that TLR4 has low affinity for allergens and suboptimal signal transduction would induce the production of Th2 inducing cytokines from antigen-presenting cells rather than Th1 inducing cytokines. In this case, part of the mechanism of the hygiene hypothesis would be a competition between allergens and bacterial PAMPs for PRRs and induction of adaptive immune responses. It is of crucial importance to determine the mechanisms that allergens induce allergic inflammation to prevent the development of allergic diseases in children.

4.4 Therapeutic Implications

The results of this work suggest important considerations in the development of therapies for atopic disease. Inflammatory cells of the nasal mucosa, their expression of TLR4 and their responsiveness to LPS are different between children and adults. In addition, neutralizing antibodies to IL-10, IL-12 and IFN-γ shows the importance of these cytokines in reducing allergic inflammation induced by LPS. Allergic rhinitis is associated with lowered IL-10 production compared to normal individuals[548]. Therapy

may focus on promoting Th1 cytokine production and IL-10 or promoting the generation of T-regulatory cells that express TLR4 if not in adults, more importantly in developing children to curb the increase in allergic disease prevalence. Aside from potentially reducing allergic symptoms, promoting TLR4 expression may be also helpful in providing protection from predisposition of developing bacterial sinusitis.

The current pharmacological therapies for allergic diseases including adrenergic agonists, anti-histamines and steroids have numerous problems associated with them. These include side effects, tolerance development, short-lasting effects and immune system suppression. Inhaled corticosteroids may even increase the severity of allergic diseases by suppressing Th1 development and enhancing Th2 cytokine synthesis such as IL-4 and IL-5[626, 627]. An alternative to pharmacotherapy is allergen immunotherapy. This is the practice of administering gradually increasing doses of allergen to a patient to ameliorate the symptoms associated with subsequent exposure with the specific causative allergen[628]. Allergen immunotherapy is effective at reducing IgE-mediated disease but requires numerous injections over the course of 3 to 5 years[629]. Allergen immunotherapy is highly effective at reducing IgE-mediated disease. It is one of the few immunomodulatory therapies used routinely for treatment of an inflammatory disease. Rhinitics and allergic asthmatics on immunotherapy can tolerate seasonal exposure to allergens with decreased symptoms, an effect that can last three years after treatment completion[630]. Unfortunately, as allergens are used, there is a chance of IgE-mediated systemic reactions and anaphylaxis. The mechanisms of allergen immunotherapy are not clear. Current understanding focuses on the deviation from a Th2 phenotype to a Th1 phenotype, a decreased responsiveness of the Th2 biased cells[520] or immune regulation

by cytokines, such as IFN-γ or IL-10[631]. There is also the possibility of the other mechanisms such as the induction of IL-10 production by $CD4^+CD25^+$ T regulatory cells[632], although, in some cases, Th1 cytokines may play a greater role in immunotherapy than immunoregulatory T cells[633]. These mechanisms are similar to the mechanisms by which LPS functions to diminish allergen-induced inflammation in the nasal mucosa of atopic children. Immunotherapy possibly reestablishes the mechanisms present in children but lost in atopic adults. Perhaps attempts to increase the regulatory T cell population or activity may improve immunotherapy. One method would be to stimulate tumor necrosis factor receptor superfamily 9 (TNFRSF9), as stimulation of the mouse homolog 4-1BB can induce the proliferation of $CD4^+CD25^+$ T regulatory cells *in vitro* and *in vivo*[634]. Another possibility is increasing the recruitment of Th1 cells from the circulation or to increase their local differentiation with cytokines such as IL-12 or IFN-γ.

It is possible that combination of PAMPs to immunotherapy may increase the efficiency, shorten the duration and avoid potential side-effects of the treatments. This treatment has as its aim a deviation of Th2 cytokine responses to Th1 cytokine responses. The PAMPs are used as adjuvants, which are substances that enhance the immune responses to an antigen. One adjuvant used for immunotherapy is derived from LPS and consists of 3-deacylated monophosphoryl lipid A (MPL). MPL can promote Th1 responses primarily through induction of IL-12 production[635]. When MPL was conjugated to grass pollen allergen, after only four preseasonal injections, reduced symptoms and IgE production were seen in patients with hay fever[636]. Similarly, the TLR9 ligand CpG DNA, when conjugated to purified ragweed and injected in patients

with allergic rhinitis, could reduce symptom scores to allergen during ragweed season, reduce nasal eosinophilia, Th2 cytokine production and could increase IFN-γ positive cells[637]. Unfortunately, for these therapies to be successful, adequate PRR expression is necessary and if Th2 cytokines reduce PRR expression, then allergic inflammation may reduce the potential benefit of these therapies. It is possible that to promote PAMP conjugated allergen immunotherapy, Th2 cytokines should be reduced prior to the treatments. Allergen immunotherapy is also performed by sub-cutaneous injections, and perhaps localized nasal immunotherapy may provide greater and faster therapeutic benefits and be more effective than by altering systemic immune responses to the allergen. In addition intranasal administration of PAMPs may be potential treatment option to protect atopic children from developing lifelong allergic inflammation. Investigation of these avenues may lead to development of alleviation of symptoms if not a complete protection from allergic inflammation.

4.5 Future Studies

The findings of this work set the stage for further research. Here presented are future studies that can be conducted to further the work of this thesis:

1. A full characterization of the $TLR4^+$ $CD4^+CD25^+$ T cells found in the nasal mucosa of children or the $TLR4^+CD4^+$ T cells found in the peripheral blood of children would be interesting. It is necessary to determine whether these cells possess the other proteins of the LPS receptor, MD2 and CD14, as well as the necessary signal transduction components for LPS responsiveness. In addition, a determination of what genes are activated upon activation of the cells with TLR4 ligands would be necessary. The

mechanisms by which TLR4 is regulated in these cells, or what can cause these cells to proliferate would also yield potential information to promote TLR4 expression in allergic adults.

