

Regulation and function of
Toll-like receptor 4 in allergic diseases

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Abstract

The hygiene hypothesis states that exposure to microbes limits the development of allergic diseases, whereas the lack of microbial stimulation of the immune system early in life would promote their development. The beneficial effects of microbes against allergies would be mediated by pathogen-derived molecules interacting with Toll-like receptors (TLR), and interfering with allergic T 'helper' lymphocyte type-2 (Th2) inflammation seen in asthma and rhinitis. Few studies investigated whether allergies interfere with the expression and function of Toll-like receptor-4 (TLR4), the receptor for bacterial lipopolysaccharide (LPS). We hypothesized that a history of allergies and Th2-type mediators affect the expression and the function of TLR4 on peripheral leukocytes and the airway epithelium. Secondly, we hypothesized that GATA-3, a transcription factor driving the Th2 phenotype, regulates TLR4 expression of airway epithelial cells in allergic conditions. Peripheral leukocytes from a paediatric cohort of atopic and non-atopic subjects were discriminated based on LPS-induced TLR4 surface expression, activation of its signalling pathways, and expression of anti-inflammatory interleukin (IL)-10. Monocytes from atopics were significantly less responsive to LPS than cells from non-atopic children, while CD4 T lymphocytes failed to respond. The TLR4 expression on monocytes diminished as a function of age in atopic, but not in non-atopic subjects. In the nasal mucosa of adults, a

reduced epithelial TLR4 expression was seen in tissues from subjects with allergic rhinitis compared with controls. Concomitantly, tissues from rhinitis patients displayed an increased Th2-type inflammatory profile. We also detected a reduced TLR4 and an increased GATA-3 expression in bronchial epithelial cells from asthmatic adults compared with non-asthmatics, as well as in epithelial cells cultured under pro-allergic conditions. Finally, epithelial cells over-expressing GATA-3 gene slightly reduced TLR4 expression, whereas those in which endogenous GATA-3 was silenced significantly augmented it. These data suggest a direct regulation of epithelial TLR4 by GATA-3. In summary, this work demonstrates that a history of atopy or allergy, as well as the Th2 inflammatory milieu, reduce the ability of atopic individuals to respond to LPS in circulating monocytes of children and airway epithelial cells of adults. This thesis project provides a new perspective to TLR agonists-based therapies that aim at preventing allergic disorders. Our data stress that the preventive effect of LPS would occur early during childhood or the absence of atopy since in atopic adolescents and adults with on-going allergic diseases displayed blunted TLR4 expression and/or function.

Abrégé/Résumé

L'hypothèse d'hygiène stipule que l'exposition à des microbes limite le développement des maladies allergiques, bien qu'un manque de stimulation du système immunitaire par des microbes durant l'enfance fait la promotion de leur développement. Les effets anti-allergiques des microbes seraient dus à l'interaction de composants microbiens avec les récepteurs Toll-like (TLR), interférant avec l'inflammation allergique dite T 'helper' lymphocyte type-2 ou Th2 observée dans l'asthme et la rhinite. Il existe actuellement peu d'études sur la relation entre les allergies et la diminution de l'expression fonctionnelle de TLR4, récepteur du lipopolysaccharide (LPS) bactérien. Nous avons donc émis l'hypothèse qu'un historique d'allergies et/ou des médiateurs de type Th2 affectent l'expression et la fonction de TLR4 dans des leucocytes périphériques ainsi que dans l'épithélium des voies respiratoires. De plus, dans des conditions allergiques nous proposons que le facteur de transcription pro-Th2 nommé GATA-3 pourrait réguler l'expression de TLR4 dans l'épithélium des voies respiratoires. Des leucocytes périphériques ont été obtenus d'une cohorte pédiatrique de sujets atopiques et non-atopiques et ont été différenciés sur la base de l'expression de TLR4, de l'activation de voies de signalisation et de l'expression de l'interleukine (IL)-10 dite anti-inflammatoire, en réponse au LPS. Les monocytes de sujets atopiques ont significativement moins répondu au LPS

que ceux de patients non-atopiques, alors que les lymphocytes T CD4 n'ont pas répondu. L'expression de TLR4 sur les monocytes diminue en fonction de l'âge des sujets atopiques, et non chez les sujets non-atopiques. De plus, nous observons dans la muqueuse nasale de patients adultes avec la rhinite allergique, une corrélation entre une expression épithéliale de TLR4 réduite et une forte inflammation de type Th2 comparativement aux sujets contrôles. Nous avons aussi détecté en parallèle de cette faible expression de TLR4 un fort niveau de GATA-3 dans les cellules bronchiales épithéliales de sujets asthmatiques adultes comparé aux cellules non-asthmatiques, ainsi qu'au sein de cellules cultivées en conditions pro-allergiques. Nous confirmons le rôle de GATA-3 dans la régulation de l'expression épithéliale de TLR4 par la surexpression ou la réduction du gène GATA-3 dans des cellules épithéliales. En résumé, ce travail démontre qu'un historique d'atopie ou d'allergies, de même que le milieu inflammatoire de type Th2, réduisent la capacité des monocytes périphériques d'enfants ainsi que des cellules épithéliales des voies respiratoires d'adultes à répondre au LPS. Ce projet de thèse fournit une nouvelle perspective aux thérapies basées sur l'utilisation d'agonistes des TLR visant à prévenir les maladies allergiques. Nos résultats démontrent que l'effet préventif du LPS se produirait tôt durant l'enfance ou en l'absence d'atopie étant donné que des adolescents atopiques ou des adultes avec des maladies allergiques présentent un défaut dans l'expression et/ou fonction de TLR4.

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List of Abbreviations

AHR	Airway hyperresponsiveness
APC	Antigen-presenting cell
ASM	Airway smooth muscle
BAL(F)	Bronchoalveolar lavage (fluid)
BCG	Bacillus Calmette-Guerin
BCR	B cell receptor
BEBM	Bronchial Epithelium Basal Medium
cAMP	Cyclic adenosine monophosphate
COPD	Chronic pulmonary obstructive disease
CRS	Chronic rhinosinusitis
DC(s)	Dendritic cell(s)
DMEM	Dulbecco's modified Eagle medium
DTT	Dithiothreitol
EAACI	European Academy of Allergology and Clinical Immunology
ECM	Extracellular matrix
ECP	Eosinophil cationic protein
EDTA	Ethylenediaminetetraacetic acid
EGF(R)	Epidermal growth factor (receptor)
EMTU	Epithelial-mesenchymal trophic unit
ERK	Extracellular signal-regulated kinase
FcεRI	Fc-epsilon receptors I
FEV ₁	Forced expiratory volume in 1 second
FITC	Fluorescein isothiocyanate
FSC	Forward Scatter
FVC	Forced vital capacity
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
GATA-3	GATA-binding protein 3
G-CSF	Granulocyte colony-stimulating factor
GFI-1	Growth factor independent-1
GI	Gastrointestinal

GINA	Global initiative for asthma
GM-CSF	Granulocyte macrophage colony-stimulating factor
GPI	Glycophosphatidylinositol
HDM	House dust mite
HRCT	High resolution computer tomography
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IKK	Inhibitor of NF- κ B (I κ B) proteins kinases
IL	Interleukin
IL-1Ra	IL-1 receptor antagonist
IRAK	IL-1R-associated kinase
IRF3	Interferon regulatory factor 3
ISAAC	International Study of Asthma and Allergies in Childhood
IVIG	Intravenous immune globulin
I κ B	Inhibitor of NF- κ B
JAK/STAT	Janus kinase
JNK	c-Jun N-terminal kinase
LBP	LPS binding protein
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
MAPK	Mitogen-activated protein kinase
MBP	Major basic protein
MCP	Monocyte chemoattractive protein
mDC	Monocyte-derived dendritic cells
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MyD88	Myeloid differentiation primary-response protein 88
NFAT1	Nuclear factor of activated T cells 1
NF- κ B	Nuclear factor κ B
NHBE	Human Bronchial Epithelial

NO(S)	Nitric oxide (synthase)
NRF2	Nuclear factor-erythroid 2-related factor 2
OVA	Ovalbumin
PAMPs	Pathogen-associated molecular patterns
PARs	Protease-activated receptors
PBMC	Peripheral blood mononuclear cells
PC ₂₀	Provocative concentration 20%
pDC	Plasmacytoid dendritic cells
PE	Phycoerythrin
PGE	Prostaglandin E ₂
PI3K	Phosphatidylinositol 3-kinase
PRRs	Pattern recognition receptors
PUFAs	Polyunsaturated fatty acids
REL	Reticuloendotheliosis viral oncogene homolog protein
RIP1	Receptor-interacting protein 1
SEB	<i>Staphylococcal</i> enterotoxin B
siRNA	Small interfering RNA
SIT	Specific immuno-therapy
SMAD	Sma and Mad-related
SNPs	Single nucleotides polymorphisms
SOCS	Suppressor of cytokine signalling
SSC	Sideways scatter
STAT	Signal transducers and activators of transcription
TAB	TAK1-binding protein
TACE	Tumour necrosis factor- α converting enzyme
TAK1	Transforming growth factor- β -activated kinase 1
TANK	TRAF family member-associated NF- κ B activator
TARC	Thymus and activation regulated chemokine
TBK1	TANK binding kinase 1
TCR	T cell receptor
TGF	Transforming growth factor
Th	T 'helper'(cell or lymphocyte)

TIR	Toll/IL-1R
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRADD	Tumour necrosis factor receptor type 1-associated death domain
TRAF6	Tumour necrosis factor receptor-associated factor 6
TRAM	TRIF-related adaptor molecule
T-reg	Regulatory T (cell or lymphocyte)
TRIF	TIR domain-containing adaptor-inducing interferon- β
TSLP	Thymic stromal lymphopoeitin
VCAM	Vascular adhesion molecule
WAO	World Allergy Organization
WHO – ARIA	World Health Organization – Allergic rhinitis and its impact on asthma

Chapter 1: Introduction

1.1. Allergic diseases: general concepts

1.1.1. History and definitions

Sporadic cases of allergic diseases, including anaphylaxis to foods as well as respiratory symptoms and hay fever date back to Ancient Egypt, Greek and Roman antiquity. Hippocrates, in *Corpus Hippocraticum*, was the first to write the term 'asthma' to describe respiratory symptoms of spasms and obstruction. The term asthma comes from the Greek word *aazein* meaning 'to gasp to breath' (1). Sir John Floyer was one of the first to publish a monograph on the disease in the *Treatise of Asthma*, in 1698 (2). Describing his own recurrent respiratory symptoms (3), Floyer associated these manifestations with various risk factors including exercise, inhaled fumes and tobacco smoke, poor air quality, damp households, swamps. Major contributions to our understanding of allergic diseases of the airways came from British physicians of the 19th century. During the 1860s, asthma was officially defined by Henry Hide Salter as "paroxysmal dyspnoea of a peculiar character, generally periodic with intervals of healthy respiration between the attacks" (4). Based on these early reports, there was however no evidence that asthma morbidity i.e. breathlessness, could be due to an allergic reaction.

The first clinical description of allergic disease, namely hay fever, was made by John Bostock in 1819. Bostock then described his own seasonal affliction, which took place during spring and was characterized by nasal discharge, sneezing episodes, itching, difficulty breathing and severe malaise (5). The aetiology of symptoms remained unknown until Charles Blackley, who also suffered from seasonal hay fever, first associated his reactions to pollen when inhaling preserved grass pollen samples during winter (6). Blackley further studied the mechanisms underlying hay fever by applying pollen to scarified skin. Such epicutaneous challenge with the allergen caused an immediate wheal and flare reaction on Blackley's forearm and was the precursor of the skin prick tests (7) which are still in use in allergy clinics today. Blackley also performed the first inhaled challenges with pollen and established the basics of inhaled pollen dose thresholds inducing nasal and lung symptoms that went to be described as hay asthma (8-10).

The term 'allergy' was first used in 1906 by the Austrian paediatrician Clemens von Pirquet who described the incidence of hypersensitive reactions upon repeated injection with horse serum or smallpox vaccine. von Pirquet termed this phenomenon 'allergy' from the Greek words *allos* ("changed") and *ergos* ("reaction") (11). Historically, all hypersensitive reactions to antigens could be called allergies. The World Allergy Organization (WAO) defines hypersensitivity as a number of "objectively reproducible symptoms or signs

initiated by exposure to a defined stimulus at a dose tolerated by normal persons” (12). In 1963, Gell and Coombs established the current classification of hypersensitive reactions which distinguishes 4 types of hypersensitive reactions (Type I to IV) based on the differential immune mechanisms involved (13, 14). In their classification, Gell & Coombs restricted the word allergy to Type I hypersensitivity reactions which are typically mediated by antigen- or allergen-specific immunoglobulin (Ig) E or IgE. Recently, the European Academy of Allergology and Clinical Immunology (EAACI) defined allergy as “a hypersensitivity reaction initiated by immunological mechanisms” which are either antibody- or cell-mediated. Besides, allergens are defined as “antigens which cause allergy” by reacting with IgE, or even IgG in some cases of anaphylaxis (12).

1.1.2. The roots of allergy: atopy, IgE and sensitization

Atopy can be defined as the syndrome of likelihood to develop hypersensitive reactions to allergens (15). The term comes from the Greek word *atopos* (“placeness”) and refers to the widespread distribution of disease manifestations. Hypersensitive reactions to allergens can indeed manifest at various sites including the skin (atopic dermatitis or eczema), the conjunctiva (allergic conjunctivitis), the gastro-intestinal tract (food allergies), the internal areas of the nose (allergic rhinitis or hay fever), and the pulmonary bronchi (asthma). In spite of the etymology of atopy which refers somewhat to disease

localization, atopy is more generally defined as “that form of immunological reactivity of the subject in which reaginic antibody (e.g. IgE) is readily produced in response to ordinary exposure to common allergen of the subject’s environment” (16). Accordingly, the modern definition emphasizes the ‘inheritable’ nature of the immunological reactivity to allergens described by Cooke and collaborators (17) rather than the presence of hypersensitivity symptoms. The EAACI states that “atopy is the personal and/or family tendency, usually in childhood or adolescence, to become sensitized and produce IgE antibodies in response to ordinary exposure to allergens, usually proteins” (12).

Allergen sensitization refers to a sequence of events initiated upon recognition of an antigen by B cell receptors or BCRs of a naive B cell, which ultimately leads to the production of antigen- i.e. allergen-specific IgE. Antigenic stimulation through BCR does not normally trigger production of immunoglobulins of the IgE isotype. In atopic individuals, B cells acquire the ability to produce IgE through a cascade of events including antigen-specific rounds of proliferation leading to clonal expansion of allergen-specific B cells and immunoglobulin isotype class switching events that initiate IgE production (18). The Ig isotype class switching takes place under specific conditions and necessitates the interaction of B cells with T ‘helper’ lymphocytes and the cytokine mediators they release. The T lymphocytes driving the IgE production were previously activated by an antigen-presenting cell (APC) in an allergen-specific manner (Diagram 1).

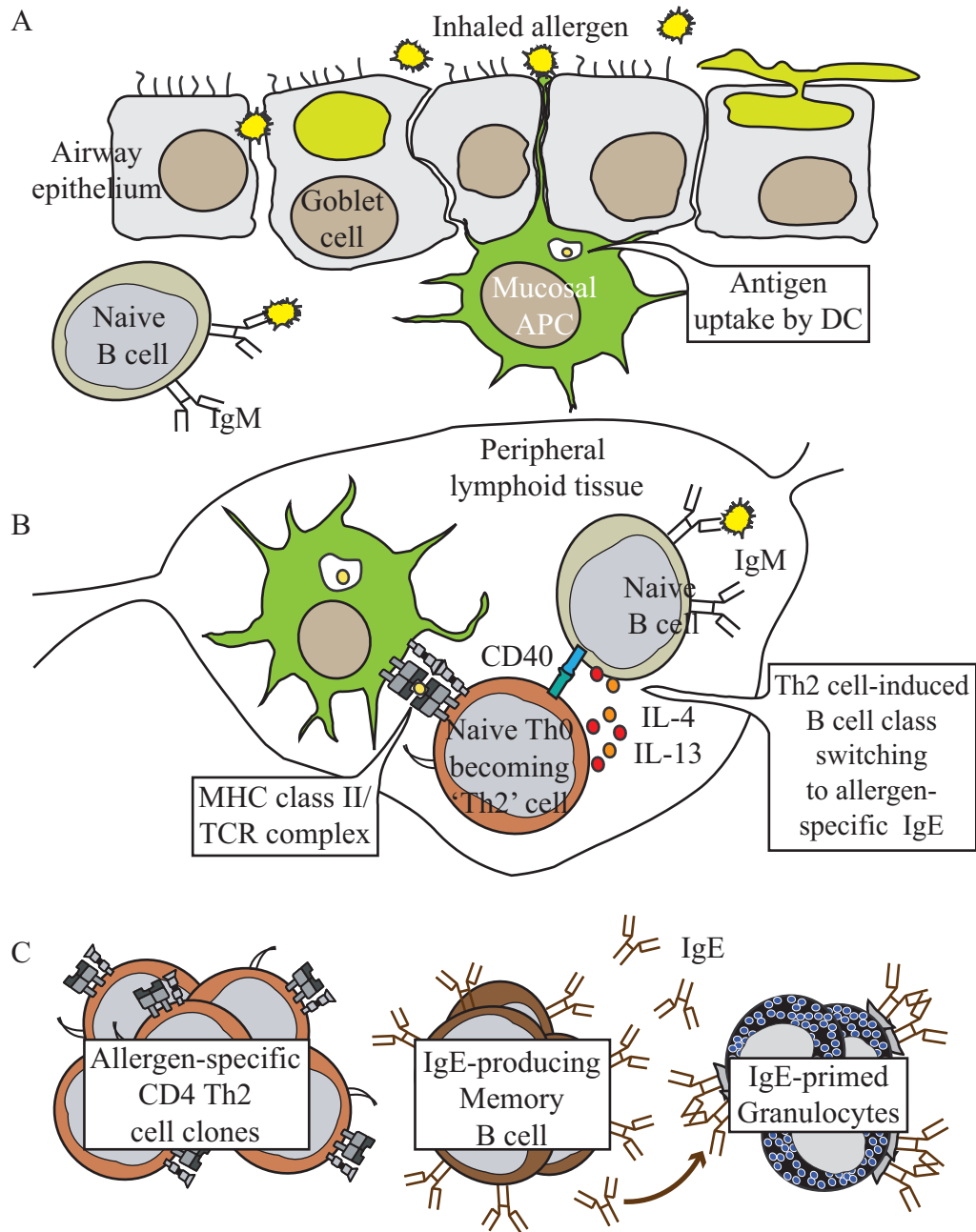


Diagram 1. Allergen sensitization. Minute amounts of allergen are taken up by mucosal antigen-presenting cell (APC) and/or activate naive B cell in an immunoglobulin-dependent manner (A). Once migrated in peripheral lymphoid tissue, APC presents allergen to T 'helper' lymphocytes and drive their differentiation into Th2-type cells that induce B cell isotype class switching to IgE, through CD40/CD40L interaction and the effect of IL-4 and IL-13 cytokines (B). These processes and allergen exposure eventually give rise to allergen-specific IgE-producing memory B cells and Th2-type lymphocytes populations (C), as well as to IgE-mediated priming of mucosal granulocytes that orchestrate early responses to further allergen exposure.

Elevated serum IgE levels in allergic diseases have long been described in the literature (19). The allergen sensitization process also includes the binding of allergen-specific IgE molecules to their cognate high-affinity receptors, called Fc-epsilon receptors I (FcεRI), mainly at the surface of mast cells and basophils (20-22). Such IgE-mediated cell sensitization brings about the priming of mast cells and basophils in atopic individuals and carries the promise of a magnified immune response to the next allergen exposure. The binding of an allergen to the IgE:FcεRI complex triggers an inflammatory response considered to be the hallmark of Type I hypersensitivity reactions and will be discussed in detail in section 1.2.1. The fact that B cell activation and IgE production depend on interaction with T 'helper' cells underlines the critical role of APC-mediated allergen-specific T cell responses in the development of Type I hypersensitivity reactions.

The reasons why allergens render T 'helper' lymphocytes capable of inducing IgE production in atopic individuals but not in normal individuals remain elusive. The nature and concentration of antigens, the genetic predispositions of atopic individuals, as well as the presence of environmental factors or immunological adjuvants are all factors that may contribute to an exaggerated adaptive response to allergens. Common aeroallergens include seasonal pollen molecules or spores from various sources, as well as perennial indoor particles including companion animal dander and house dust components such as mite

feces and cockroach-derived antigens. Interestingly, exposure to elevated concentrations of cockroach or mite-derived allergens is associated with increased risk of sensitization, whereas this risk inversely correlates with the concentration of exposure to animal-related allergens (23-26). These studies suggest that additional immuno-modulatory factors may come into play and influence the development of allergic disorders (27) (sections 1.3.3.2.3. and 1.3.4.).

1.1.3. Airway allergic diseases

The development of respiratory allergic diseases, including asthma and rhinitis, depends on: 1) aeroallergen exposure, and; 2) sensitization leading to aeroallergen-specific IgE generation. Further inhalation of aeroallergens then may trigger IgE-mediated inflammatory responses causing respiratory symptoms in the upper airways (nasal cavity) in the case of allergic rhinitis, and/or in the lower (bronchi and bronchioles) in allergic asthma.

1.1.3.1. Allergic rhinitis

Allergic rhinitis, or hay fever is clinically defined as a symptomatic Type I hypersensitivity disorder of the upper airway or more precisely the nasal conduits (28). Symptoms of hay fever develop as a consequence of an allergen-induced inflammatory response causing mucosal edema and mucus production. Clinical symptoms mainly feature nasal itching or pruritus, sneezing, increased mucus secretions or rhinorrhea, and nasal congestion. These symptoms are often

associated with lacrimation, sinusitis and post-nasal drip or sore throat/pharyngitis. Upper airway symptoms in rhinitis are reversible either spontaneously or upon treatment. In contrast with aeroallergen-specific IgE-mediated rhinitis symptoms, non-allergic forms of rhinitis also exist and are triggered by infectious agents including bacteria and viruses, occupational exposures to irritants and use of certain drugs. According to the World Health Organization – Allergic rhinitis and its impact on asthma (WHO ARIA) Workshop (28), allergic rhinitis is subdivided into “intermittent” and “persistent” disease based on the frequency of symptoms. “Intermittent” disease refers to symptoms that are present for less than 4 days a week or for less than 4 weeks, whereas “persistent” disease means that symptoms are present more than 4 days a week and for more than 4 weeks. Furthermore, allergic rhinitis is also classified as either “mild” or “moderate-severe” depending on the severity of symptoms. Patients with “moderate-severe” disease show one or more of the following conditions: sleep disturbance, impairment of daily activities and /or sport, impairment of school or work. Patients with none of the previous features are classified as “mild”.

1.1.3.2. Allergic asthma

According to the Global initiative for asthma (GINA), asthma is defined as a chronic inflammatory disorder of the lower conducting airways characterized by recurrent episodes of wheezing, breathlessness, chest tightness, and coughing

(29, 30). As for allergic rhinitis in the nose, symptoms of allergic asthma develops as a consequence of exposure to aeroallergens which triggers an IgE-mediated inflammatory response (section 1.2.1.). The allergen-induced inflammation is globally perceived as the cause of impaired lung function or bronchoconstriction (31). In asthma, the impaired lung function mainly results from the contraction of airway smooth muscle (ASM) cells surrounding the bronchi. Aside from bronchoconstriction, bronchial edema and inflammation, mucus overproduction as well as structural changes of the airway wall termed airway remodelling (section 1.2.2.) are all considered to contribute to airflow obstruction in asthma (Diagram 2a). Remodelling of the asthmatic airway translates into thickening of the airway wall, and positively correlates with disease severity (32). Airway wall thickening likely results from repair processes that follow chronic inflammatory insult. The remodelling would contribute to the relative irreversibility and persistence of airflow limitation observed in moderate to severe disease treated with bronchodilators (33-36).

The clinical spectrum of allergic asthma mainly includes the development of respiratory symptoms, lung function impairments, limitations of daily activities, sleep disturbances as well as the use of drug therapies. Asthma can be defined as allergic or non-allergic based on the co-existence of atopy, aeroallergen sensitization and/or other allergic disorders. The most common classification is based on disease severity, where patients are classified as 'mild', 'moderate' or

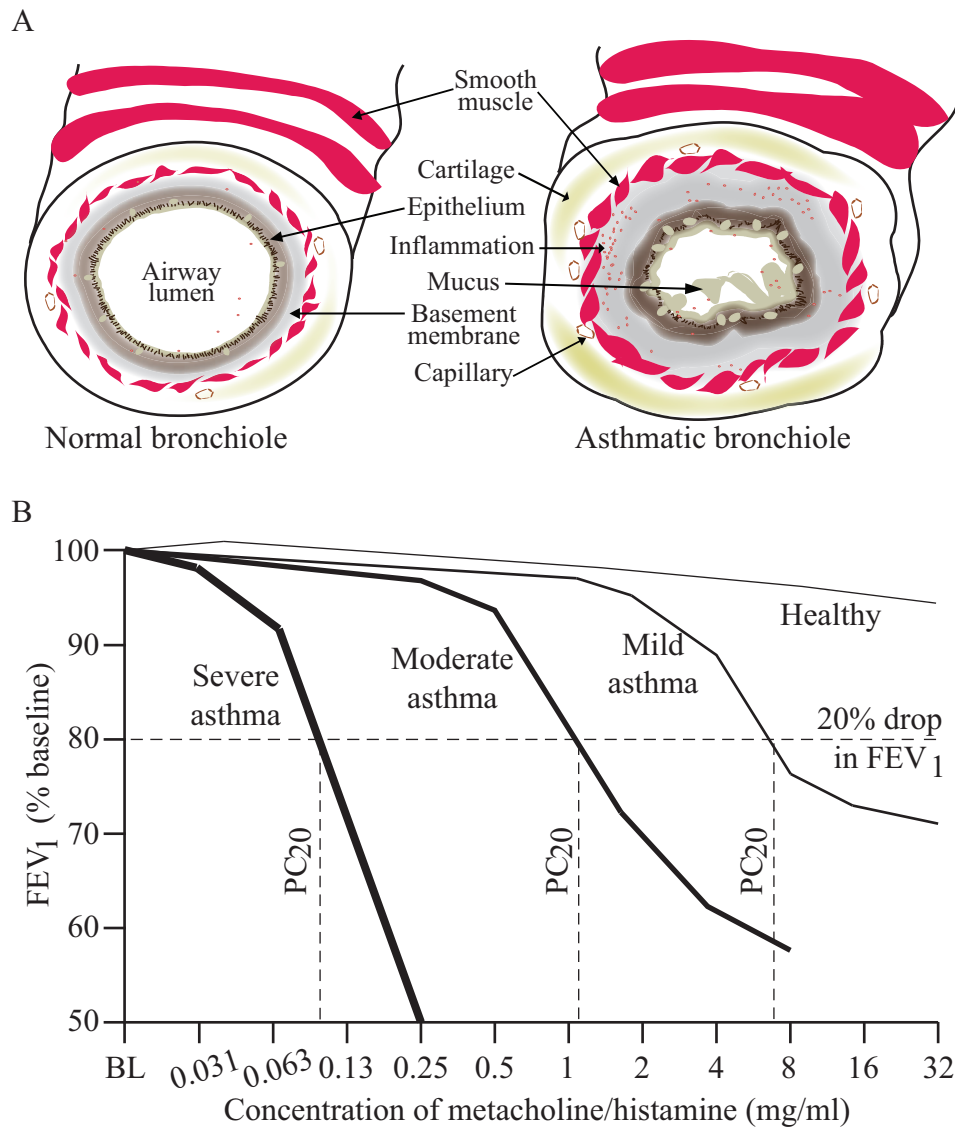


Diagram 2. Airway narrowing and hyperresponsiveness in asthma. In A, a schematic representation of the normal and asthmatic bronchiole, depicting the airway narrowing in asthma. Airflow limitation is due to airway smooth muscle contraction, mucus hypersecretion, bronchial wall edema and inflammation, as well as progressive structural changes that thickened the airway wall (termed airway remodelling). In B, airway hyperresponsiveness is depicted as the change in FEV₁ and PC₂₀ values to increasing doses of inhaled bronchoconstrictor stimuli in mild, moderate and severe asthma compared with normal control (Adapted from reference 44).

'severe' asthmatics depending on the intensity of treatment required to achieve asthma control (37, 38). These severity subcategories also include the level of symptoms, airflow limitation and lung function variability (39-41). Airflow limitation is determined by means of lung function tests or spirometry, and is expressed as the ratio of the forced expiratory volume in 1 second (FEV_1) to the forced vital capacity (FVC, i.e. the amount of air expired after maximal inspiration). Airflow limitation is characterized by reduced FEV_1 /FVC ratios due to decreased FEV_1 values, whereas the FVC value remains constant. Airflow limitation is usually induced via bronchoprovocation using agonists such as inhaled metacholine or histamine which trigger contraction of bronchial smooth muscles (42). The dose of administered bronchoconstrictor causing a 20% fall in FEV_1 is defined as the 'provocative concentration 20%' or PC_{20} (43). This PC_{20} value reflects the notion of airway hyperresponsiveness (AHR), and is inversely associated with asthma severity (Diagram 2b). The concept of AHR can be defined as the tendency of asthmatic airways to narrow excessively and more easily in response to triggers that have little or no effect in non-asthmatic individuals. AHR consists of a combination of an amplified airway reactivity or hyperreactivity, defined as a greater reduction in airway lumen to a given dose of bronchoconstrictor stimulus, and airway hypersensitivity, corresponding to luminal reductions occurring at a lower dose of ASM constrictor agent compared with non-asthmatics (44, 45). The AHR episodes "are usually associated with widespread, but variable, airflow

obstruction/limitation within the lung that is often reversible either spontaneously or with treatment” (29).

1.2. Pathophysiology of allergic airway diseases

1.2.1. Mechanisms of Type I hypersensitivity and allergic inflammation

Type I hypersensitivity reactions comprise two phases, the early and the late-phase, both characterized by symptoms that appear following allergen sensitization. The cellular and molecular mechanisms leading to allergen-specific IgE production involve T ‘helper’ lymphocyte-mediated B cell activation and immunoglobulin isotype class switching towards IgE. The T ‘helper’ lymphocytes inducing IgE production display a specific phenotype, referred to as type-2 or Th2 phenotype (46). The differentiation of T ‘helper’ lymphocytes into Th2 cells requires adequate antigen presentation to T cells by APCs which internalized antigens at mucosal sites (47) and migrated to regional lymphoid tissues (48-50). The sensitization process also includes the ‘priming’ of granulocytes (mast cells and basophils), which are coated with IgE bound to their cognate high-affinity receptor (FcεRI) awaiting the next allergen challenge (51) (Diagram 1).

The early-phase takes place within minutes and develops when allergens recognized by IgE molecules cross-link at the surface of IgE receptor-expressing cells, effectively activating them (Diagram 3). Activated mast cells and basophils release a wide variety of preformed and granule-stored mediators through a process called degranulation, and the release of newly formed mediators

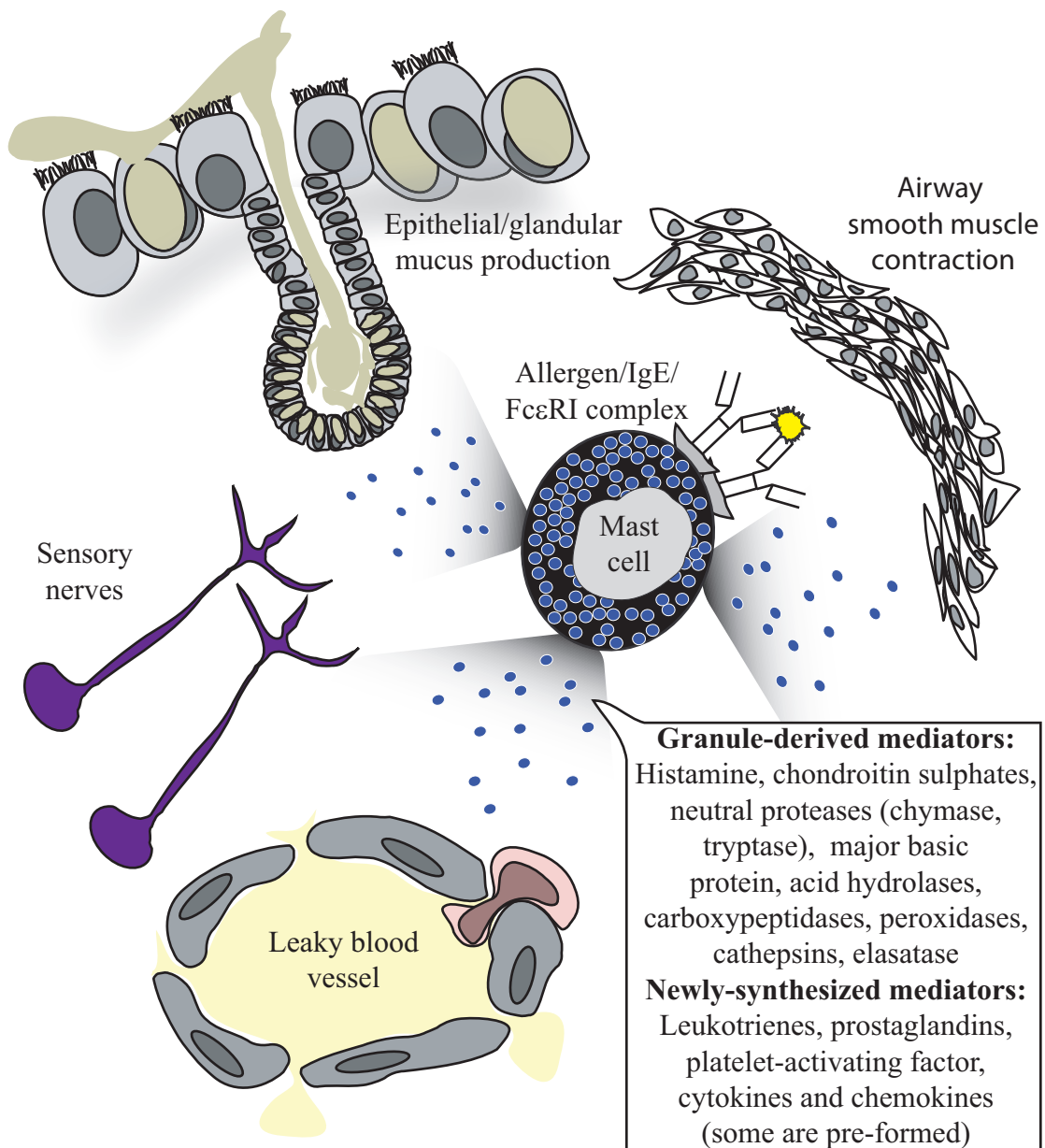


Diagram 3. Early-phase of the Type I hypersensitive reaction. This early phase takes place within minutes after allergen exposure, and is triggered by cross-linking of allergens to allergen-specific IgE-primed mast cells (and basophils) in the airway mucosa. Allergen/IgE complexes are bound to FcεRI receptors which mediates a signaling cascade leading to rapid production of a variety of inflammatory mediators and degranulation/release of pre-formed mediators contained in granules outside cells. The resulting early phase symptoms include include itching due to local nerve sensitization, mucus overproduction and release by epithelial cells, vascular leaking and tissue edema, and smooth muscle contraction.

includes histamine, several proteases, cytokines, chemokines and lipid mediators such as cysteinyl leukotrienes and prostaglandins (49, 52, 53). The mixture of pro-inflammatory mediators quickly released in the nasal or bronchial mucosa is responsible for triggering symptoms of the early-phase. In allergic rhinitis, symptoms include pruritus or itching due to nerve cell reactivity, nasal congestion due to mucus secretion, erythema and swelling due to vasodilatation and mucosal edema (54). Similar symptoms develop during the early-phase of an allergic asthma episode, but may also be accompanied by rapid airflow limitation and breathlessness due to bronchoconstriction of ASM cells and airway hyperresponsiveness. The early-phase typically subsides within a few hours but is seen as the initiating trigger for the development of a magnified and long-lasting late-phase reaction later on.

The late-phase normally develops 4 to 12 hours following the early-phase. Symptoms are typically more prominent than their earlier counterpart, and are associated with increased morbidity as they can last for several days. The late-phase develops as a result of the biological activity of mucosal cells of the nasal cavity or the bronchi which secrete degranulation products during the early-phase on. These pro-inflammatory mediators exert differential effects on structural cells including epithelial and endothelial cells, as well as on resident immune cells including macrophages and dendritic cells. Activated endothelial cells release chemokines to attract leukocytes and express elevated levels of adhesion

molecules therefore facilitating leukocyte attachment, rolling and extravasation through the capillaries in a process called diapedesis. The leaky endothelial barrier is coupled to the establishment of a chemoattractive gradient within the submucosa and towards the airway lumen as a result of endothelial and epithelial cell-derived chemokine production. These elements translate into delayed but massive recruitment and activation of peripheral leukocytes into the airway submucosa. Infiltrating leukocytes include allergen-specific lymphocytes (mainly cluster of differentiation 4 or CD4+ T 'helper' cells, but also B cells), granulocytes (mainly eosinophils, but also basophils and neutrophils), APCs such as dendritic cells, monocytes and macrophages (55), and hematopoietic stem cells (Diagram 4) (56-60). The most prominent infiltrating cell type associated with late-phase allergic reactions are the allergen-specific CD4-expressing T lymphocytes, and activated eosinophils (61-63). CD4 T 'helper' lymphocytes infiltrating the site of allergic inflammation display a skewed Th2-type phenotype (46, 59, 61, 64). Th2 lymphocytes are characterized by the production of a specific array of cytokines and are seen as the main orchestrators of allergic inflammation, as detailed in section 1.2.3.1.1. Activated eosinophils recruited to the airways release a vast array of mediators, including proteases, and therefore participate in the tissue damage/repair process which can lead to structural remodelling and thickening of the airway wall (65). The activation of leukocytes and the local stem cell differentiation into effector cells (66, 67) leads to the production of mediators

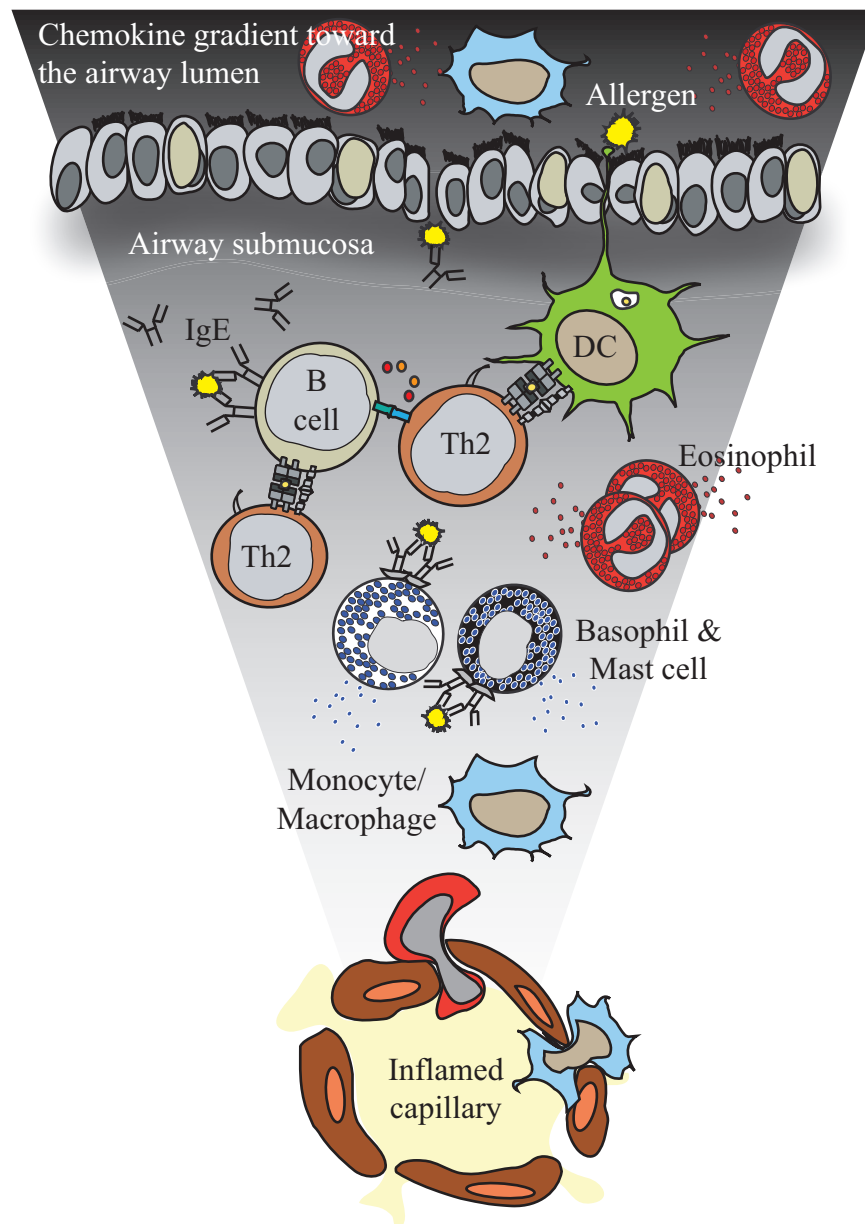


Diagram 4. Late-phase of the Type I hypersensitive reaction. The inflammatory response taking place here results from the massive recruitment and activation of immune cells: Th2-type lymphocytes and IgE-producing B cells, activated eosinophils and macrophages, degranulating basophils and mast cells. These cells invade the airway submucosa and/or lumen in response to gradients of chemokines. In a positive feedback loop, the lasting inflammation exacerbates the airway narrowing and hyperresponsiveness through bronchial edema, mucus hypersecretion, sensory nerve sensitization, structural cell damage and most importantly smooth muscle contraction.

perpetuating the pro-inflammatory condition as well as airflow limitation. In contrast with the early-phase reaction which depends on tissue resident mast cell and IgE-mediated events, it is the magnitude and nature of the cellular influx into mucosal tissues, as well as their release of inflammatory mediators, which drive the amplified and prolonged clinical manifestations of the late-phase. The inflammation goes on until allergen elimination and/or until negative-feedback counter-regulatory and/or repair mechanisms take over.