2. It would be also important to determine whether Th2 cytokines have an impact on other components of the LPS receptor or LPS signal transduction events. Also it would be of interest to determine if other TLRs have reduced expression in the nasal mucosa of allergic adults. It would also help in showing different potential PAMPs that would benefit the early immune system against the production of allergic disease. This would help to determine which ligands would function most efficiently in adjuvant modified immunotherapy.

3. The regulation of TLR4 and other TLRs needs to be further elucidated. Further characterization of the mechanism by which STAT6 functions to inhibit TLR4 is needed. In addition, Tyrphostin AG 490 may cause unrecognized nonspecific effects on the cells and another method, such as STAT6 antisense oligonucleotides or small interfering RNA (siRNA), to inhibit STAT6 is required to confirm these results. Numerous potential transcription factor binding sites were observed during computer analysis of the upstream region of TLR4. This included potential GATA3 binding sites. GATA3 may play an important role in the regulation of TLR4 in chronic allergic rhinitis. In addition, other potential regulators of TLR4, such as the novel Th2-associated cytokine IL-33, and their mechanisms of action need to be better characterized.

4. Perhaps of greatest interest is the elucidation of the mechanisms by which allergens function to cause sensitization and activation of primary Th2 immune responses. This is especially important, since increases in allergic disease incidence is a world-wide

phenomenon. It is possible that a PRR plays a role in binding to allergen and can induce the necessary IL-4 to prime Th2 immune responses. Potentially TLR4 or other TLRs may play a role in the recognition of allergen and this possibility needs to be investigated.

4.6 Summary and Conclusion

In summary, this thesis demonstrates the ability of bacterial LPS to prevent local allergen-induced allergic inflammation in the nasal mucosa of atopic children. This was shown to occur by down-regulation of local Th2 cytokine production and induction of Th1 cytokine and IL-10 production. LPS also caused the proliferation and activation of $TLR4^{+}$IL-10 producing cells and $CD4^{+}CD25^{+}$ cells. These events are dependent upon local tissue constituents and independent of systemic recruitment of inflammatory cells, and require TLR4 for this process to occur. In addition, $CD3^{+}$ cells were shown to be one of the major cell types expressing TLR4 in the nasal mucosa of children. Moreover, it was shown that responses to LPS and expression of TLR4 are significantly different between children and adults, especially adults with allergic rhinitis. Allergic inflammation may downregulate TLR4 expression as IL-4 and STAT6 can downregulate TLR4 in an *in vitro* model and IL-4 can downregulate peripheral blood T cell expression of TLR4. Activation of STAT6 led to binding of STAT6 to an upstream region of the TLR4 gene, where it may mediate the inhibitory actions of IL-4. TLR4 is an important bridge between the innate and adaptive immune systems and its activation may provide protection against the development of atopic diseases by promoting the maturation of the immune system. This provides one of the potential molecular mechanisms of the hygiene hypothesis, where bacterial components promote immunoregulatory responses to allergen.

On the other hand, in adults, Th2 cytokines may promote allergic inflammation by downregulating components of the innate immune system; aside from stimulating pro-inflammatory mediators, IL-4 can downregulate the innate immune receptor TLR4, potentially affecting the immunoregulatory effect of LPS in allergic disease.

5.0 Research Compliance Certificates

6.0 Contributions of Authors

The following original manuscripts, which have been published in peer-reviewed scientific journals and are part of the thesis work of Pierre-Olivier Fiset:

1. Pierre-Olivier Fiset, Meri K. Tulic, Petra S.A. Skrablin, Samuel M. Grover, Séverine Létuvé, Bruce D. Mazer, and Qutayba Hamid. STAT6 downregulates Toll-like Receptor-4 Expression of a Monocytic Cell Line. (Clin Exp Allergy. 2006 Feb;36(2):158-65.)

2. Meri K. Tulić, Pierre-Olivier Fiset, John J Manoukian, Saul Frenkiel, François Lavigne, David H Eidelman, Qutayba Hamid. Role of toll-like receptor 4 in protection by bacterial lipopolysaccharide in the nasal mucosa of atopic children but not adults. Lancet. 2004 May; 363(9422):1689-97.

The following are the contributions of each author in each paper:

1. Pierre-Olivier played the greatest role in production of this manuscript. He designed the experiments, participated in the development of the cell model for this work, and played a major role in the experimental procedures and data acquisition. Mr Fiset also analyzed the results and put the manuscript together. Dr Meri Tulic played a role in theory behind this manuscript and in the editorial process. Ms. Petra Skablin, Mr Samuel Grover and Dr Séverine Létuvé assisted Mr Fiset in performing some of the experiments for this manuscript. Dr Bruce Mazer helped in supervision of the work and the editorial process. Dr Qutayba Hamid as senior author oversaw the work for this manuscript and the editorial process.

2. Dr Tulic played the greatest role in production of this manuscript but Mr Fiset made significant contributions. Dr Tulic led the experiments, played a role in development of the model, acquired data and put the manuscript together. Mr Fiset helped significantly in development of the model, played a significant role in developing the theory and experimental design for this work. He also acquired data, helped analyze it, helped in production of the figures, participated in writing the manuscript and the editorial process. Dr John Manoukian, Dr Saul Frenkiel and Dr François Lavigne participated in this study by providing nasal biopsies for the experiments from the Montreal Children's Hospital, from the Jewish General Hospital and from the Nôtre Dame Hospital, respectively. Dr David Eidelman helped in supervision of the work and the editorial process. Dr Qutayba Hamid as senior author oversaw the work for this manuscript and the editorial process.

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