1.2.2. Airway remodelling

In asthma, the late-phase inflammation causes prolonged airflow obstruction. Airway narrowing is due to the mucus hypersecretion, microvascular leaking (edema) and bronchial smooth muscle contraction. However, allergen-induced inflammatory responses in the airways are associated with a variable degree of tissue damage. Chronic tissue damage in turn commands proper repair which causes thickening of the airway wall leading to airflow limitation (68). The first evidence of structural changes in the asthmatic airways came from Huber and Koessler (69) who made post-mortem examinations of bronchial tissues from patients who died from severe asthma. In addition to massive inflammation of the airway wall and the partial or total occlusion of the bronchial lumen by mucous plugs, the authors noted a consistent thickening of the airway wall compared with control subjects (69, 70). Thicker airway wall was seen both in the small and large airways i.e. bronchioles and bronchi (70-72). The airway wall area

measurement, performed using high resolution computer tomography (HRCT), was recently established as an indicator of thickness (73). The airway wall area is increased in asthmatics compared with controls (74), and also successfully predicts airway hyperresponsiveness in patients (73). Studies in bronchial tissue of asthmatic children and adults similarly reported associations between the airway wall thickness and disease severity (68, 75).

The concept of airway wall thickening is referred to as airway remodelling; an umbrella term regrouping the semi-permanent and permanent (i.e. dynamic and progressive) structural changes taking place in response to various extrinsic and intrinsic stimuli. Airway remodelling is often described as a protective but dysregulated mechanism in response to chronic inflammatory insult. The airway remodelling regroups the following histological features (Diagram 5): 1) inflammation (infiltration of airway lumen and submucosa by leukocytes) (56, 58, 64); 2) epithelial changes including goblet or mucus-producing cell hyperplasia, overproduction of mucus and inflammatory mediators, increased epithelial damage and exaggerated repair processes leading to thickening (76-78); 3) subepithelial fibrosis: thickening of basement membrane or lamina reticularis, due to dysregulated production and regulation of extracellular matrix (ECM) proteins (79-83); 4) airway smooth muscle (ASM) changes: hypertrophy, hyperplasia and possibly migration towards the airway lumen, phenotypic changes with regards to contractility and mediator production (84-87); 5) vascular changes: submucosal

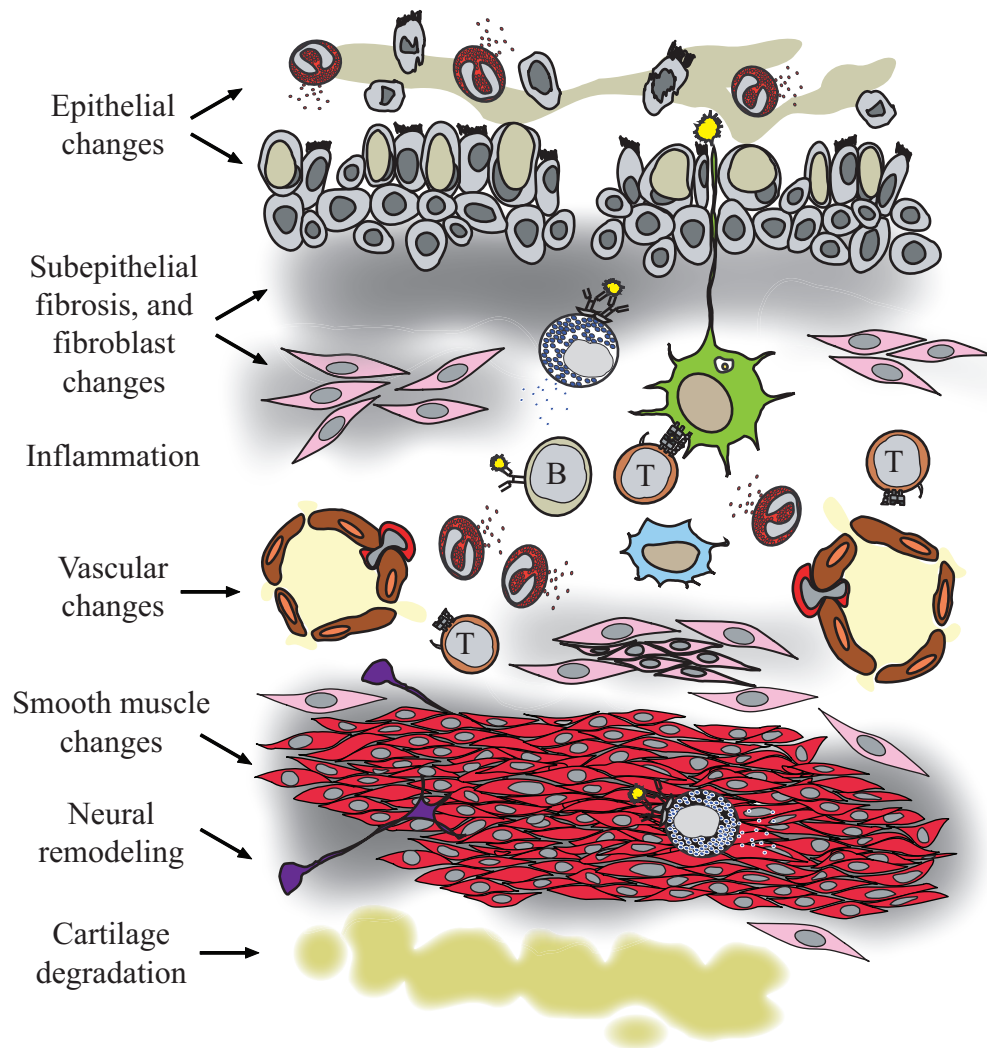


Diagram 5. Airway remodelling in asthma. Schematic representation of reversible or partially reversible structural changes taking place the bronchial wall of subjects with asthma. Such changes affect the epithelium (damage/repair, mucus, thickening), subepithelial fibrosis (excessive extracellular matrix deposition), submucosal infiltration and activation of inflammatory cells, angiogenesis and vascular leakage, changes in the airway smooth muscle layer (due to hypertrophy, hyperplasia and migration), neural remodelling and cartilage degradation.

angiogenesis and vascular permeability (88, 89), and; 6) neural remodelling (release of nerve growth factors, hyperinnervation, and neuronal hyperexcitability) (90-92); and 7) loss of cartilage integrity: collagen degradation favouring airway obstruction) (93, 94). Despite these features of remodelling being primarily and extensively described in the asthmatic bronchi, some also apply to the nasal conduits of rhinitis patients. Notably, these include the inflammatory cell infiltrate, epithelial changes as well as subepithelial fibrosis (95).

1.2.3. Immune cell players

The present section briefly overviews some of the immune cell types whose role in allergic inflammation will be further discussed in this thesis. We will particularly focus on the differentiation and the biology of Th2-type lymphocytes, which play a critical role in orchestrating allergic-type immune responses. Furthermore, we will also consider the role of antigen-presenting cells and airway epithelial cells as they both served as experimental models for this thesis.

1.2.3.1. T 'helper' lymphocytes and lineages

Allergic rhinitis and asthma have been associated with increased numbers of nasal and bronchial mucosa infiltrating CD4-expressing T lymphocytes (56, 57, 96-98). Increased allergen-specific T 'helper' cells are the major cell type infiltrating the bronchoalveolar tissue (fluid and mucosa) of asthmatic subjects, in particular those suffering from late-phase allergic reactions i.e. acute asthma

exacerbations (99, 100). The proportion of circulating 'activated' CD4 T cells (those expressing the interleukin-2 receptor α or IL-2R α chain) was shown to be increased in symptomatic asthmatics compared with asymptomatic asthmatics, and to correlate with measures of airflow limitation (63, 100, 101). This activated CD4 T cell population also correlates with eosinophilic inflammation (64, 102). In contrast, decreased numbers and proportion of peripheral T 'helper' lymphocytes are seen in atopy and in allergic airway diseases (103, 104).

The CD4-expressing T lymphocytes are seen as the coordinators of adaptive immunity. T 'helper' (Th) cells are now classified into four lineages: Th1, Th2, Th17 and regulatory T (T-reg) cells. This classification is based on lineage-specific phenotypic characteristics including the cytokines they produce, cell surface markers they express and the type of immune responses they orchestrate (Table 1). Regulatory T cells are distinguished from other lineages based on their ability to suppress antigen-specific activation of effector Th1, Th2 and Th17 cells. All lineages arise from mature but naive CD4 T (i.e. Th0) cells which travelled from the thymus to secondary lymphoid organs (mainly lymph nodes) where they first encounter cognate T cell receptor (TCR) antigen bound to major histocompatibility complex (MHC) class II molecules on an APC. Extensive research on the molecular processes leading to Th cell differentiation and commitment to the different lineages has revealed their adaptive, self-promoting, counter-regulatory and plastic nature. Type 1 (Th1) and type 2 (Th2) cells were

Lineage	Discovery	Function	Featured cytokine produced	Immune responses involved in	
				Physiological	Pathological
Th1	1986 (46)	Effector	Interferon (IFN)- γ	Infections by intra-cellular pathogens	Autoimmunity
Th2		and memory T cells	IL-4, IL-5, IL-13	Infections by extra-cellular pathogens	Allergies
Th17	2006 (105)		IL-17A, IL-17F	Infections (various pathogens)	Autoimmunity
T-reg	1985-95 (106, 107)	Immuno-suppression	IL-10, TGF- β	Homeostatic control, negative feedback	Auto-immunity, Allergies

Table 1: List and summarized features of T ‘helper’ lymphocyte lineages

first categorized by Mossman et al (46) based on the distinct and counter-regulatory set of cytokines they produce. The T-reg and Th17 subsets were recently described and more subtypes are likely to be discovered (108). The concept of Th lineage plasticity remains relatively obscure, but recent studies stress that the ability of Th cells to be converted into other phenotypes depend on their differentiation state (109, 110).

1.2.3.1.1. Type-2 or Th2-type lymphocytes

Type-2 immunity or Th2-type immune reactions normally occur in response to extracellular pathogens, mainly helminthic parasites. Th2-type responses are the central element in atopy and allergic diseases since they favour the production of antigen-specific IgE (111, 112). Activated Th2-type lymphocytes produce a wide range of cytokines from the Th2 cytokine cluster including IL-4, IL-5, IL-9 and IL-13, but also IL-25 (also called IL-17E) (113); the latter not being found in the Th2 cytokine gene cluster. Th2-type responses are associated with prominent humoral responses, principally mediated by antigen-specific IgE or IgG4 isotypes (114). Th2 cells promote allergen sensitization as they induce immunoglobulin isotype class switching of B lymphocytes to produce IgE (18). IgE sensitization ensures early-phase reactions upon further allergen exposure. This is followed by the recruitment of numerous leukocytes including Th2 cells and eosinophils to the site of inflammation. The Th2-type cytokines play divergent and important roles. The production of allergen-specific IgE by B cell

along with Th2 cell differentiation is largely driven by IL-4 (115-117), whereas the IL-3, IL-5 and granulocyte macrophage colony-stimulating factor (GM-CSF) act as differentiation and maturation factors on late-phase effector granulocytes, mainly eosinophils (118-120). While IL-4 appears to be essential for initiating Th2-type responses, IL-5 chiefly drives eosinophilic inflammation. IL-13 plays a prominent role in the effector phase of the allergic response through its multiple effects on differing cell types (121). IL-13 induces IgE production by B cells and chemokine production by airway structural cells, contributes to AHR by promoting ASM contraction, promotes epithelial cell mucus production and the production of ECM proteins by fibroblasts. The main functions of pro-allergic Th2-type cytokines are summarized in Table 2.

The association of a late-phase allergic reaction with activated T cells committed to the Th2 phenotype became clearer upon the characterization of IL-4 and IL-5 mRNA-expressing T lymphocytes in the bronchoalveolar lavage fluid (BALF) of atopic asthmatic subjects compared with controls (111). Such observations in the lung were preceded by the demonstration of Th2 that had infiltrated the skin of allergic individuals at sites of allergen-induced late-phase reactions, and expressed gene transcript for IL-3, IL-4, IL-5, GM-CSF (61). Massive infiltration of bronchial and nasal tissue by Th2 cytokine-expressing cells was also confirmed in allergic individuals exposed to inhaled allergens (64, 122-124). Studies correlated the numbers of CD25⁺ CD4⁺ T 'helper' lymphocytes

with numbers of eosinophils and IL-5 mRNA+ cells in the BALF. T cells were shown to be a primary source of IL-4 and IL-5 mRNA in allergen-induced rhinitis

Cytokine	Immunological functions
IL-3	✓ Eosinophil differentiation, maturation and activation ✓ Mast cell differentiation / maturation (mastocytosis)
IL-4	✓ Th2-type cell differentiation ✓ B cell isotype switching and IgE production ✓ Goblet cell metaplasia (mucus hypersecretion) ✓ Subepithelial fibrosis ✓ Inflammatory cell recruitment by inducing chemokine production and adhesion molecule expression ✓ Airway smooth muscle hyperplasia (remodelling) and contraction (AHR)
IL-5	✓ Eosinophil differentiation, maturation and activation ✓ ASM hyperplasia (remodelling) and contraction (AHR)
IL-9	✓ Th2-type cell differentiation ✓ Mast cell differentiation / maturation (mastocytosis)
IL-13	✓ B cell isotype switching and IgE production ✓ Goblet cell metaplasia (mucus hypersecretion) ✓ Subepithelial fibrosis ✓ Inflammatory cell recruitment by inducing chemokine production and adhesion molecule expression ✓ ASM remodelling and contraction (AHR)
IL-17E (IL-25)	✓ Th2-type cell differentiation
GM-CSF	✓ Eosinophil differentiation, maturation and activation

Table 2: Main Th2-type cytokines driving the immunopathological features of airway allergic diseases

(125, 126). Similarly, a majority of IL-13-expressing cells in bronchial tissue of asthmatic subjects are T cells and a majority of IL-13-immunoreactive cells also coexpressed IL-4 (127, 128). IL-13 levels and mRNA expression by cells in the BALF of allergic subjects is significantly increased upon airway segmental allergen challenge (129). IL-13 mRNA levels are also elevated in homogenized bronchial tissue from asthmatics (regardless of atopic status) compared with both atopic controls and non-atopic controls (130). The numbers of IL-13 mRNA+ cells in lung tissues of allergic asthmatics peaked at 24h after allergen inhalation and correlated strongly with eosinophil, T cell, and macrophage (but not neutrophil) recruitment during the late-phase response (128). Numbers of IL-4, IL-5 and IL-13-expressing cells were similarly increased in nasal tissue of allergen-challenged patients with allergic rhinitis (131). In addition to mucosal tissue and BALF, the Th2 bias can be detected in sputum samples as well as in peripheral in blood cells (123, 132, 133). Neonatal CD4 T cell-derived IL-13 production is also elevated in children who develop allergic disease later in life, and is associated with disease severity (134). The overproduction of IL-9 in the airways of asthmatics is also mainly due to T cells (135).

Although mild-to-moderate allergic asthma phenotypes are associated with Th2-mediated inflammation (136), the inflammation in severe asthma appears to be more complex and would not consist of strict Th2-type profile. More prominent Th1-type inflammation exists in the lung tissue of subjects with those who suffer

from severe asthma compared with moderate disease, however the numbers of IL-4-expressing cells are increased in moderate compared with severe asthma sufferers (137, 138). This opposing Th1/Th2 balance in moderate vs. severe asthma likely reflects the counter-regulatory nature of Th1/Th2 phenotypes (139). However, it has been argued that stable asthmatics display a Th2 immune bias but might display more of a mixed Th1/Th2 cell activation during exacerbations (140). Recent studies also integrated the role Th17-type responses in discriminating severe asthma patients based on the persistence of airflow obstruction (141). There is indeed increasing evidence of a role for Th17 cells in severe asthma, as depicted by their numbers of IL-17A and F-expressing cells in the airways of severe asthmatics compared to those with mild or moderate disease (142, 143). Despite the relative uncertainty regarding the exclusive role of type-2 immunity in subsets of severe asthmatic patients, the above-mentioned findings squarely established the central role of Th2 cells in orchestrating allergic inflammation in asthma and rhinitis (136).

1.2.3.1.2. Differentiation and regulation of Th2-type lymphocytes

This section summarizes our current understanding of the differentiation of Th2 cells. It will then integrate the self-promoting and counter-regulatory nature of Th2-type cells, which are central to the pathogenesis of allergies. In conjunction with antigenic stimulation via their TCR, several factors influence naive CD4 T cell differentiation into one of the above-mentioned lineages. These factors

include the type of APC, the strength of the antigenic signal, the presence of co-stimulatory molecules and the existing cytokine microenvironment. The differentiation of T 'helper' cells into phenotypic lineages is controlled by lineage-specific transcription factors and chromatin remodelling agents which together regulate gene expression (144). Chromatin remodelling agents rearrange the nucleosomes (DNA packaging units), rendering specific DNA sequences available to DNA sequence-specific binding proteins called transcription factors. These proteins regulate locus-specific recruitment of RNA polymerase II, gene transcription into mRNA and further translation into proteins e.g. cytokines.

The differentiation of T 'helper' lymphocytes into Th2 cells via weak TCR interaction with antigen:MHC II complexes (145, 146) from the surface of plasmacytoid dendritic cells (pDC) (147-149). Weak TCR activation can either be due to low concentrations of cognate peptides (150-152) or altered antigen integrity (153). APC-derived Th2-promoting co-stimulatory signals also include the engagement of OX40 ligand (OX40L) (154-156) and/or of Notch ligand Jagged (157, 158) onto their respective OX40 or Notch receptors on T cells. Antigenic stimulation of developing Th2-type cells activates kinases which, in turn increase intracellular Ca^{2+} levels and activate the calcium/calcineurin signal transduction pathway (159-161). Calcineurin dephosphorylates the nuclear factor of activated T cells 1 (NFAT1) which then translocates to the nucleus where it triggers the expression the GATA-binding protein 3 (GATA-3) (152, 158, 162); the

master regulator of Th2 differentiation (163, 164). Furthermore, TCR-mediated antigenic stimulation also induces the expression of IL-4, the prototypical Th2-type cytokine, along with IL-2, but not interferon- γ (IFN- γ) (161, 165, 166).

The presence of IL-4 is mandatory for Th2-type cell differentiation and immune responses (167-170). IL-4 signals through a heterodimeric receptor composed of the common cytokine-receptor gamma chain (γ c; CD132) subunit and the IL-4R α chain (171), both present on naive Th cells. Signalling through IL-4R share similarities with IL-12 as they both activate Janus kinase/signal transducers and activators of transcription (JAK/STAT) signal transduction pathways. IL-4R signalling results in further recruitment and tyrosine phosphorylation of Jak1 and Jak3, followed by STAT6. Phosphorylated STAT6 proteins homodimerize which enables nuclear translocation and binding to consensus sequences of target genes. IL-4-mediated STAT6 activation up-regulates the expression of Th2-specific cytokines including IL-4 and IL-5, and reduces IFN- γ and IL-12R β 2 expression (167, 172, 173). The numbers of STAT6-expressing cells is elevated in the nasal mucosa of allergen-challenged patients with allergic rhinitis, but also in the lung tissue of allergic asthmatics compared with controls (174, 175). STAT6 expression in the nasal mucosa of rhinitis patients correlates with IL-4 expression (174). STAT6 activation is also necessary and sufficient to induce GATA-3 expression (162, 173, 176).

Interleukin-2 signalling is also required for Th2 differentiation (167, 177). IL-2 induces early IL-4 production (177), and also contributes to maintaining it (152, 168). IL-2 signals through the IL-2 receptor complex which comprises the IL-2R α chain (CD25), the IL-2R β (CD122) and the common γ c (178). Signalling through IL-2R sequentially recruits and activates JAK1/JAK3, and then STAT5A. Activation of STAT5A promotes cell growth and survival through the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways as well as through STAT5A homodimerization and translocation to the cell nucleus (179). Nuclear STAT5A homodimers also bind the IL-4 gene promoter and favour its expression (180).

Beside IL-2, other type I cytokines including IL-7 and IL-15 (181), IL-6 (182), thymic stromal lymphopoeitin (TSLP) (156, 183, 184), IL-25 (also called IL-17E) (113, 185) and the IL-1-type cytokine IL-33 (186-188) also promote Th2 cell differentiation. The IL-33R complex contains ST2, a receptor previously shown to be critical for Th2-mediated immune responses to develop (187, 189, 190). IL-33 protein promotes expression of IL-5 and IL-13 by CD4 T cells (188, 191), and also acts as a chemoattractant for Th2, but not Th1 cells (192). TSLP signals through the TSLP receptor TSLPR/IL-7R α -chain (IL-7R α) complexes and promotes Th2 differentiation via the STAT5A pathway (183, 193). IL-25 is released by a wide variety of cell types. IL-25 signals through IL-17BR (IL-25R) (194) on naive Th cells where it induces early IL-4 release (185). On memory Th2

cells, IL-25 maintains Th2-type cytokine production in an IL-4-independent manner, by directly inducing GATA-3 (195).

As seen earlier, GATA-3 expression is mainly induced via the IL-4/STAT6 axis (196), but also upon TCR-mediated antigenic stimulation. GATA-3 is considered the main regulator of type-2 immunity since its ectopic expression in T cells increases its own expression and drives the expression of Th2-type cytokines (197). The transcription factor GATA-3 comprises zinc-finger motifs recognizing 'WGATAR'-containing consensus DNA sequences, where W denotes A or T, and R denotes A or G nucleobases (198, 199). GATA-3 regulates the transcription of several genes found in the Th2 cytokine gene cluster (197), located on chromosome 5q31.1 and which includes the IL-3, IL-4, IL-5, IL-9, IL-13 and GM-CSF genes (200-202). GATA-3 directly induces the transcription of IL-4 (203, 204), IL-5 (205), IL-10 (206) and IL-13 (207). The number of GATA-3-expressing cells is increased in the BAL fluid and the bronchial tissue of patients with asthma (208), and in allergen-challenged allergic rhinitis patients (131) compared with non-allergic controls. GATA-3 expression in the lung of asthma patients correlates with IL-5 expression (208). GATA-3 mediates critical steps in the CD4⁺ T cell commitment as it favours Th2 and limits Th1 differentiation (209). As well, GATA-3 limits STAT4 levels and IL-12 rather than down-regulating IL-12R β 2 (210). Furthermore, the master regulator in Th1 cells, T-bet, directly acts on GATA-3 expression and function to limit Th2 cell differentiation (211). Despite

the fact that GATA-3 expression is mandatory and sufficient for Th2 polarization, it is not sufficient for driving Th2 cell proliferation (212). This step depends on the IL-4/STAT6 pathway, which up-regulates the expression of the growth factor independent-1 (GFI-1) (213). The latter induces IL-2/STAT5A-mediated proliferation and inhibits Th17 and T-reg cell differentiation (213, 214). Taken together, TCR activation, IL-4 and IL-2 are considered as the main triggers driving the development of Th2-type immunity. These extracellular signals are coupled to the intracellular roles of GATA-3, STAT6, STAT5A and GFI-1 which mediate Th2 cell differentiation and proliferation (152, 177, 215).

1.2.3.2. Antigen-presenting cells

The mounting of a Type I hypersensitive allergic reaction requires allergen-specific IgE production and a Th2-type response. Such adaptive immune bias depends on the instruction by allergen-presenting cells expressing MHC class II molecules (48). The type of APC, the concentration and duration of antigenic signal, the presence of co-stimulatory molecules and the cytokine milieu are all factors influencing the differentiation of naive CD4 T lymphocytes. While DC-mediated TCR activation without CD28 engagement on T cells leads to anergy (i.e. failure to mount antigen-specific proliferation and activation), CD28 engagement promotes T cell survival, IL-2-mediated proliferation and effector lineage commitment. Cytokines also play roles in this process: IL-6 and tumour

necrosis factor (TNF)- α promote Th2 (216, 217), IL-12 promotes Th1 (218), and IL-10 promotes T-reg cell differentiation (219).

Antigen-presenting cells provide an important bridge to the innate arm of immunity as they respond to a wide variety of pathogen-associated molecular patterns (PAMPs) via the pattern recognition receptors (PRRs) they express (220). As well, innate immune signals can greatly influence CD4 T cell-mediated adaptive immune responses by modifying the phenotype of APCs. Some of these innate immune pathways influencing Th2-type immune responses to allergens will be reviewed later (section 1.3.4.4 and 1.3.4.5) Antigen-presenting cells regroup different cell types, including dendritic cells, macrophages and B lymphocytes, together considered as professional APCs. Non-professional APCs regroup other immune cells and structural cells, which occasionally express MHC class II molecules (221). Macrophages and B cells accomplish other immune functions aside from antigen presentation (as phagocytes and antibody-producing cells, respectively) while dendritic cells are the most dedicated APCs driving both cell-mediated and humoral arms of adaptive immunity (222, 223).

Although they were first identified in lymphoid tissues (224, 225), dendritic cells (DCs) infiltrate all organs and tissues (225, 226). Dendritic cells even localize close to or within epithelial cell layers lining mucosal tissues such as the skin, the gastro-intestinal tract and the airways (227-229). Their dendrites access the extracellular milieu, where they sense and take up potential antigens by

phagocytosis. Tissue-resident DCs remain highly mobile and can migrate to other tissues within hours. They migrate through the blood circulation and home in lymphoid tissues where they present antigens to lymphocytes by the MHC class II complex (230-233). Two main subtypes of DCs have been described to date, namely the myeloid or monocyte-derived DC (mDCs) and the plasmacytoid DCs (pDCs) which both originate from lymphoid and myeloid progenitors (234, 235). The classification of DC subtypes based on cell precursors, *in vivo* localization, surface markers and function remains a constantly-evolving field of research (236). Additionally, studies in murine models greatly outnumber those on human cells, which has also generated a great deal of confusion due to subtle inter-species variations.

Dendritic cells are critical for the allergen-specific Th2-type immune response (232, 233). The migration of allergen-presenting DCs to lymph nodes depends on a C-C motif chemokine 19 (CCL19 or macrophage inflammatory protein-3 β) gradient, which attracts DCs through its receptor called CCR7 (50, 232, 237). The antigen presentation can also take place directly in the submucosa where DCs activates tissue-infiltrating T cells (48, 238, 239). In this context, DCs also play crucial roles in the effector or late-phase of allergic reactions. In inflammatory conditions or during a late-phase allergic reaction in the airways, DCs are actively recruited from the bloodstream and invade the airway lumen and mucosa (240-244). Recent studies reported that both mDCs

and pDCs quickly infiltrate the airway lumen (i.e. sputum) upon inhaled allergen challenge of subjects with allergic asthma (244). Once in the airways, activated DCs up-regulate their expression of co-stimulatory molecules and cytokines which influence T cell differentiation (48, 245). Furthermore, allergen-stimulated DCs also release chemokines preferentially attracting Th2-type cells, including the chemokines CCL17 (thymus and activation regulated chemokine or TARC (246)) and CCL22 (macrophage-derived chemokine) (247, 248), as well as eosinophil chemoattractants (249).

Monocytes consist of myeloid phagocytes exclusively found in peripheral blood. Monocytes act as circulating sentinels of the innate immune system which phagocytose and dispose of foreign molecules, and respond to PAMPs through the PRRs they express. Under normal conditions, monocytes migrate to tissues where they differentiate to replenish the populations of macrophages or dendritic cells (250). Under inflammatory conditions, greater numbers of monocytes infiltrate the tissues and similarly differentiate in order to play important roles bridging innate (PAMP recognition and phagocytosis) and adaptive immunity (antigen presentation). While circulating monocytes resemble lymphocytes as they express the CD4 marker (251), they do not express either CD3 nor TCR. In allergic diseases, studies describing the differential phenotype of monocytes from individuals with allergic airways disease compared with those of controls yields controversial data. Monocytes from patients did not differ from those from

controls in terms of their adhesion molecule expression which enable endothelial adhesion and extravasation (252, 253), but may express more of the CD11c integrin; a common DC marker (254), and release more chemokines (like CCL3, also called macrophage inflammatory protein-1 α) (255). Monocytes also produce the immune-suppressive IL-10 cytokine, known for its anti-inflammatory effects (256). Whether monocytes expressing the IgE receptor (Fc ϵ RI) play a significant role in allergies remains unclear (257, 258).

Macrophages are tissue-resident monocytes that act as scavenger cells (phagocytes) and mediator-secreting cells upon recognition of foreign molecules via their numerous PRRs. Nonetheless, maturation and activation of macrophages provide these cells with the ability to present antigens to T lymphocytes. In the airways of asthmatic patients, macrophages also display high levels of cell surface co-stimulatory molecules and DC markers, suggesting they regulate adaptive responses to allergens (259). Despite the fact that their numbers in the asthmatic airway typically increase 48h post-allergen challenge (55), the role of macrophages as APCs is not yet fully understood. Macrophages are described as suppressors of adaptive immunity and the function of DCs as APCs (245, 260, 261). Macrophage depletion from the lung leads to enhanced immune responses to subsequent exposure to harmless antigens in normal animals (262). In a murine asthma model, animals with macrophages-depleted lungs displayed worsened Th2-type allergic responses compared with non-

depleted littermates (263). Adoptive transfer experiments revealed the critical role of lung macrophages in the IFN- γ -mediated suppression of Th2-type inflammation, which was absent in macrophage-depleted asthmatic animals (263). Macrophages also suppress pro-allergic immune responses via interactions with DCs, in a nitric oxide-dependent manner (260). Macrophages from allergic asthmatic patients may display an altered phenotype; producing more or less IL-10 than controls (264-267). Macrophages from allergic asthmatics induce IL-5 production by T 'helper' cells, a phenomenon not seen using cells from controls (268). Taken together, it appears that normal macrophages play immuno-suppressive roles, and that these functions may be dysregulated in the context of allergies.

The contribution of B lymphocytes to the antigen-presentation effort remains unclear. Although B lymphocytes sense antigens through their BCR and express MHC class II molecules to potentially activate T cells, there is little evidence supporting their role as APCs in humans (269). Nonetheless, B cells localize both in the epithelium and in the submucosa where they likely interact with antigens and infiltrating T cells (270). In an animal model of allergic asthma, B cell numbers in the draining lymph nodes increased following allergen exposure (271), and B cell to T cell interactions via MHC class II and TCRs were detected (272). Studies led in a B cell-deficient mouse model of experimental

asthma supported the existence of antigen-presenting B cells, influencing Th2 cytokine production via expression of CD40, CD80 and CD86 (273, 274).

Finally, other cell types have been described as non-professional APCs. These include endothelial cells (275, 276), epithelial cells (277, 278), mast cells (279, 280), eosinophils (281-284) and neutrophils (285). These cells may present antigens to memory or activated T cells and therefore contribute to secondary immune responses (e.g. late-phase allergic reactions), but their ability to activate naive T cells remains to be proven (230, 286). Nonetheless, these cells can greatly influence the mounting of adaptive immune responses by releasing mediators, including cytokines (281, 287).

1.2.3.3. Epithelial cells

The airway epithelium forms a protective and interactive barrier between the airway lumen and the tissues. It is the largest body surface in contact with the outside world, with an estimated area of 100 m², exposed to approximately 10,000 L of inhaled air per day (288). In addition to gases, the lung epithelium is the first cell to come in contact with inhaled chemicals, particulate matter, microbes and allergens. More than a strict physical barrier, the airway epithelium is considered an important immune player that contributes to the initiation and maintenance of inflammatory responses (289). Epithelial cells continuously sense the airways' microenvironment, respond to a wide variety of intrinsic and extrinsic

stimuli through receptors they express, and secrete numerous mediators which influence the immune system.

Cells forming the epithelium include basal cells onto which are attached both ciliated and non-ciliated (mucus secretory) columnar epithelial cells, named Clara and goblet cells, respectively. The barrier function of the epithelium is maintained by the cilia of Clara cells, the mucus layer as well as the tight junctions between epithelial cells. Ciliated Clara cells cover most of the epithelial surface, where they produce surfactant and ensure the mucociliary clearance as their cilia propel the mucus (and the entrapped particles) out of the airway. In the large airways including the trachea and the nasal cavity, the mucus is secreted both by goblet cells lining the airways and by subepithelial multicellular glands reaching the lumen through small ducts (290). In the bronchi or small airways, mucus is released only from goblet cells. The mucus is mainly composed of high molecular weight glycoproteins called mucins, such as MUC5AC and MUC5B gel-forming proteins (291, 292). The luminal epithelial cells are interconnected via tight junctions that limit paracellular transport and ensure the impermeability of the cell layer to inhaled molecules and luminal fluids. Tight junctions, also called *zonula occludens*, are created by the interaction of several proteins that associate at the cell membrane and cytoskeleton of adjacent cells (288). Epithelial integrity also relies on cadherin-mediated interactions between adjacent columnar cells and/or basal cells, called desmosomes. Basal cells attach to the

underlying basement membrane via integrin-mediated interactions with the extracellular matrix named hemidesmosomes (293).

In allergic rhinitis and asthma, both the upper (nasal) and lower (bronchial) epithelium undergo numerous phenotypic changes, which have both structural and functional impacts. One of the most prominent alterations noted is the presence of overabundant mucus secretion; a hallmark in allergic disease in the airways (294). Mucus overproduction in asthma and rhinitis is associated with epithelial remodelling including: increased numbers of goblet cells (hyperplasia) (295), increased mucin production, production and release on a single-goblet cell basis (294), as well as frequent columnar cell metaplasia or differentiation into goblet cells. The numbers of airway goblet cells increases with disease severity (294), but there are also reports of increased submucosal gland size contributing to mucus overproduction in the large airways. Goblet cells spread more distally in the peripheral airways of asthmatics, where they are not normally observed (296). Post-mortem analyses of the lung tissues from patients who died from asthma revealed small airway congestion by mucus plugs (71, 76, 297). In asthma, mucus hypersecretion is associated with sputum overproduction and decreased lung function during acute exacerbations (298, 299). In the upper airways of patients with rhinitis, mucus hypersecretion presents at the same time as symptoms of rhinorrhea and post-nasal drip.

Early reports comparing bronchial tissues and sputum samples from allergic asthmatic patients to those of healthy controls documented extensive epithelial damage. Epithelial shedding or desquamation (i.e. basement membrane denudation) were seen both in the large and small airways through microscopic and histological observations of the mucosa (295, 300, 301) and was also evidenced by the detection of columnar cell clusters (Creola bodies) in sputum or BAL samples of patients (302-304). The surface of denuded or epithelial cell-free lamina was greatly increased in asthma (305). The prominent bronchial epithelial damage in both allergic and non-allergic asthma compared with controls is also reflected by altered or absent desmosomes and hemidesmosomes in asthmatic subjects (295, 306). Nonetheless, several studies failed to observe significant epithelial damage or differences in the epithelial ultra-structure between tissues from asthmatics compared with controls. Some argued the asthma-related epithelial alterations could be an artefact of tissue handling and processing (307-309). These discrepancies likely reflect dynamic epithelial alterations in the natural course of allergic diseases, which include inflammatory episodes followed by aberrant repair processes further described in this section. Nevertheless, the notion of increased airway epithelial damage in allergic disease remains well-accepted.

Several studies have described the mechanisms leading to disruptions of the epithelium integrity. Both intrinsic (inflammatory mediators, growth factors)

and extrinsic stimuli (chemical pollutants, inhaled allergens, infectious agents) can significantly alter the barrier function and damage the airway epithelium. Among the extrinsic factors, allergens themselves decrease transepithelial electrical resistance (310), an indicator of barrier integrity. Pollens and house dust mite (HDM) contain several allergenic proteins with known proteolytic activity. These proteases can directly cleave tight junction proteins and cause cell desquamation (311, 312). Epithelial shedding can, in turn, facilitate the allergen's access to mucosal dendritic cells and bring about sensitization (310, 313, 314). Furthermore, Der p 1, 3 and 9 proteases derived from the house-dust mite *Dermatophagoides pteronyssinus* activate epithelial cells through protease-activated receptors (PARs, namely PAR-2) and trigger the release of the mediators IL-6, IL-8, GM-CSF and eotaxin/CCL11 (315-317). In animal models of experimental asthma, the proteolytic activity of allergens was necessary and sufficient to induce *in vivo* Th2-type allergic immune responses (247, 313, 318). Particulate matter and chemicals associated with air pollution, as well as infectious agents, also damage airway epithelial cells. Cigarette smoke (319) and urban pollution-derived particulate matter (320) cause epithelial cell DNA damage and induce cell death through the effects of reactive oxygen species. Similarly, detergents with chlorine derivatives, oxidants such as superoxide anions and ozone (321-323), and reactive nitrogen reactive species similarly damage epithelial cells and decrease epithelial permeability (324-326). Respiratory

infections caused by viruses have long been described as triggers of asthma exacerbations, both in children and adults (327, 328). Viral infections can kill epithelial cells (329, 330) and trigger an inflammatory response that destroys the invading pathogens (331) but also likely contribute to the epithelial damage in the asthmatic airways. The infiltration of airway lumen (64, 332) and subepithelium (58, 332) by activated immune cells leads to the release of numerous mediators which directly or indirectly alter epithelial cell functions. During the early-phase reaction, activated mast cells and basophils degranulate and release mediators including tryptase, which activate PAR-2 in the same way allergen-derived proteases do (333). Activated eosinophils release cationic protein (ECP) (334, 335) and major basic protein (MBP) (336) which induce epithelial cell apoptosis. Activated eosinophils and epithelial cells produce TNF- α which, either alone (337) or together with T cell-derived IFN- γ , trigger a pro-apoptotic signal in epithelial cell cultures (334, 338). Combined TNF- α and IFN- γ also destroy cell-cell interaction structures such as desmosomes. Taken together, several extrinsic and inflammatory stimuli are directly harmful to epithelial cells.

Studies have also demonstrated intrinsic differences between asthmatic and normal epithelial cells (77). There is evidence that the asthmatic epithelium, but not epithelial cells from normal controls, exhibit an endogenous defect in antioxidant mechanisms, suggesting that asthmatic cells are more susceptible to oxidative damage (339, 340). Similarly, studies demonstrated the reduced ability

of bronchial epithelial cells from patients with allergic asthma, but not from controls, to mount an anti-viral immune response (341). Epithelial cells from allergic asthma patients, but not from normal controls, also display a biased phenotype resulting in exaggerated production of pro-inflammatory mediators (Table 3). Epithelial cells from asthmatic individuals are also unique in that they display constitutive activation of the signal transducer and activator of transcription 1 (STAT1) (342), and an increased expression of STAT6 (343). These transcription factors respectively play prominent roles in the differentiation of T 'helper' lymphocytes into Th1 or Th2 cells (344). STAT1 and STAT6 are respectively activated downstream of IFN- γ and IL-4/IL-13 signalling, suggesting that both Th1 and Th2-type immunity play roles in the activation of epithelial cells. Caramori et al intriguingly documented the expression of the Th2-type transcription factor GATA-3 by airway epithelial cells, but its role in these cells remains unknown (345). Through STAT1, IFN- γ induces epithelial cell release of various chemokines, including TARC/CCL20 which attracts CXCR4-expressing Th2 cells (346), and RANTES (347) which attracts memory T cells (348), basophils and eosinophils (349, 350). STAT6 mediates IL-4/IL-13 signalling responsible for up-regulating the expression of chemokines including the eosinophil chemoattractant eotaxin. Th2 cytokines either up-regulate chemokine expression alone or in combination with TNF- α (351-354). Epithelial STAT6 expression is also associated with increased IL-8 expression (343), a neutrophil

Class of mediators	Mediators
Adhesion molecules	ICAM-1, VCAM-1
Acute phase proteins	Serum amyloid A
Antimicrobial peptides	α and β -defensins, such as HBD2
Chemokines	Eotaxin-1/CCL11, Eotaxin-2/CCL24 and Eotaxin - 3/CCL26, GRO α , IL-8/CXCL8, IP-10/CXCL10, MCP-1/CCL2, MIP-3 α , RANTES/CCL5
Cytokines	GM-CSF, IL-1 β , IL-5, IL-6, IL-10, IL-11, IL-13, IL-16, IL-18, IL-25, IL-33, oncostatin M (OSM), TGF- β 1 and TGF- β 2, TNF- α , TSLP
Ectoproteases	Disintegrin and metalloproteinase domain-containing protein 8 and 33 (ADAM8 and ADAM33)
Extracellular matrix proteins	Fibronectin
Growth Factors	BDNF, b-FGF, EGFR, EGF, HB-EGF, IGF-1, NGF, PDGF, SCF
Arachidonic acid metabolites	15-hydroxyeicosatetraenoic (HETE), 12,15-diHETE, 8, 15-diHETE, lipoxins, PGE ₂
Reactive oxygen and nitrogen species	Superoxide (O ⁻), nitric oxide (NO)
Vasoactive peptides and related proteins	Endothelin-1 (ET-1), kinin receptors B1 and B2

Table 3: Airway epithelial cell-derived mediators

chemoattractant. Bronchial epithelial cells from asthmatic individuals endogenously produce elevated levels of IL-8 and GM-CSF (355). Epithelial cell-specific STAT6 expression is mandatory for IL-13-mediated airway hyperresponsiveness and mucus production, consistent with a central role for the STAT6 axis in Th2-type diseases like asthma (356). These data are also in line

with the fact IL-4 and IL-13 indirectly induce mucin (MUC5AC) production by airway epithelial cells (356-358).

Additional cytokines including IL-1 β , TNF- α (359), IL-9 (360, 361), IL-17A (362) and transforming growth factor β 2 (TGF- β 2) (363, 364) induce mucus production by bronchial epithelial cells. Other intrinsic and extrinsic factors also induce mucin expression including: neutrophil elastase (365), reactive oxygen species (366), ozone (367), cigarette smoke chemicals (368, 369), bacterial-derived molecules or PAMPs (367, 370), and viral infections (371). Mucus production mainly depends on the epidermal growth factor receptor (EGFR) pathway, in which the above-mentioned triggers activate the TNF- α converting enzyme (TACE). The latter cleaves and releases membrane-bound EGFR ligands from epithelial (372, 373) and immune cells (374). EGFR signalling directly mediates mucin gene up-regulation (375, 376).

Aside from inducing mucus production and epithelial damage, the inflammatory insult also brings about mediators to regenerate the epithelium. Epidermal growth factor receptor (EGFR) is one of these mediators that serves both as a primary regulator of mucus production (375), but more importantly of epithelial cell proliferation (377, 378) and wound repair (378). Interestingly, several studies demonstrated an up-regulated expression of EGFR (379, 380) and EGFR ligands (380-382) in the asthmatic compared with non-asthmatic bronchial epithelium. Despite this over-representation of elements suggesting

extensive epithelial repair in the airways of asthmatic patients, asthmatic bronchial epithelial cells display reduced EGF-induced EGFR phosphorylation, and reduced proliferative response (383). As well, the asthmatic epithelium is apparently unable to proliferate and repair properly upon damage compared with normal epithelial cells. These data support the concept of dysregulated repair mechanisms in the airway epithelium of patients with asthma compared to non-asthmatic controls (384).

Another key mediator involved in epithelial remodelling is the transforming growth factor- β (TGF- β), whose expression increases in the asthmatic airways and is associated with disease severity (385-387). Eosinophils (385, 388), macrophages and virtually all structural cells express TGF- β (389). Studies have associated epithelial damage of the asthmatic epithelium with increased expression and release of TGF- β compared with epithelium from the normal controls (303, 304, 334, 390-392). Under normal conditions, TGF- β participates in wound healing as it has proliferative effects on epithelial cells via the SMAD (Sma and Mad-related) protein-mediated pathways (393). However, inflammatory conditions also activate TGF-mediated MAPK pathways which induce cell apoptosis (394) and may cause epithelial cell desquamation in asthma (395). Interestingly, TGF- β also impairs the EGFR-dependent proliferative response as it inhibits EGF-induced MAPK activation and up-regulates cyclin-dependent kinase (cdk) inhibitors (392). Finally, Th2-type cytokines but not Th1-type

cytokines favour TGF- β expression by cultured bronchial epithelial cells (396, 397). These data likely explain the dual role of TGF- β , first as promoting wound healing under normal conditions, but otherwise as interfering with epithelial repair under inflammatory conditions.

The impact of the inflammatory milieu on mucus production (398) and epithelial damage (334, 399, 400) further reinforce the idea that airway epithelial remodelling are consequences of allergic inflammation (Diagram 5). However, epithelial loss has been detected not only in adults, but also in children with allergic asthma compared with control children (401-403). The latter suggest that epithelial changes may take place early in the natural history of allergic asthma, consistent with intrinsic differences seen between the asthmatic and normal epithelium. This inherently biased epithelial phenotype in patients with asthma and rhinitis reflects the altered expression of pro-inflammatory transcription factors discussed earlier. Studies have shown that the asthmatic airways display features of the immature or developing lung, referred to as dysregulated epithelial-mesenchymal trophic unit (EMTU) (404, 405). The EMTU refers to bidirectional interactions between epithelial cells, the basement membrane made of extracellular matrix proteins, and the underlying mesenchymal cells called fibroblasts (405). Epithelial cells express a wide variety of mediators including TGF- β s, EGFR and its ligands, which auto-regulate the development and maintenance of airway wall structure. These mediators greatly influence epithelial

morphology and repair, mucus production, epithelial to mesenchymal cell transition (i.e. fibroblast differentiation), ECM protein release and basement membrane formation (406, 407).

1.3. Epidemiology and etiology of airway allergic diseases

1.3.1. The allergy epidemic

In the previous sections, I overviewed our current understanding of the cellular and molecular mechanisms underlying the pathogenesis of allergic diseases, with a particular focus on allergic rhinitis and asthma. When looking back to the epidemiology of allergies in a more general fashion, the past decades have witnessed a rising trend in the incidence of allergic diseases worldwide (408). In the United States of America, the prevalence of asthma increased about 75% between the 1960s (409) and the mid-1990s. The asthma rates in children under age 5 have increased strikingly by 160% from the 1980 to 2003, passing from 3.6% to approximately 5.8% (410). In Canada, numbers are similar with a reported increase in prevalence from 2.5% to 11.2% between 1978 to 1995 in children below 15 years of age (411). Among adults, asthma rates increased from 2.3% in 1979 to above 8.4% in 2003 (412). Since then, the percentage of asthmatic Canadian adults remained relatively stable and above the 8% mark (413). Geographically, numerous studies conducted in the context of the International Study of Asthma and Allergies in Childhood (ISAAC) have repeatedly demonstrated the predominant increase in asthma prevalence in

westernized countries, including Western Europe, North America and Australia; in contrast with developing countries of Eastern Europe and Asia (408, 414, 415). These results match similar geographical variations in the prevalence of atopic eczema and allergic rhinoconjunctivitis (literally rhinitis with 'itchy-watery' eyes), further reinforcing the international patterns (28, 414). While few data are available in Canada, the prevalence of rhinitis in the USA is estimated at 10 to 30% of adults and 40% of children (28, 416, 417). Although the asthma mortality rates in Canada have significantly declined since 1985 and now cause approximately 1 death per 100,000 Canadians (412), asthma is responsible for over 50,000 admissions to Canadian hospitals per year (418). In the USA, asthma accounts for more than 500,000 hospitalizations each year (419), including more than 200,000 emergency room visits (420). The high and increasing prevalence of airway allergic diseases in our societies also implies a significant socioeconomical burden (421). According to the National Heart, Lung and Blood Institute, the annual expenditures for health care, drugs and reduced productivity due to asthma are estimated to be more than \$20 billion US (29). In Canada, the total annual cost of asthma care reaches \$600 million CAN (422). The costs of asthma treatment management also proportionally rise with disease severity (421, 423).

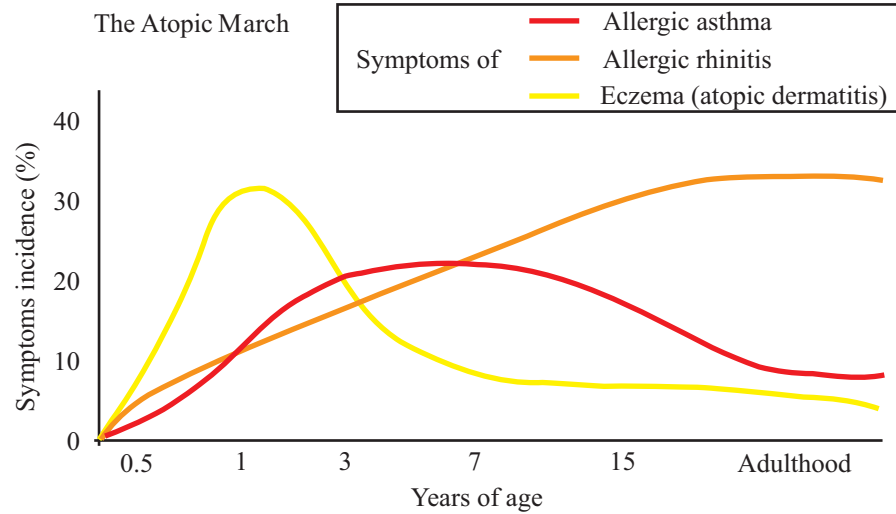
1.3.2. The 'atopic march' and the united airway theory

As discussed earlier, atopy or the tendency to produce IgE antibodies and to become sensitized upon exposure to innocuous proteins has been widely associated with the development and incidence of allergic diseases such as eczema, rhinoconjunctivitis (often referred to as rhinitis) and asthma (12, 424). Several studies indeed describe the extent to which the development of allergic diseases such as asthma, is attributable to atopy (425). In a longitudinal study conducted on newborn children until 13 years of age, sensitization to perennial allergens was associated with a diagnosis of asthma at school age and impaired lung function (426). In contrast, children with wheeze but no atopy lost their symptoms and retained normal lung function at puberty. Using both skin prick tests and IgE levels as indicators of sensitization and atopy, meta-analyses of epidemiological data reported that a majority (>60%) of asthmatic children and adults are also atopic (425) and have allergies (427). After correcting for the proportion of non-asthmatic atopic subjects, about 40% of asthma cases remained attributable to atopy (425). In a paediatric cohort with or without rhinitis, children with rhinitis displayed increased diagnosed rates of allergic sensitization, asthma and dermatitis (428-431). A large number of longitudinal studies of newborn children with atopic histories have documented the high propensity to progress from one atopic disease to another (for example from atopic dermatitis to rhinitis and asthma) up to the age of 22 years-old (432-437). Furthermore, childhood allergic rhinitis was associated with a significant risk of developing

asthma later in life (438). The co-incidence of atopic diseases also led to the emergence of the concept of the 'atopic march', describing a progression of atopic disorders, commonly evolving from atopic eczema in young infants to rhinitis and finally asthma in older children and adults (424, 439) (Diagram 6a).

The fact that asthma and rhinitis co-exist in the same patients has been confirmed by several studies, on many continents (440-445). The incidence of asthma can reach 80% among subjects with rhinitis, whereas it remains below 10% in individuals without rhinitis (446-448). These numbers reflect a much higher risk of developing asthma in rhinitis patients (431, 443, 449). The similarities between asthma and rhinitis pathophysiology, along with an epidemiological link and the physiological connection between the upper and lower airways have led to the emergence of the concept of 'one airway-one disease', also known as the 'united airway' or 'integrated airway' theory (28, 450-454). Undoubtedly, the upper and lower airways are functionally complementary, as the nasal cavity conditions the air to be inhaled throughout the lower respiratory tract (455) (Diagram 6b). Although they have different embryologic origin (456), the upper and lower airways show similar structure and histology, with the exception of an absence of smooth muscle in the upper airway wall. Structural similarities between the nasal and bronchial airway wall include the ciliated columnar epithelial lining and mucous glands, the vasculature and innervations. The question of whether or not asthma and rhinitis reflect

A



B

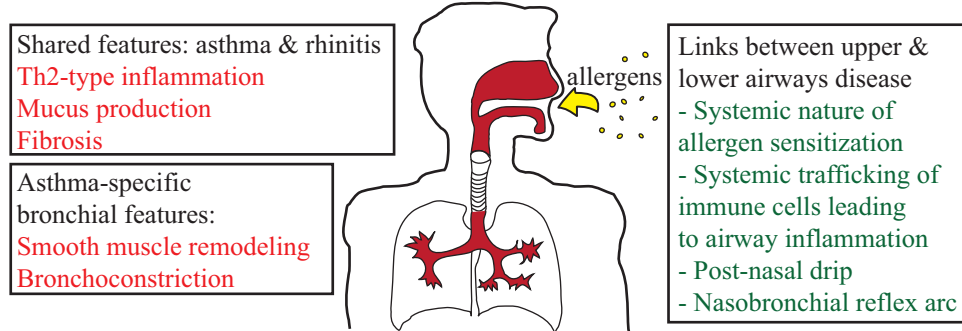


Diagram 6. The atopic march and the ‘united airway’ theory. The atopic march refers to the typical co-existence of atopic disorders in a same patients, diseases which tend to progress from skin to airway manifestations from childhood to adulthood, respectively (A). The diagram was based on references 424 and 439. The frequent co-incidence of and similarities between allergic asthma and rhinitis led to the emergence of the ‘one airway one disease’ theory (B). This theory refers to shared pathophysiological features of the diseases: shared and disease-specific features are presented in red, while mechanistic hypotheses explaining a link between the two diseases are presented in green.

pathophysiologic manifestations of a single disease at different locations or two different disorders is still debated (457). The inflammatory component appears to be relatively similar in both diseases, as chronic allergen-specific responses lead to vasodilatation, mucus production and a bi-phasic inflammatory response. Although airway remodelling may be more prominent in the lower airways (456), features of remodelling including epithelial shedding, thickening of the basement membrane and the presence of myofibroblasts are present in both airway diseases and compartments (95, 458). In terms of therapeutic approaches, the efficacy of intranasal therapies as a treatment for bronchial symptoms and asthma is still debated (452, 459).

Potential explanations to the 'united airway theory' include the post-nasal drip, the nasobronchial reflex arc and the systemic allergic response hypothesis. The post-nasal drip hypothesis postulates that inflammatory and secretory responses in the nose likely enable the transit of inflammatory cells and/or mediators from the upper to the lower airways (460). In the case of the nasobronchial reflex arc, few studies suggested that allergen-driven nasal inflammation may trigger bronchial hyperresponsiveness through a reflex arc involving trigeminal afferent and vagal efferent fibres (461). Finally, the systemic hypothesis refers to the atopic march: the likeliness of atopic individuals to develop disorders at different locations (e.g. skin, airways, gut). Longitudinal epidemiological studies indeed documented the linkage between skin

sensitization (atopic dermatitis) (424, 439), or gastro-intestinal sensitization (food allergies) (462) with airway diseases such as rhinitis and asthma. In these medical conditions, local sensitization and/or challenge can lead to allergic-type inflammatory responses at distant sites, reflecting systemic integration of cells and/or mediators into the circulation, and the transfer of sensitization to other organs (451, 453, 463-465). Interestingly, house dust mite-sensitized patients with dermatitis developed immediate bronchial hypersensitivity response to inhaled dust mite allergen similarly to those with HDM-induced asthma (463). Animals studies also demonstrated that epicutaneous allergen sensitization predisposes the animals to both nasal (466) or bronchial inflammation and hyperresponsiveness following aerosolized allergen challenge (465, 467-469). Nasal biopsies isolated from asthmatic patients without rhinitis exhibited increased infiltration by eosinophils compared with tissues from healthy controls (453). Similarly, rhinitis non-asthmatic patients undergoing segmental endobronchial allergen challenge present both nasal pulmonary symptoms, along with lung tissue eosinophilia (451). The extent of Th2-type nasal inflammation also correlated with nasal and bronchial symptoms in patients with only seasonal allergic rhinitis (470, 471). Furthermore, patients with concomitant asthma and rhinitis display exaggerated nasal and bronchial symptoms compared with patients with persistent asthma alone (472). KleinJan et al demonstrated both nasal and bronchial allergic inflammation following intranasal allergen challenge

in a mouse model of rhinitis (473). Finally, the systemic inflammation hypothesis can also be supported by the concept of increased numbers of peripheral hematopoietic stem cells, the precursors of effector cells such as eosinophils and basophils, in atopy subjects, patients with allergic diseases and animal models (67, 474, 475).

1.3.3. Causes of the allergic epidemic

While their prevalence was reportedly limited at the beginning of the 19th century, the rising incidence of allergic diseases in the last 200 years has reached epidemic proportions (476). This rapid increase in allergic disorders along with the relative confinement of the epidemic to developed countries likely reflects changes in the environment rather than genetic factors (477). Part of this rising prevalence of allergies may also be attributable to improvements in diagnosis, increased awareness and reporting of symptoms (478). Allergic asthma and rhinitis both result from hypersensitive immune responses to allergens, complex immune interactions and physiological consequences in the airways. The etiology of allergic rhinitis and asthma is therefore necessarily polygenic and multifactorial as it integrates both genetic and environmental factors.

1.3.3.1. Genetic predispositions

Henry Hide Salter was the first to propose that asthma had a heritable component in the 19th century (4). However, the genetic component of allergic

diseases was empirically demonstrated 50 years later by Cooke & van der Veer, who, using skin tests, established the increased propensity of children with hypersensitive relatives to develop atopy (17, 479). More than a century after Salters' work, it is well-recognized that not only asthma, but also eczema, rhinitis, food allergy and the atopic state all have a strong genetic basis. This was evidenced by studies in twins reporting higher concordance rates for allergic diseases (asthma, rhinitis, eczema) and atopy in monozygotic compared with dizygotic twins (479-481). Twin studies have been supported by familial inheritance studies from parents to children (482): approximately 40% of children from asthmatic parents develop asthma during childhood, and this before the age of 6 (433).

There is a great deal of published literature identifying specific genes or genetic polymorphisms associated with allergic diseases and phenotypes (483-488). The two main approaches commonly used to identify genetic effects on a disease are as follows: the candidate gene approach and the positional cloning. The first approach starts off with a known gene, whose alleles are screened throughout a population of allergic cases and controls. In this approach, polymorphisms of a given gene (i.e. genotype) may ultimately be associated with an increased or decreased risk of developing a disease. Through positional cloning, researchers usually first perform genome-wide scans looking for chromosomal regions containing one or more disease predisposing genes in a

cohort of high-risk individuals or families (489). The selected regions are further dissected in search of genetic variants (e.g. mutations, alleles, single nucleotides polymorphisms or SNPs) associated with disease. Although they are more expensive, the advantages of a genome-wide association studies and positional cloning is that it can be applied blindly, does not imply prior knowledge of the disease, phenotype or the genes to be found (i.e. it is not hypothesis-driven).

Considering the common immunological basis of atopy and allergic disorders, it is easily conceivable that genes predisposing individuals to develop an allergic disorder like rhinitis account for the increased propensity of the same individual to develop another allergic disease like asthma, later in life (438). A comprehensive review of genes associated with asthma and atopy phenotypes was recently published by Bosse & Hudson, in 2007 (483). Several of these asthma and allergy-associated genes play prominent roles in the allergen-specific (adaptive) immune response. They include the beta (β) subunit of the high affinity IgE receptor ($Fc\epsilon R1\beta$) (490), members of the Th2 cytokine gene cluster including IL-4 (491, 492), IL-13 (493-495), but also the Th2-type transcription factors STAT6 (496, 497) and GATA-3 (498) which favour Th2-type differentiation. Variants within the gene sequences of various inflammatory mediators (cytokines and chemokines) and/or receptors were also widely associated with asthma and atopy phenotypes (487, 499).

1.3.3.2. Environmental factors

The rapid change in the incidence rates of allergies in the past decades likely indicates changes in the environment or lifestyle rather than a genetic drift. Initiated in 1991, the International Study of Asthma and Allergies in Childhood (ISAAC) globally documented the rising incidence of self-reported, questionnaire-based symptoms of allergic diseases (500). The first phase of the ISAAC reported worldwide variations in the incidence of allergic asthma, rhinitis (i.e. rhinoconjunctivitis) and eczema in about 750,000 teenagers representing more than 50 countries (414). In particular, these results highlighted the increased rates of allergic diseases in westernized countries (414). The first phase also provided a framework for further investigation of the factors that might explain these regional differences and appropriate longitudinal surveys in the second (501) and third phase, respectively (502). Besides genetic factors, the present section will briefly address some of the environmental factors that may explain the allergy epidemic in the developed world.

1.3.3.2.1. Pollution and occupational exposures

Industrialization and urbanization has occurred throughout the world but more prominently in western countries since the end of the 18th century, greatly elevating the ambient pollutants (gases) and inhaled particulate matter (both organic and non-organic), especially in urban areas. Interestingly, the increasing prevalence of hay fever coincided with the industrial revolution both in Europe

and America (476). Furthermore, exposure to increased levels of particulate matter ($<10\text{ }\mu\text{m}$ in size) has reproducibly been associated with more frequent asthma exacerbations across the world (503, 504). The main contributor to air pollution in urban areas remains road traffic, which emits vast quantities of diesel exhaust particles, sulphur dioxide (SO_2), nitrogen dioxide (NO_2) and ozone (O_3). The proximity to high-traffic roads, as well as traffic counts, were epidemiologically linked and correlated with increased asthma rates, active asthma symptoms, cough and wheeze (505, 506). Diesel exhaust particles, when studied as an agonist in human exposure experiments, animal models and *in vitro* experiments, reproducibly displayed strong adjuvant effects, as they skew the immune system toward IgE production and a Th2-type phenotype (507-510). Although the biological effects of O_3 , NO_2 and SO_2 can hardly be dissociated from those of particulate matter, elevated ambient levels of these gases robustly decrease lung function, augment acute responses to allergens in atopic individuals and increase the frequency of bronchoconstrictions among asthmatics (504, 511). Taken together, exposure to air pollutants is definitely associated with worsened symptoms and more frequent allergic disease exacerbations, but it may also influence the immune system and favour the development of allergic phenotypes.

Smoking habits and exposure to smoke are also considered as significant risk factors in the development of allergic diseases. Cigarette and tobacco smoke

consists of a mixture of more than 5,000 chemicals (512, 513). These chemicals damage cells, induce inflammatory responses but also impair various aspects of both innate and adaptive immune systems (514). Chronic smoking is widely associated with lung cancer, chronic obstructive pulmonary disease (COPD) and respiratory infections (514-517). With regards to allergies and asthma, there is epidemiological evidence supporting the fact that maternal exposure to tobacco smoke during gestation (433, 518) as well as early life exposure to environmental smoke (519, 520) are significant risk factors for childhood wheeze, poor lung function, respiratory infections and physician-diagnosed asthma (521, 522). The mechanistic explanation of the trans-generational effect of smoking on asthma development in the offspring of smoking mothers remains unclear. Recent reports suggest it could involve epigenetic processes including DNA methylation and histone modification patterns transferred *in utero* (523, 524). Another study conducted in mice (525) suggested that AHR in the offspring of smoking mothers is due to an up-regulation of muscarinic receptors (for acetylcholine) and of a phosphodiesterase (an enzyme that cleaves anti-inflammatory cyclic adenosine monophosphate [cAMP]) (526). Pre-natal exposure to smoke induces remodelling of the offsprings' airways (527), but does not influence allergen sensitization (528-531), nor favour the development of Th2-type phenotypes or IgE production (527, 532), unless the parents were themselves allergic (therefore reflecting the genetic basis of atopy) (533). In contrast, early life (post-natal)

exposure to environmental smoke may increase the rates of allergen sensitization (518). A recent meta-analysis documented that smoking is more prevalent among asthmatic subjects compared with non-asthmatics and that it is a significant risk factor for developing asthma (534, 535). Smoking is also associated with poor response to glucocorticoid anti-inflammatory drugs and poor asthma control, possibly due to neutrophilic inflammation (534, 536). In conclusion, despite maternal smoke exposure would promote the development of respiratory illnesses of progeny *in utero*, the mechanisms underlying the immune-modulatory effects on atopy and allergic diseases remain unclear.

1.3.3.2.2. Obesity and diet

In addition to the allergy and asthma epidemic described earlier, there is also an obesity epidemic worldwide (537). In the USA, the prevalence of obesity, as defined by a body mass index (BMI) greater than 30 kg/m² in height, has more than doubled from 1980 to 2006, passing from 15 to 34% (538, 539). Similarly to what is observed in asthma, obesity rates in the US have increased particularly among children, passing from 5.5% in 1980 to 16.3% in 2006 for children aged 2 to 19 (410, 411, 540-542). Cross-sectional studies reported that the prevalence of asthma augments as a function of the body mass index (BMI), both in children and adults (543-546). In contrast, the atopy rates did not significantly associate with BMI data (547). Longitudinal studies demonstrated that obesity precedes asthma in most cases (543, 548-550). Taken together, the previous data

reinforce the idea that obesity is a risk factor for asthma rather than as a consequence of asthma. Furthermore, obesity in asthmatics is associated with poor asthma control compared with lean asthmatics (551, 552). As a corollary, both surgical and diet-induced weight loss improved asthma control and diminished symptoms (553, 554). Nevertheless, the association between obesity and allergic phenotypes or AHR remains unclear. Obesity rates may correlate with skin test reactivity or atopy, but not with peripheral eosinophil counts or airway inflammation as assessed by sputum (547, 555, 556). The number of obese women with detectable allergen-specific IgE levels was 3 times that of non-obese women (557), a phenomenon not seen in men. These results probably reflect the fact that atopy (558-560) and AHR (559) rates increase with increasing BMI more prominently in women than men. However, other studies failed to detect any association between BMI and AHR, asthma, rhinitis, eczema, or allergen-specific IgE levels (561, 562). Chen et al indeed reported that obesity and asthma are more strongly associated among non-allergic than allergic adults (563, 564). Mechanistically, the adipocytes are increasingly seen as immune players secreting a wide variety of inflammatory mediators (TNF- α , IL-6, IL-8, etc.) and adipokines (such as leptin). This likely reflects the state of systemic inflammation associated with high BMI which in turn influences asthma pathogenesis (565). In summary, asthma is more prevalent among obese individuals and obesity apparently worsens asthma control.

The causes of obesity are multiple and include diet, under-exercising, genetics, behavioural and psycho-social factors (541). Obese individuals indeed tend to exercise less and to consume a less healthy diet (566, 567). Several studies further described dietary implications in obesity (568) but also on the immune system (569). Both maternal (pre-natal) and early life (post-natal) diet tendencies can greatly affect the development of atopic phenotypes and account for increasingly prevalent allergic diseases in children (570). Elevated consumption of trans fatty acids, typically associated with westernized diets, was associated with an increased risk of childhood allergic asthma, rhinitis and eczema (571). Recent declines in the consumption of omega-3 polyunsaturated fatty acids (n-3 PUFAs), found in oily fish, was linked with rise in allergic diseases. Despite high consumers of n-3 PUFAs display less wheeze and asthma in paediatric cohorts (572, 573), not all preventive studies on dietary supplementation reproducibly confirmed these results (569). Nonetheless, fish oil supplementation during pregnancy reduces the risk of atopy in infants and allergen-induced inflammatory responses by neonatal immune cells (574, 575). The consumption of antioxidant-containing foods is protective against allergic disorders in those who consume them (576). Reduced maternal consumption of fruits and vegetables containing the antioxidants vitamin C and E, carotene, riboflavin and pyridoxine, but also zinc and selenium was further associated with increased risk of impaired lung function, asthma and allergen sensitization in the

progeny (577-579). It is now well-established that some dietary nutrients significantly impact on the development of allergic diseases through epigenetic modifications. For example, folic acid or dietary folate intake during pregnancy is associated with increased risk of allergic airway diseases in children (580-582). In addition to inherited genetic predispositions, these epigenetic modifications alter gene expression and might further influence the T effector/T-reg cell differentiation and/or lineage balance (581, 583). Finally, there is also evidence that breastfeeding reduces the risk of allergic sensitization, allergic diseases and AHR in children (561, 584), although other studies failed to reproduce these results (585). These protective effects would likely be driven by breast milk-derived PUFAs, immunoglobulins and anti-inflammatory cytokines that are transferred to the neonates (586-589). Taken together, these data reflect the relatively controversial role of dietary factors and nutrients in the development of allergies.

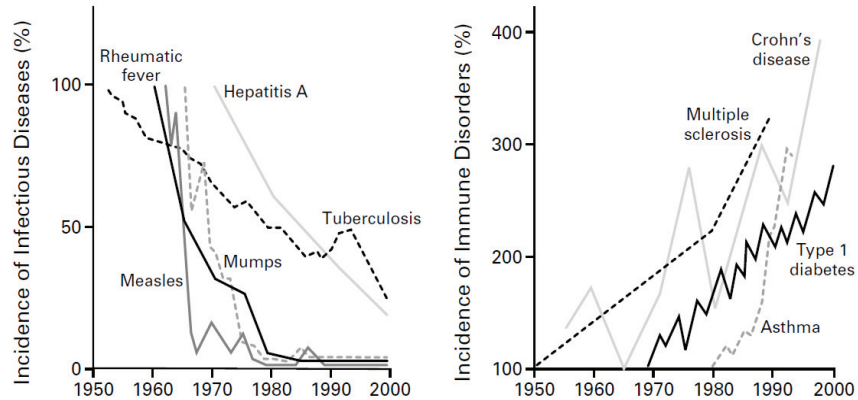
1.3.3.2.3. The hygiene hypothesis

Aside from the genetic predisposition of certain individuals to develop atopy and allergic diseases, one of the possible and probably the most documented explanation of the allergy epidemic is the hygiene hypothesis. This hypothesis originated from a study by David Strachan, in 1989, who documented the inverse relationship between the prevalence of hay fever in children and young adults and their respective number of siblings (590). Strachan

hypothesized that: "...declining family size, improvements in household amenities, and higher standards of personal cleanliness have reduced the opportunity for cross infection in young families. This may have resulted in more widespread clinical expression of atopic disease..." (590). The dramatic increase in the incidence of allergic disorders, but also autoimmune diseases, was indeed paralleled by a significant decrease in the prevalence of infectious diseases (591), likely reflecting improved preventive and therapeutic strategies (e.g. vaccines and antibiotics) and hygienic standards in the westernized world (Diagram 7a) (591). Strachans' pioneer hypothesis preceded a burst of epidemiological and experimental studies, either supporting or challenging the hygiene hypothesis (592-594). More than 20 years later, the hygiene hypothesis remains a plausible explanation to the rising prevalence of allergies (590, 594). It is now defined as a concept where a reduced exposure to microbes, increasing hygiene or limited exposure to immunological stimuli may under-stimulate the developing immune system of children, leading to dysregulated and hypersensitive responses to innocuous self and non-self antigens (591, 594, 595). Without ignoring the genetic factors predisposing some individuals to atopic diseases, the hygiene hypothesis robustly integrates temporal, geographical and environmental factors characterizing the allergy epidemic (595-597).

The protective effect of having siblings on the development of allergies has been well-documented (598-602), and extends to children who attended daycare

A



B

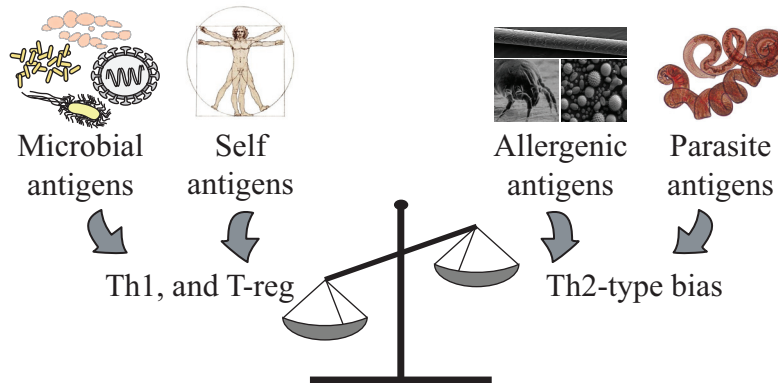


Diagram 7. The hygiene hypothesis. Inverse relation between the incidence of prototypical infections diseases (A, left graph), and the incidence of immune disorders (A, right graph), from 1950 to 2000. The diagram was reproduced with permissions from reference 591. The hygiene hypothesis states that early life exposures to microbial antigens would educate the immune system and, like self antigens, promote more tolerant responses to foreign and innocuous antigens (i.e. allergens) (B). The missing immune deviation theory postulates that microbes would drive Th1-type responses counter-regulating the Th2-type immune bias seen in allergies, whereas reduced immune suppression theory suggests that microbial education of the immune system may favor the development of tolerogenic regulatory T (T-reg) cells.

and were similarly protected against allergic disorders (598, 603-606). This reinforces the importance of genetics in the development of atopy and shows how environmental factors can influence allergic disease manifestations. Both the sibling and day care effect were associated with protective effect of sharing microbes early in life (590, 607). Accordingly, a recent longitudinal cohort study reported a reduced risk of developing childhood rhinitis among children that were either exposed to siblings, had viral infections or underwent tonsillectomy early in life (608). Children with type 1 diabetes mellitus (609) or who had contracted the measles (610) displayed a reduced incidence of asthma, rhinitis and eczema symptoms compared with uninfected children. Delayed-type hypersensitivity to *Mycobacterium tuberculosis* was not only a predictor of low rates of atopy, asthma and rhinitis symptoms (611), but also low serum IgE levels compared with tuberculin unresponsive school-age children (612). The risk of developing asthma by age 7 was decreased by 50% in children who had documented colds during their first year of life (613). These trends were also mirrored by an increased risk of developing asthma in children who underwent antibiotic treatment during infancy (614-616). In 1976, a study comparing white to native Canadians showed that allergic diseases were more common among the urbanized whites, whereas rural natives displayed lower allergy rates but elevated rates of helminth infections (617). While there is evidence for the protective effects of poor sanitation and gastrointestinal (GI) tract infections on

the incidence of allergic disorders (591, 618, 619), studies have shown some infections (especially lower respiratory tract viral infections) conversely trigger wheezing and reduced lung function in children and induce exacerbations in patients with already established asthma (619-623). These observations provide an intriguing paradox to the hygiene hypothesis (594), and whether infections contribute to the development of atopy is still debated today (624-626).

Epidemiological studies comparing the prevalence of asthma and allergies around the world localized the allergy epidemic mainly in westernized countries, where higher hygiene standards have been implemented, as opposed to the 'less hygienic' and more rural developing countries (414, 591). Probably the best example reflecting this situation are studies comparing East and West Germany soon after the fall of the Berlin Wall and reunification. Asthma and AHR were shown to be more prevalent in modern and industrialized Munich compared with rural areas of East Germany such as Dresden and Leipzig (501, 522, 627, 628). A recent meta-analysis and epidemiological studies further linked this protective effect against allergies and asthma to the farming environment and microbe contaminations (629-631). The farming environment is associated with higher rates of orofaecal or food-borne infections, including hepatitis A virus infections (619, 632). In separate studies, atopy in adults (632) and childhood rhinitis rates (633) were both reduced among individuals that were seropositive for the hepatitis A viral antibody compared with those who had never been exposed to

the virus. The consumption of unpasteurized milk, a vector for food-borne infections or GI colonization by pathogenic and non-pathogenic bacteria, similarly protects against allergic disorders (630, 634, 635). There even seems to be a protective hygiene gradient among farmers as lower rhinitis and asthma rates were observed in children of families with full-time farming activity and exposure to livestock compared with those of families with part-time farming activity (636, 637). In line with the idea that there is a protective effect of livestock exposure on developing allergic disorders, pet ownership has also been shown to be protective against developing childhood asthma and rhinitis, as well as against pollen sensitization (638-640).

1.3.4. Mechanisms of the hygiene hypothesis

The immunological mechanisms underlying the hygiene hypothesis remain incompletely understood. Two main mechanistic interpretations of the hygiene hypothesis have been formulated: namely the 'missing immune deviation', and the 'reduced immune suppression' theory (595, 641). The first theory proposes that microbes overcome allergic phenotypes by skewing the immune system towards reciprocal Th phenotypes (e.g. Th1, which suppresses Th2). The second theory postulates that microbial stimulation would activate regulatory T cells, which are in turn responsible for suppressing allergen-specific responses (Diagram 7b).

The missing immune deviation theory suggests that a reduced opportunity for infections, especially during infancy, may limit the development of the Th1 branch of adaptive immunity, and therefore promote a pro-allergic Th2 immune bias. The immune system of newborns is indeed skewed toward Th2-type immunity, and lacks functional Th1-type immunity (642). This bias is interpreted as an evolutionary mechanism limiting potentially deleterious Th1/Th17-type responses *in utero*, therefore promoting survival of the foetus (643, 644). This bias in the newborns' Th1-type adaptive anti-microbial defences is also partially compensated for by the supplementation of *in utero* transfer of immune mediators to the foetus, via maternal-placental circulation and later on by lactation (645). This theory is also supported by the reciprocal or counter-regulatory nature of Th1 and Th2 lineages. A lack of microbial stimulation early in life could then lead to a by-default bias toward a Th2-type phenotype, predisposing to subsequent IgE production against innocuous allergens (591, 646) and the atopic march. In support of this deviation theory, foetal IgE levels inversely correlate with the ability of cord blood mononuclear cells to produce the Th1-type anti-microbial IFN- γ cytokine (647). It has long been observed that two distinct immune responses elicited by different antigens tend to inhibit each other. This has led to the antigenic competition/homeostasis theory: a variant of the immune deviation theory. In support of this theory, individuals who develop delayed-type a hypersensitivity (DTH) Th1-type response to *Mycobacterium tuberculosis*

following immunization with bacilli Calmette-Guerin (BCG) develop less allergies than those not over-reacting to the BCG vaccine (611, 612). Taken together, the immune deviation theory proposes that a lack of microbial stimulation of the developing immune system may lead to impaired Th1 effector functions and result in a bias toward hypersensitive Th2-type immunity.

The reduced immune suppression theory suggests that the reduced microbial burden would fail to promote homeostatic immuno-suppressive functions of regulatory T (T-reg) cells. Allergic diseases are increasingly linked with defective T-reg cell-mediated suppression of effector Th cell lineages (648, 649), likely reflecting the bias towards pro-allergic Th2-type effector lineage (650). It has therefore been proposed that exposure to microbes could induce the development of T-reg cells, potentially inhibiting responses to distinct antigens (i.e. allergens), a phenomenon referred to as bystander suppression (595). Interestingly, it was recently reported that women living in a farming environment during pregnancy increase the numbers of circulating T-reg cells and improve the suppressive functions of these cells in their babies (651). These data support the concept that *in utero* exposures to a more rural or farming lifestyle serve as a natural model of immunotherapy against allergies, as demonstrated previously (652). Microbes consecutively trigger pro-inflammatory and then immuno-suppressive immune functions, the latter contributing to the resolution of inflammation after the threat subsided (653). It is therefore conceivable that

microbe-derived broad spectrum immuno-suppression can limit allergen-induced inflammation, through the effects of T-reg cells and their regulatory cytokines (e.g. TGF- β and IL-10), even at distant anatomical sites. The potential use of gastrointestinal probiotics, which are non-pathogenic microorganisms, which exert a positive influence on host health and physiology (654) has generated promising, but still equivocal results for the treatment and/or prevention of allergic disorders, such as atopic dermatitis (655-658). Nonetheless, several reports on the anti-allergy effects of probiotics (e.g. *Lactobacillus* strains) demonstrated the induction of immuno-suppressive T-reg cells (659, 660).

1.3.4.1. Bacterial endotoxin: lipopolysaccharide

The epidemiological basis of the hygiene hypothesis documents the inverse association between a farming environment, greater family size, exposure to livestock and pets and atopy or allergy rates, especially among children (592, 661, 662). It also appears that infections and microbes are inversely associated with allergy rates, and may skew the developing immune system away from Th2-type hypersensitive reactions to allergens. Before mounting an adaptive immune response against microbe-derived protein antigen, our immune system initially recognizes generic microbial patterns or pathogen-associated molecular patterns (PAMPs), through conserved and ubiquitously expressed pattern recognition receptors (PRRs). One particular microbe-derived molecule has been strongly associated with the above described allergy-

preventing conditions. This PAMP has become and remains a strong candidate to explain the hygiene hypothesis: it is called bacterial endotoxin or lipopolysaccharide (LPS). The following sections will summarize the biology of this endotoxin, the physiological and immunological responses to LPS through its receptor, and a particular focus will be made on its effects in the context of allergies and the hygiene hypothesis.

The discovery of endotoxin followed the emergence of the 'germ theory' by Louis Pasteur and Robert Koch, in the second half of the 19th century. In this theory, Pasteur and Koch demonstrated that specific germs or microbes were responsible for the symptoms of infectious diseases such as tuberculosis and cholera (663, 664). Soon after, Brieger reported that not only the germs themselves but also secreted products obtained from their culture supernatants, termed 'toxins' (e.g. diphtheria and tetanus toxins), can induce pathological symptoms (665). The term 'endotoxin' was defined by Richard Pfeiffer, in 1894, after he showed that substances released in the lysates of *Vibrio cholera* could kill inoculated animals, by sepsis (666). Septic shock is the result of a systemic infection and inflammatory state, leading to multi-organ failure in fatal cases. After inoculated animals died, regardless of whether they had been immunized or not, endotoxin was defined as a non-peptidic (i.e. heat-stable) insoluble toxic substance for which immunization provided no protection. Endotoxin also appeared to be well-preserved among various bacterial genera (667, 668).

Endotoxin or lipopolysaccharide is known as a protective component of the external leaflet of the outer bacterial wall of Gram-negative strains (669-671) (Diagram 8a). LPS is composed of a distal polysaccharide portion called the O antigen, an oligosaccharide 'core' and a membrane which anchors the hydrophobic lipid A domain (672-674). The lipid A domain is the only portion of endotoxin which is recognized by and potently activates the immune system (675) (section 1.3.4.4). One of the earliest examples is the endotoxin from heat-killed Gram-negative bacteria, which has potent fever-inducing or pyrogenic properties (676). The lipid A portion is composed of a diglucosamine diphosphate headgroup and associated acyl chains. The structure of lipid A moieties is highly variable, with regular intra- and interspecies variations in the composition of the headgroup and the acyl chains (677). The number, length and saturation of the acyl chains are indeed key to explaining the biological activity lipid A variants (678-680). The prototypical lipid A structure often referred to is that of the *Escherichia coli* species (669, 673), and differs slightly from that of the *Salmonella* species including *S. minnesota* (681) (Diagram 8b).

1.3.4.2. Toll-like receptor 4: the LPS receptor

Our understanding of how exposure to microbes and LPS modulate the immune system has rapidly evolved in the past 15 years, following the discovery of innate immunity pattern recognition receptors (PRRs). These receptors sense pathogen-associated molecular patterns (PAMPs) and potentially regulate both

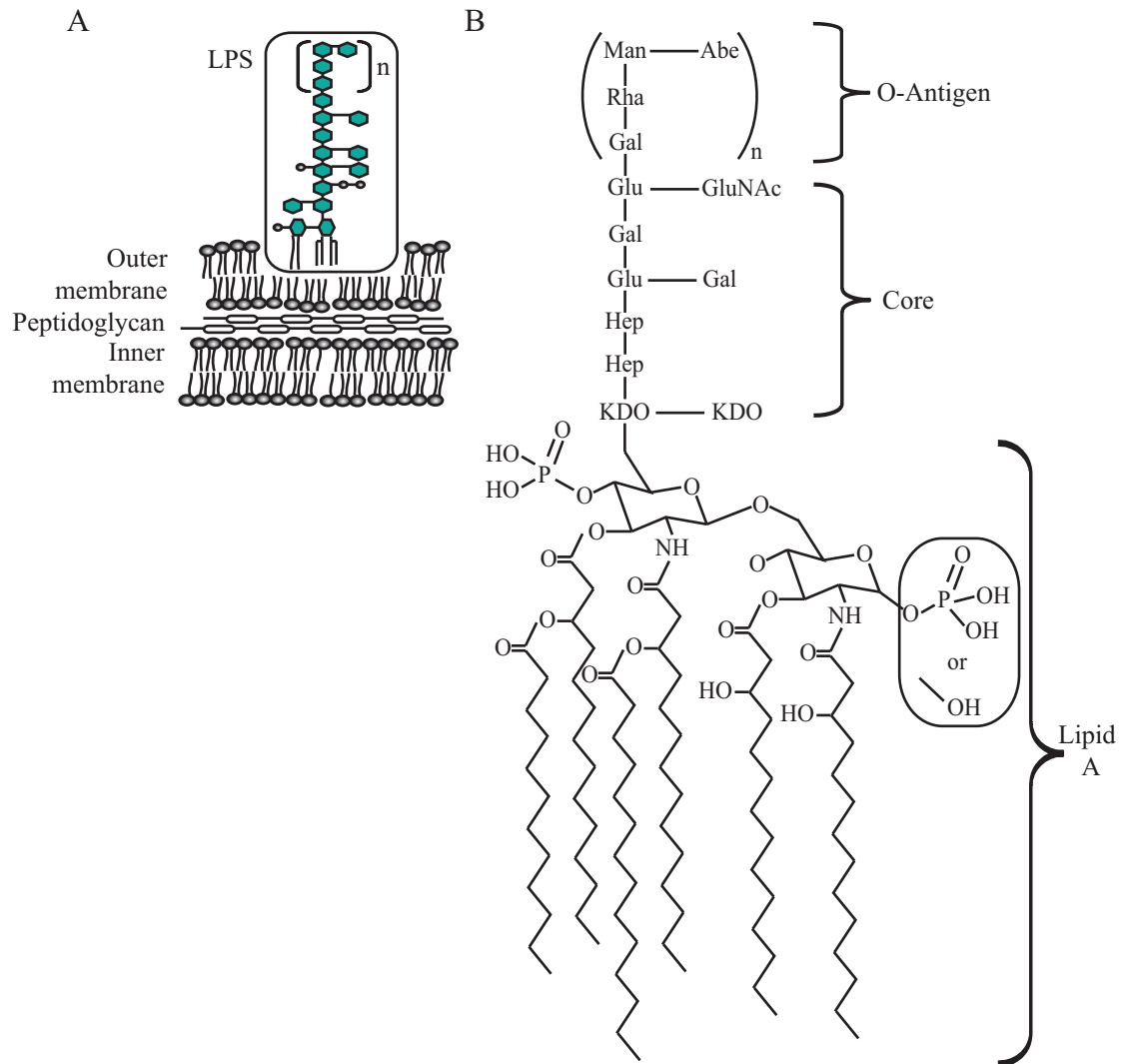


Diagram 8. Gram-negative bacteria lipopolysaccharide (LPS) structure. The LPS molecule is part of the external leaflet of the bacterial wall (A). LPS is composed of a distal O antigen, a oligosaccharide core, and a membrane-anchored immunostimulatory Lipid A domain, also called endotoxin (B). The lipid A moiety contains a diglucosamine headgroup complemented by 4 to 8 acyl chains. There is a high inter-species variability in the structure of LPS. For example, the right-end glucosamine monophosphate of Lipid A is substituted by an hydroxyl group differentiate *Escherichia coli*-from *Salmonella minnesota*-derived endotoxin (see inset in B). Adapted from references 672 and 677.

innate and adaptive immune responses (682). The concept of a LPS receptor emerged from studies of the 1960s and the 1980s describing different strains of mice that were, respectively, highly susceptible to bacterial infections and hyporesponsive to LPS (namely the C3H/HeJ and the C57BL/10ScCr) (683-687). When experiments involving the crossing of two strains of hyporesponsive mice revealed no change in the LPS sensitivity, it was suggested that this phenotype was due to a mutation residing within a same locus; called the *Lps* locus.

In 1985, a new protein named Toll (meaning 'great' in German) was described in the fruit fly *Drosophila melanogaster*. In addition to its role in the dorsoventral polarity during embryonic development (688, 689), the Toll protein was further reported to play roles in anti-fungal and anti-bacterial defense (690-692). The cytoplasmic domain of *Drosophila* Toll protein was also found to share homologies with sequences of the interleukin-1 receptor responsible for cytokine-mediated signal transduction and activation of the nuclear factor κ B (NF- κ B) cascade (693, 694). This common domain was cloned and named Toll/IL-1R (TIR) domain-containing transmembrane receptor. Sequence homology studies further described the first mammalian Toll homolog, and soon to be named Toll-like receptor 1 (TLR1) (695, 696). The first human Toll homolog (hToll) was identified and cloned by Medzhitov et al, in 1997 (697). Although its ligand was unknown at the time, human cells transfected with a constitutively active hToll mutant activated the NF- κ B pathway and induced the expression of IL-1, IL-6, IL-

8 as well as T cell-co-stimulatory molecule CD80 (697, 698). This work was quickly followed by the identification of other human gene sequences homologous to the *Drosophila* Toll, named Toll-like receptor (TLR) 1 to 5, respectively (699). The Toll-like receptors (TLRs) now consist of the better-characterized class of innate immunity PRRs (682, 691). Up to now, a total of ten functional and structurally similar TLRs have been identified in humans (700). TLRs recognize evolutionarily-conserved PAMPs from bacteria, fungi and viruses, as well as a variety of synthetic molecules and host-derived DAMPs (danger-associated molecular patterns) (Table 4).

The identification of the Lps locus as Toll-like receptor 4 (TLR4) resulted from positional cloning studies on the LPS-susceptible C3H/HeJ mouse strain, conducted by Poltorak et al, in 1998 (701, 702). These studies demonstrated that the high susceptibility to LPS was due to a point mutation in the cytoplasmic domain of the mouse *tlr4* gene. These findings were confirmed by the discovery, in the LPS-hyporesponsive C57BL/10ScCr mice, of a deletion in the same *tlr4* gene (703, 704). These results were confirmed by transfecting the human TLR4 gene into the Human Embryonic Kidney 293 (HEK293) cell line which does not endogenously express it (705). In these experiments, only TLR4-transfected cells responded to LPS by activating the NF- κ B signalling pathway, and the effect could be abolished by an LPS antagonist. Hoshino et al confirmed that TLR4 is responsible for LPS sensitivity as they generated TLR4 gene mutants animals

TLRs	Ligands	Ligand origin
TLR1/2	Tri-acyl lipopeptides	Bacteria and mycobacteria
	Soluble factors and Porin PorB	<i>Neisseria meningitidis</i>
	Outer surface protein A (OspA)	<i>Borrelia burgdorferi</i>
TLR2	Lipoproteins/lipopeptides	Various pathogens
	Di-acyl lipopeptides	Synthetic compounds
	Peptidoglycan and Lipoteichoic acid	Gram-positive bacteria
	Lipoarabinomannan	Mycobacteria
	Phenol-soluble modulin	<i>Staphylococcus epidermidis</i>
	Glycoinositolphospholipids	<i>Trypanosoma cruzi</i>
	Glycolipids	<i>Treponema maltophilum</i>
	Porins	<i>Neisseria</i>
	Atypical lipopolysaccharide	<i>Leptospira interrogans</i> and <i>Porphyrromonas gingivalis</i>
	Hemagglutinin	Measles virus
	Zymosan	Fungi
	Hyaluronan, and Heat-shock protein 70 (HSP70)	Host
TLR3	Double-stranded RNA	Viruses
	Polyinosinic:polycytidylic acid	Synthetic compound
TLR4	Lipopolysaccharide	Gram-negative bacteria
	Taxol	Plants
	Fusion protein (protein F)	Respiratory syncytial virus

Table 4: The human Toll-like receptor (TLR) family, and their specific ligands

TLR4	Envelope protein	Mouse mammary-tumour virus (MMTV)
(continued)	Fungal hyphae	<i>Aspergillus fumigatus</i>
	Heat-shock protein 60 (HSP60)	Host and <i>Chlamydia pneumoniae</i>
	HSP70, Fibrinogen, High-mobility group box 1 (HMGB1), Surfactant protein A, Minimally modified-low-density lipoprotein, Oligosaccharides of hyaluronic acid, Polysaccharide fragments of heparan sulphate, Type III repeat extra domain A of fibronectin, α A crystallin and HSPB8	Host
TLR5	Flagellin	Bacteria
TLR6/2	Di-acyl lipopeptides	Mycoplasma
	Lipoteichoic acid	Gram-positive bacteria
	Zymosan	Fungi
TLR7	Imidazoquinoline (anti-viral agent), Loxoribine, and Bropirimine	Synthetic compounds
	Single-stranded RNA	Viruses
TLR8	Imidazoquinoline, resiquimod (R-848)	Synthetic compounds
	Single-stranded RNA	Viruses
TLR9	CpG-containing unmethylated DNA	Bacteria, viruses, yeasts, insects
TLR10/1 or 2	Likely TLR1 and 2 ligands	Unknown

Table 4: The human Toll-like receptor (TLR) family, and their specific ligands (continued)

displaying phenotypes similar to C3H/HeJ animals upon LPS exposure (698). Further evidence came from human studies identifying specific TLR4 gene polymorphisms with increased susceptibility to infections by Gram-negative bacteria, septic shock, and impaired LPS responsiveness (706-708). Among all TLRs, the LPS receptor TLR4 is undoubtedly the most studied.

The TLR4 gene (also called hToll and CD284) is located on the chromosome 9 at position i.e. 9q33.1 and encodes 839 amino acid long single-pass type I membrane protein. The TLR4 protein shares two core domains with other TLRs: namely an intracellular TIR domain necessary for signal transduction, and extracellular leucine-rich repeat (LRR) core domains are complemented by a short transmembrane domain, as well as by a short hypervariable region between the two LRR modules (Diagram 9a). Variability in the extracellular domains reflects inter-species differential ligand binding properties, in contrast with the evolutionarily-preserved TIR domain shared with all TLRs (672). The LRR ectodomains of TLR4 forms (LRR) modules which enable ligand binding (697). These horseshoe-shaped solenoid-type structure typical of all TLRs and which enables ligand binding (709).

Despite the fact that TLR4 mutations abrogate LPS sensitivity *in vivo* and LPS is closely associated with TLR4 as determined by crystallography (710), the lipid A portion of endotoxin only slightly binds to TLR4, and rather robustly binds to an accessory protein called MD2 (also called lymphocyte antigen 96) (135)

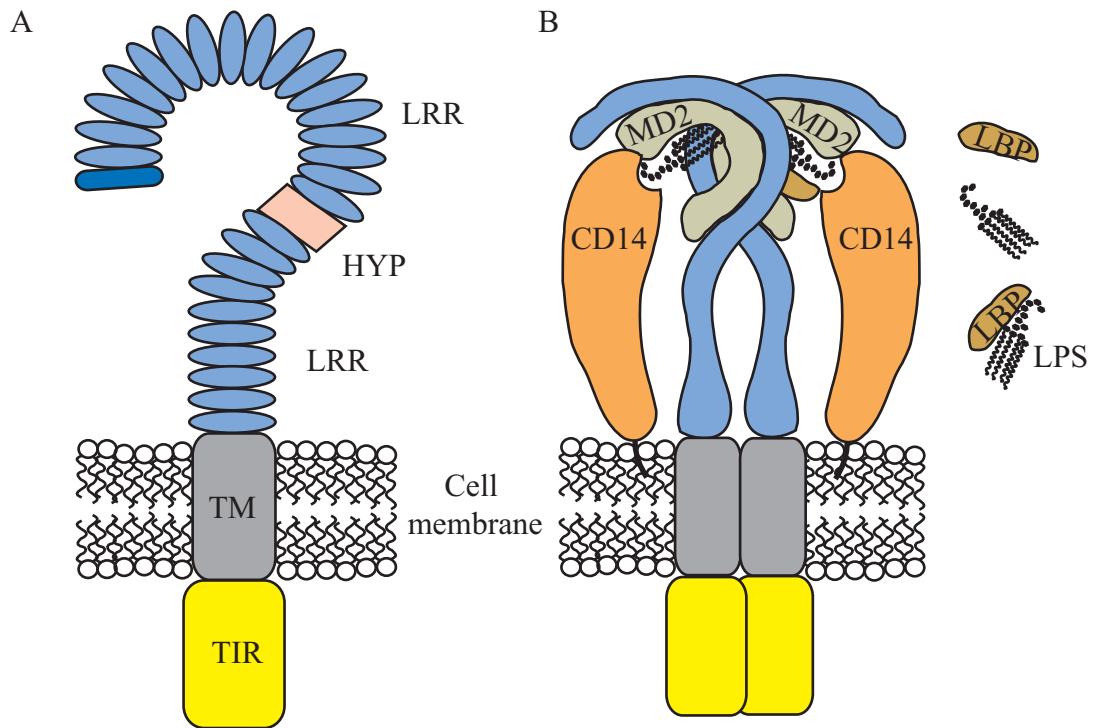


Diagram 9. The Toll-like receptor 4 (TLR4) structure and complex. In A, the different structural domains of TLR4 include the cytoplasmic Toll/IL-1 receptor (TIR) domain which serves for signal transduction, the single-pass transmembrane domain (TM), and extracellular leucine-rich repeat domains (LRR) separated by a hypervariable region (HYP). In B, the LRR domains typically form a horseshoe-like structure which receive LPS bound to MD2, CD14 and LPS binding protein (LBP). Sources: references 710 and 717.

(Diagram 9b). Furthermore, the LPS delivery to MD2/TLR4 complexes is performed by a glycosylphosphatidylinositol (GPI)-anchored LPS-binding protein called CD14 (711), in collaboration with a serum glycoprotein called LPS binding protein (LBP) (712). Binding of LBP to LPS enhances the sensitivity of phagocytes e.g. macrophages to LPS (713, 714), as it catalyzes LPS transfer to CD14 (711, 715, 716). The CD14 molecule is considered as a LPS co-receptor (711, 715, 716) despite the fact it similarly binds other microbe-derived molecules (717). CD14 exists under two forms: a membrane-bound form which uses a GPI anchor (mCD14) but also a soluble form which enables LPS responsiveness of CD14-deficient cells (718-721). The mCD14 neither possesses transmembrane nor cytosolic domain. CD14 therefore does not directly transduce any signal. In contrast with LBP and CD14 which are both constitutively found in serum and blood plasma (722), MD2 is not normally detected in body fluids and is apparently only secreted under widespread inflammatory conditions (e.g. sepsis) (722). MD2 is ubiquitously expressed by most TLR4-expressing cells, and associates with TLR4 in the Golgi apparatus and endoplasmic reticulum (723, 724). The association between MD2 and TLR4 is necessary for TLR4 translocation to the cell surface (725). However, airway epithelial cells were shown to only express MD2 at low levels, a mechanism limiting excessive deleterious inflammatory responses to inhaled LPS (726). Nevertheless, soluble MD2 can enable LPS signalling in TLR4- and CD14-expressing cells lacking membrane-associated

MD2 (722, 725). LPS binding the TLR4 complex triggers the assembly of a m-shaped multimer containing two copies of the TLR4-MD2-LPS complex arranged in a symmetrical fashion (Diagram 9b) (710, 727).

In humans, TLR4 is expressed by a wide variety of hematopoietic cells and non hematopoietic cell types. Depending on the cell type, TLR4 expression can either be confined in the intracellular compartment, while most hematopoietic cells express it mainly on the cell surface. TLR4-expressing hematopoietic cells include monocytes and macrophages (728), immature dendritic cells and myeloid DCs (729, 730), mast cells (731), basophils (732), neutrophils (728), eosinophils (733), B cells (246, 734), activated CD4⁺ T lymphocytes (735-737). Besides, most structural cells, including airway resident cells, also TLR4; namely epithelial cells (upper nasal airways and lower bronchial airways) (738, 739), fibroblasts (740, 741), endothelial cells (742) and smooth muscle cells (743). Expression of TLR4 is highly variable between these different cell types. Interestingly, epithelial cells of mucosal organs like the gastrointestinal tract (intestinal epithelium) (744) and the airways (bronchial epithelial cells) (738, 745) exhibit intracellular (Golgi-associated) TLR4 expression, a confinement that reflects a protective strategy limiting permanent receptor activation upon binding with intestinal flora-derived or inhaled LPS (746). Interestingly, a soluble form of TLR4 (sTLR4), which serves as a decoy LPS receptor, was recently detected in human saliva (747).

1.3.4.3. LPS recognition by TLR4 and signal transduction

The TLR4 complex recognizes LPS with a high sensitivity and reportedly influence a mammalian immune system in the picogram (per kg of body weight) range (748). The primary targets of LPS are monocytes and endothelial cells in the periphery, and both macrophages and epithelial cells in the lung (749). As mentioned previously, LPS physically binds to MD2 rather than TLR4. Delivery of LPS to the MD2/TLR4 complex via the LBP/CD14 interaction enhances LPS sensitivity (714, 724, 750). Upon binding, LPS mediates conformational changes of the MD2 molecules, which triggers dimerization of TLR4-MD2-LPS complexes and signal transduction (751, 752). The dimerization of TLR4-MD2 complexes is now seen as an essential step for receptor activation (753, 754). This conformational change is mandatory for the recruitment of downstream adaptor molecules at the Toll/IL-1R (TIR) domain and the induction of early-phase signal transduction (Diagram 10). This early-phase TLR4 complex activation leads to the recruitment of the TIR domain-containing adaptor protein (TIRAP, also called Mal), which serves as a sorting adaptor that subsequently attracts the myeloid differentiation primary-response protein 88 (MyD88) (755) and activate MyD88-dependent signal transduction (756-758). On the other hand, LPS binding to the receptor also triggers the internalization of the receptor complex (759, 760), in a dynamin and clathrin-dependent manner (761). The LPS/TLR4 complex is then trafficked throughout the endosomes where it activates delayed-phase signal transduction pathways (Diagram 10). This delayed-phase signalling depends on

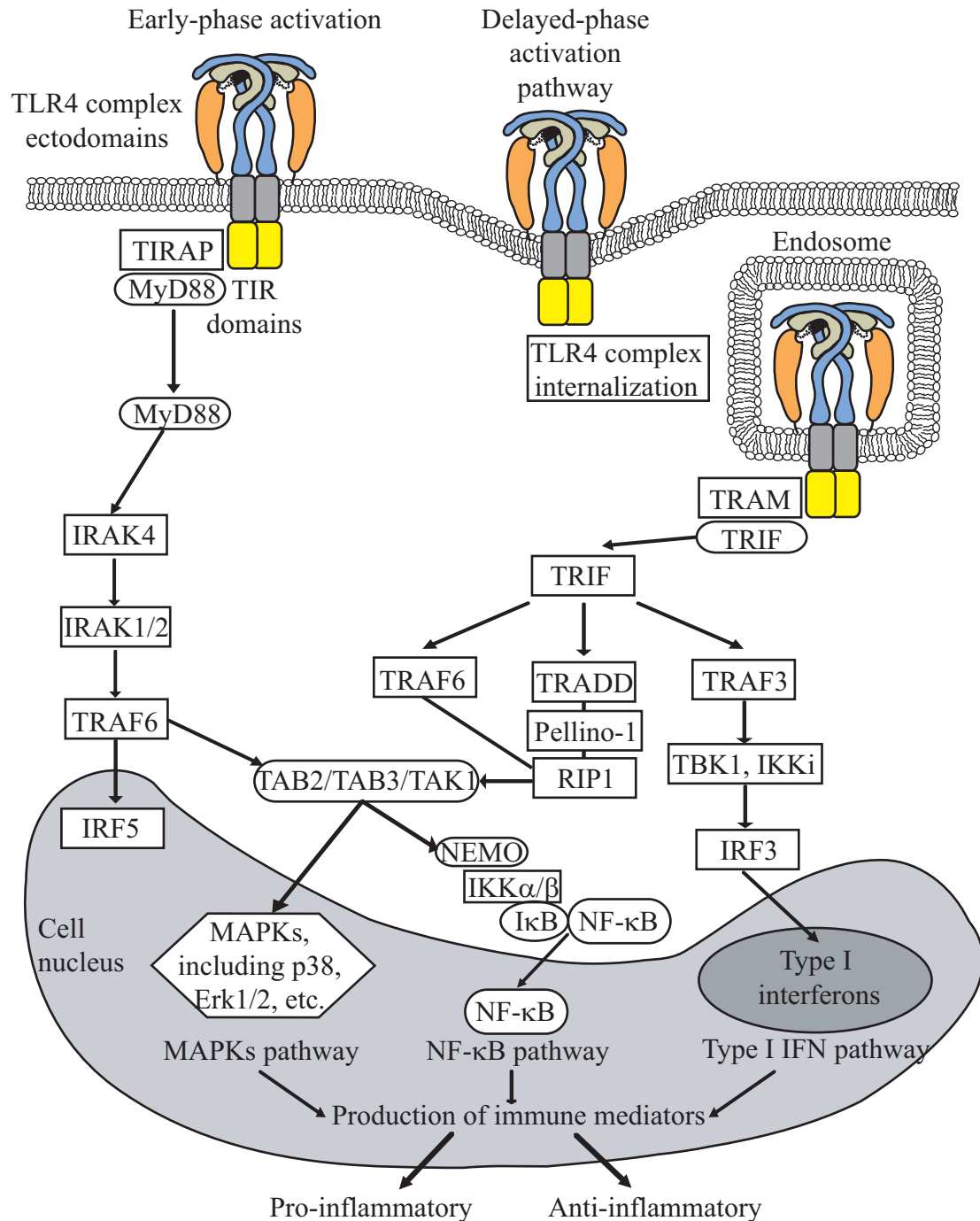


Diagram 10. Summary of LPS-induced TLR4 complex-mediated signalling pathways. Two main pathways were identified. The Toll-IL-1 receptor (TIR) domains recruits and activates the cytosolic TIRAP and MyD88 adaptor proteins (left side) during the early phase activation. This pathway culminates with the activation of TRAF6 which drives IRF5, MAPKs and NF-κB pathways. Then, the TLR4 complex is internalized into endosomes before to recruit/activate the TRAM and TRIF adaptors that culminates with the activation of both MAPKs and IRF3 through several pathways. Both MyD88 and TRIF pathways leads to the production of various pro- and anti-inflammatory mediators.

the recruitment and activation of the TIR domain-containing adaptor-inducing interferon- β (TRIF) (762) by the TRIF-related adaptor molecule (TRAM) (763, 764) (763-766). The TLR4 receptor complex is further recycled in the Golgi apparatus (744, 757).

The MyD88-dependent signalling pathways involve the recruitment and activation of the IL-1R-associated kinase 4 (IRAK4) (767). IRAK4 kinases then sequentially recruit and phosphorylate the serine/threonine kinase molecules IRAK1 and IRAK2 (768). Activated IRAK1/2 proteins further associate and activate the tumour necrosis factor receptor-associated factor 6 (TRAF6) which mediates signalling for both the TNF-receptor and the TLR/IL-1R superfamilies. TRAF6 consists of an E3 ligase that catalyzes polyubiquitination of various targets (including itself) via ubiquitin-conjugating enzymes (769). Polyubiquitinated TRAF6 then recruits TAK1 (transforming growth factor- β -activated kinase 1) (770), and its regulatory components TAK1-binding protein 2 (TAB2) and TAB3. Polyubiquitinated TRAF6 then binds the inhibitor of NF- κ B (I κ B) proteins kinases (IKK) gamma (IKK γ , also called NEMO). Furthermore, TAK1 phosphorylates the IKK β , another important regulator of the I κ B complex and NF- κ B activation (771, 772). Activated IKK β and IKK γ further phosphorylate I κ B proteins, a process that targets them for degradation by the 26S proteasome, and release the NF- κ B transcription factors. The NF- κ B family of transcription factors includes 5 members: the reticuloendotheliosis viral oncogene homolog A (REL-A), REL-B,

cytoplasmic (c-) REL, p50 and p52; which function as homo- and heterodimers. The most common dimer is the p65/p50 and is normally sequestered in the cytoplasm due its binding to I κ B proteins. Phosphorylation-induced degradation of I κ Bs enables NF- κ B proteins to enter the nucleus and promote target gene transcription (773). In addition to activating the IKK complex, TAK1 also activate a variety of MAPKs including Extracellular signal-regulated kinases 1 and 2 (ERK1 and 2, also called p44 and p42, respectively), p38 and the c-Jun N-terminal kinase (JNK). These kinases further activate other transcription factors including AP-1, an association between JNK and the proto-oncogene c-Fos. The MyD88-mediated pathway therefore culminates with the activation of transcription factor NF- κ B and MAPKs which together regulate the expression of various inflammatory mediators (774). Finally, studies using MyD88- and TRIF-deficient cells and animals demonstrated the former is necessary for early-phase responses, but not sufficient for inducing the expression of inflammatory mediators. The MyD88-dependent pathway then require functional TRIF-mediated pathway for activating NF- κ B/MAPK targets (765, 775, 776).

The TRIF-mediated signalling pathways reflect the endosomal TLR4 complex-driven and delayed-phase LPS responses (763). They begin with the TRIF recruitment and activation by TRAM in the early endosomes (763), and culminates with the activation of the interferon-regulatory factor 3 (IRF3) (776), and of NF- κ B/MAPK-mediated pathways complementing those activated in a

MyD88-dependent fashion (765, 774, 776) (Diagram 10). As seen earlier, the MyD88- and TRIF-mediated pathways cannot independently lead to NF- κ B/MAPK activation or induce an inflammatory response (i.e. mediate mediator production). However, LPS-driven activation of the TRIF signalling pathway is necessary and sufficient to phosphorylate interferon regulatory factor 3 (IRF3) and inducing the expression of Type I interferon genes (777, 778). LPS-induced TRIF pathway activation has not been well-studied yet, but it would involve the recruitment and activation of TRAF6/TAK1 molecules necessary for the NF- κ B/MAPK pathways. Besides binding TRAF6, TRIF also activates and associates with the tumour necrosis factor receptor type 1-associated death domain (TRADD) protein, the ubiquitin ligase Pellino-1 and the receptor-interacting protein 1 (RIP1) that also culminate with TAK1 and NF- κ B/MAPK pathway activation (700). Finally, the TRIF-mediated IRF3 dimerization involves the phosphorylation of the tumour necrosis factor receptor-associated factor 3 (TRAF3) (779, 780), which in turn associates with TANK (TRAF family member-associated NF- κ B activator), TANK binding kinase 1 (TBK1) and the I-Kappa-B Kinase ϵ (IKKi) (780-783). The induction of anti-viral defence via Type I interferon genes would depend on both IRF3 and NF- κ B (783, 784).

1.3.4.4. LPS-induced/TLR4-mediated immune and physiological responses

The *in vivo* toxic effects of LPS have been described since the end of the 19th century, when Pfeiffer and Centanni (666, 676) associated endotoxin

administration with sepsis or systemic inflammatory response syndrome (785). In sepsis, symptoms of dysregulated body temperature, tachycardia and hyperventilation normally appear as the immune system eliminates the invading pathogens. When infections overwhelm immune defences, the sepsis degenerates into acute severe septic shock which can be fatal. Upon injection, LPS either binds to plasma proteins (like LBP) and/or to its receptor onto host cells. Circulating LPS level normally remains low as it is usually cleared by macrophages or hepatocytes (786, 787). In humans, the plasma of healthy individuals contains low but detectable amounts of LPS (from 1 to 7 pg/ml), compared with patients with sepsis (100 to 800 pg/ml) (788-790). Cells that recognize LPS are primarily circulating monocytes (791), tissue macrophages (792), neutrophils (793), but also structural cells including endothelial cells in the periphery (794). Epithelial cells are the first exposed to LPS in mucosal tissues, such as the lungs and the GI tract (749, 795).

Early studies revealed that endotoxin mediates cytotoxic effects beneficial against tumours (796). These results further led to the discovery tumour necrosis factor- α (TNF- α) (797), to which were ascribed the *in vivo* toxic effects of LPS (798). Apart from TNF- α (799), LPS also stimulates the expression of pro-inflammatory cytokines including IL-1 β (800, 801), IL-6 (802, 803), IFN- γ (804), IL-12 (805, 806) and IL-18 (807). While IL-12 and IFN- γ contribute to skew the immune response towards a Th1-type phenotype, IL-18 likely has a dual role in

the Th1/Th2 balance (808, 809). LPS influences adaptive immune response through its effects on APCs: it promotes APC maturation through the up-regulation of MHC class II and co-stimulatory molecules including like CD40, CD80 and CD86 (810, 811). LPS also induces APC migration towards lymphoid tissues where they interact with and promote antigen-specific T cell activation and clonal expansion (811, 812). LPS may also have important, but yet poorly described, roles on B cell responses and immunoglobulin production (813). LPS stimulation mounts an immune cell battalion ready to fight potential infections: LPS drives the production of hematopoietic cell growth factors including the macrophage colony-stimulating factors (M-CSF) (814), the granulocyte colony-stimulating factor (G-CSF) (815) and the granulocyte-macrophage colony-stimulating factors (GM-CSF) (816). These mediators favour the differentiation of several effector cells. LPS also increases the generation of reactive oxygen species (817, 818), phagocytic functions (819) as well as the expression of inducible nitric oxide synthase (NOSII) and NO production (820, 821). LPS induces the expression and release of chemokines including the IL-8/CXCL8 (822, 823), the monocyte chemoattractive protein (MCP-1/CCL2) (824), and the macrophage inflammatory protein (MIP-1 α /CCL3 and MIP-1 β /CCL4) (825, 826). Finally, LPS up-regulates the expression of intercellular adhesion molecule-1 (ICAM-1) by the endothelium (827), which facilitates leukocyte adhesion and transmigration (828, 829).

In summary, LPS activates both leukocytes and resident cells and induce their release of pro-inflammatory mediators. This early response promotes the recruitment and activation of leukocytes, therefore amplifying the response in a positive-feedback loop. However, the LPS response is also associated with the induction of immuno-suppressive mediators, through a delayed recovery phase that normally follows elimination of the microbial threat. The immuno-suppressive mediators preventing exaggerated inflammation include the anti-inflammatory cytokines IL-10 (256, 805, 830-833), TGF- β (834, 835), but also the IL-1 receptor antagonist (IL-1Ra) (836, 837) and antagonistic cytokine receptors. LPS also activates the production of prostaglandin E₂ (PGE₂) (838, 839), which has potent regulatory properties (840). The kinetic of release of anti-inflammatory mediators and their contribution to the inflammatory milieu are poorly described compared to the pro-inflammatory roles of LPS.

Exposure to high doses of inhaled LPS has been studied in the context of occupational asthma. Elevated endotoxin levels detected in the baker's environment (841), the farming environment, livestock and animal facilities, saw, grain and cotton mills, textile factories, and others are associated with chronic wheezing and reduced lung function among workers (749, 842). Inhalation studies conducted in healthy individuals demonstrated that single dose exposures of around 40 μ g (or more) of LPS is required for eliciting a significant decrement in FEV₁ (843-845). LPS also induces epithelial cell production of

mucus (846). Such LPS-mediated lung function impairments are often accompanied by symptoms of fever, airway irritation, joint and muscle pain, headache, nausea and general fatigue (845). When looking at the LPS-induced fall in FEV₁ in patients with/without documented non-specific AHR (to histamine), only patients with AHR, but not those with atopy, displayed decreased FEV₁ and fever, whereas the both groups of patients had increased numbers of peripheral leukocytes (847, 848). Placebo-controlled studies demonstrated that LPS significantly decreases the lung function and causes lung inflammation in asthmatic subjects (both atopic and non-atopic), whereas it triggers limited inflammation in healthy subjects (849, 850). The LPS-induced AHR appears about 6h post-challenge and resolves within 48h (851). Regardless of the patient's lung response, LPS triggers local and systemic inflammatory responses, in a dose-dependent manner (852): it elevates serum TNF- α levels (after 1h), BALF and sputum cytokine levels (853), and the numbers of circulating and BAL leukocytes (after about 3h) (851, 854). Based on knock-out mouse experiments, the effect of LPS on endothelial cells would be sufficient for recruiting neutrophilia into the lungs upon systemic endotoxin administration (855). In contrast, LPS-responsive hematopoietic cells (in particular macrophages) would be critical for driving neutrophil recruitment to the airspace upon inhaled LPS challenge (856). Taken together, LPS inhalation studies demonstrated that low dose LPS triggers local, if not systemic inflammatory responses (857, 858), whereas only

higher doses possibly elicit clinical responses including impaired lung function (749). It also appears that LPS is a significant AHR-exacerbating factor for asthma severity as well as for other on-going respiratory disorders, like chronic bronchitis (859). In line with this, a recent meta-analysis reported that children living in high-endotoxin-level homes had an increased risk of having wheezing/AHR episodes (631).

1.3.4.5. LPS in allergies: friend or foe?

In support to the hygiene hypothesis, numerous studies associate elevated ambient levels of LPS with conditions conferring protection against the development of asthma and allergies. LPS levels were found to be higher in the farming than in the non-farming or air conditioned-urban environment (661, 860-863). Indoor LPS levels (measured in kitchen floors or mattresses) were elevated in households of individuals from the farming environment or that had contact with livestock (862). Accordingly, household LPS levels were positively associated with the numbers of people in the home (864) and pet-keeping or exposure to animals (637, 865-867), compared with homes with small family size or without animal exposures. Based on epidemiological and observational studies, LPS could limit allergies.

Gereda and collaborators provided the first *in vivo* evidence that prolonged exposure of humans to elevated LPS concentrations protects against the development of atopy in children (868). In this study, infants found to be allergen-

sensitized (<2 years of age) had been exposed to lower house-dust concentrations of LPS than age-matched non-atopic children exposed to high LPS concentrations (868). As an explanation to this phenomenon, the percentage of circulating IFN- γ -containing CD4⁺ T lymphocytes was increased as a function of house-dust LPS concentrations, suggesting the protective effect of LPS against atopy may be mediated by the induction of Th1-type immunity. In support to the above-described immune deviation theory, LPS promoted Th1-type response without directly affecting Th2-type cytokine-positive CD4⁺ cells (868). Household exposures to high levels of LPS was similarly associated with a decreased risk of sensitization to common aeroallergens in adults (869), and with decreased allergic sensitization and disease rates in children (637, 866, 870-872). On another hand, studies conducted in urban settings, on children with familial predisposition to allergies and on children with diagnosed asthma revealed that the intensity of LPS exposure correlates with early wheezing symptoms (873-876). These deleterious LPS effects on children predisposed to allergies were proven independent of lower respiratory tract infections, a common cause of wheezing during infancy. These studies reinforce that endotoxin exacerbates wheezing among children exposed to more polluted (i.e. urban) environments and those who are allergic or predisposed to atopy (631, 874), rather than contradicting the protective LPS effect against the development of allergic disorders described in large cohort studies (608, 612, 613, 862). The

latter is illustrated by the reduced risk of children raised in high-LPS-level environments to develop allergic symptoms and allergen sensitization (631). Besides, LPS was shown to worsen wheezing symptoms/AHR in atopic individuals or those with on-going respiratory illnesses including asthma (631).

Several studies highlighted the possible genetic association between LPS receptor genes and the development of allergies (487). Polymorphisms in the CD14 gene have been repeatedly associated with atopy (IgE levels) and allergic diseases (asthma and dermatitis) (877-880), while none of the CD14 variants could be associated with the incidence of respiratory infections (706). In contrast, polymorphisms in the gene encoding TLR4 are not unanimously associated with atopic asthma and disease severity (881-884). One of the most documented TLR4 gene variant, called Asp299Gly/Thr399Ile, is present in about 8% of Caucasian populations and was associated with LPS hyporesponsiveness, increased susceptibility to infections and failure to develop AHR upon LPS inhalation (707, 885). Nonetheless, there is still a controversy about the association of this polymorphism with asthma incidence (882), and disease severity (884, 886). The Asp299Gly TLR4 polymorphism is otherwise associated with increased prevalence of infection-induced asthma (706, 887). Despite discrepancies in genetic association studies, these results support a yet-to-be-defined role for TLR4-mediated innate immune functions in the development of asthma and/or allergic phenotypes.

Beside the study by Gereda et al (868) supporting the immune deviation theory, only few experimental studies using human tissues investigated how LPS may counter-regulate allergen-driven responses. Our laboratory previously implemented an *ex vivo* tissue culture model enabling stimulations of explanted nasal mucosa biopsies from allergen-sensitized individuals with allergen extracts in the presence or absence of bacterial LPS. Using nasal explants from both non-atopic and atopic children, LPS increases the proliferation and number of CD3+ T lymphocytes (736, 888). When applied alone, LPS also increased the numbers of macrophages, neutrophils and of IL-2, IFN- γ and IL-12 mRNA-expressing cells likely reflecting a Th1-type immune skewing (888). When exposed to specific allergen extracts, tissues from sensitized children mounted Th2-type inflammation characterized by increased numbers of immune cells like eosinophils, CD3-, IL-2- and CD25-expressing cells, as well as IL-4, IL-5 and IL-13 mRNA-expressing cells. In contrast, exposures to both LPS alone or LPS and allergen increased IL-10, IL-12 and IFN- γ mRNA-expressing cells and prevented allergen-induced Th2-type bias (736). Interestingly, this immune switch was abolished by neutralizing IL-10 and IL-12. LPS exposure of tissues from atopic children was associated with LPS-induced increases in the numbers of CD3, IL-10 and TLR4-expressing cells (736). The three markers colocalized in 60% of cases suggesting that TLR4-expressing CD3+ T cells would drive IL-10-dependent immuno-suppression of allergic responses. Taken together, LPS-

induced IL-10 and IL-12 likely collaborate to limit allergen-driven Th2 immunity, therefore supporting both the immune deviation and reduced immune suppression theories.

This explanted tissue model has obvious limitations. The local immune responses to LPS and/or allergens is studied in the absence of cellular recruitment from systemic circulation, and when all cells of tissues are exposed to LPS rather than only cells lining the airway lumen. Few studies documented the effect of LPS on allergic diseases using allergen challenged subjects. Eldridge et al reported that instilled LPS exacerbates nasal allergen challenge-induced inflammatory responses in a group of patients with diagnosed allergic asthma (889, 890). In this study, asthmatics that received nasal allergen challenge followed by an LPS instillation displayed an enhanced neutrophilia and eosinophilia compared with allergen instillation followed by saline challenge. These results are in line with the concept that LPS exposure worsens on-going inflammatory airway diseases (859, 873).

The vast majority of studies assessing the ability of LPS either to prevent allergies or to limit on-going disease manifestations *in vivo* were conducted in animals. These studies yielded contradictory results, reflecting important variations between models experimental approaches. Experimental allergic asthma models typically imply that the animals (e.g. mice) are sensitized to an exogenous antigen (allergen, mainly ovalbumin or OVA), therefore inducing the

generation of allergen-specific IgE. The animals are later exposed to the allergen or challenged either by inhalation or via intra-nasal/tracheal instillations which trigger allergen-specific inflammatory responses, bronchial hyperresponsiveness and even airway remodelling in chronic exposure models (891, 892). The genetic background of the mouse strains used in experimental asthma models has major influence on the allergic phenotypes (468, 893-895). Differences between OVA-induced asthma-like phenotypes in the most commonly used strains of mice (BALB/c and C57BL/6 mice) have been attributed to genetic variations in the Th2 cytokine gene cluster (896, 897). BALB/c animals develop elevated IgE levels and more prominent allergen-induced AHR than 'asthma-resistant' C57BL/6 mice (893, 898). Hollingsworth et al demonstrated that OVA-sensitized and challenged TLR4-deficient C57BL/6 mice display increased lung eosinophilia, circulating IgE levels and AHR compared with wild-type littermates (899). These data suggesting that LPS/TLR4 limit allergen-induced experimental asthma were independently confirmed: LPS administered once, before or until the last OVA challenge successfully protected C57BL/6 mice against OVA-mediated allergic inflammation and airway hyperreactivity (900). In contrast, studies conducted on 'asthma-susceptible' BALB/c mice variably reported that low-dose LPS induce or prevent intra-nasal or sub-cutaneous allergic sensitization, lung Th2-type inflammation and AHR upon challenge (901-904). BALB/c experiments revealed that co-administration of OVA along with LPS doses approximately similar to

common environmental concentrations (from 10 to 100 µg/animal) protected mice against developing Th2 immune bias to OVA, by inducing transient Th1-type immune bias (902, 903, 905) and IL-10 production by interstitial macrophages (906). Taken together, environmental LPS doses would confer protection against allergies by skewing the immune system away from allergic-type response.

In contrast, LPS doses lower than those found in the environment may fail to induce Th1-type, and lead to a by-default Th2-type response (903). These results on low-dose LPS are in line with studies in human subjects, where very low doses of inhaled LPS that fails to recruit neutrophils promote intranasal APC-driven Th2-type cytokine production (907, 908). Nonetheless, elevated doses of inhaled LPS promote Th1-type response, neutrophil recruitment and what can be described as a non-allergic asthma phenotype (138, 909). Furthermore, DC treated with high LPS doses limited Th2-type immunity (i.e. IL-4 production) *in vivo* (910). Accordingly, intravenous LPS mediates a strong allergy-/asthma-protective response in OVA-sensitized guinea pigs and mice (911, 912).

Beside the influence of the genetic background on the nature and magnitude of the allergic responses, animal studies also underlined that the nature, the concentration and the purity of allergen extracts or LPS are critical. Allergen extracts themselves can contain biologically active LPS (913), while LPS isolated from different bacterial strains can trigger different receptors (914, 915) and/or have variable biological activity (916). Most allergic sensitization protocols

also use adjuvant additives (such as aluminium hydroxide) that skew the immune system and promote Th2-type responses to peptide antigens and likely alter the LPS response (901). The timing and duration of LPS exposures (prior or during allergen sensitization) also greatly influence the allergic response. In rat and murine models of allergic asthma, single LPS administration into the airways, either prior to and until a couple of days after intraperitoneal OVA sensitization protected the animals against the development of OVA-specific IgE (917), abolished OVA challenge-induced airway inflammation and AHR (905, 918, 919). Similarly, perinatal LPS exposures during the first days/weeks of life, prior to or simultaneously with allergen exposure were also reported to limit Th2-type immune responses to allergens (920) and bronchial responsiveness (921). However, LPS exposure later after allergen sensitization did not protect but rather worsened the development of allergic asthma (919, 922). In summary, these data indicate that LPS can interfere with allergen sensitization (923) when administered early in life and prior to allergen exposure, therefore preventing further subsequent allergen-mediated disease manifestations. Early life may be considered as a potential window of opportunity for microbe-mediated education of the immune system, ensuring more tolerant or balanced responses to allergens. In comparison, late LPS exposures that come after sensitization may not protect and even exacerbate allergic diseases similarly to what is seen for allergic or asthmatic exposed to LPS (631, 876).

Recent studies elegantly established that maternal bacterial exposures confer asthma protection to the progeny, through the regulatory effects of maternal TLR signalling and mild/transient Th1-type anti-microbial inflammation (924, 925). In these experiments, pregnant wild-type or TLR2/3/4/7/9 quintuple knockout mice were exposed to a stable-derived bacterial strain (*Acetivobacter lwoffii* F78) or sham, while the asthma outcomes were assessed by applying the ovalbumin protocol on the offspring. Only newborn mice from mothers infected with the bacteria were protected from developing allergic asthma (i.e. Th2-type inflammation and AHR), as they were exposed to the mother's mild/transient Th1-type anti-microbial response. In contrast, TLR heterozygous offspring from infected TLR-deficient mothers were not exposed to the anti-microbial inflammatory milieu and developed asthma (924). The LPS from *A. lwoffii* F78 and TLR4 axis had been previously shown to induce Th1-type and reduce Th2-type responses in *A. lwoffii* F78-stimulated human monocyte-derived dendritic cells (926). Similarly, exposure of pregnant BALB/c mice to aerosolized LPS limited the development of allergic asthma in their progeny, compared with newborns from PBS-exposed mothers. In these conditions, prenatal LPS exposure suppressed OVA-specific IgE levels, Th2-type airway inflammation and hyperresponsiveness upon challenge compared with mice from sham-treated mothers (925). These studies provide direct evidence linking maternal exposure

to bacteria from the farming environment during pregnancy and TLR4-mediated protection of newborns against allergic airway inflammation and asthma (927).

Endotoxin may also confer an immune tolerance to allergens or bystander suppression through the induction of allergen-specific regulatory T (T-reg) cells. Studies indeed suggested that the protective effect of LPS in experimental allergic asthma is independent of the Th1 arm (928), as LPS-exposed IL-12/IFN- γ -deficient animals failed to develop asthma (900, 929). In support to the 'reduced immune suppression' theory, T-reg cells were shown to express the LPS receptor TLR4 (930-932). LPS also promotes proliferation and survival of CD4⁺CD25⁺ effector T cells, which include T-reg cells. LPS increases the immuno-suppressive ability of T-reg cells, as assessed in co-culture experiments with activated effector T cells (931, 933). Exposure of newborn mice to intra-nasal LPS during the first days of life not only prevented the development of OVA-induced experimental asthma but simultaneously increased the numbers IL-10-producing regulatory T lymphocytes (921). Synthetic analogs of LPS administered as a sub-lingual immunotherapy to prevent experimental asthma in the OVA protocol also limited Th2-type responses, by inducing both T-reg-type and Th1-type cytokines (934). Finally, experiments conducted on germ-free and control animals either fed with LPS-free or LPS-rich diet demonstrated that gut microbiota and exposure to LPS promote both Th1 and T-reg phenotypes, and limit systemic Th2 cytokine production (935).

Evidence of the role of LPS against the development of allergies further came from food allergy models. Bashir et al used three different mouse strains lacking functional TLR4 and compared them to MHC-matched wild-type controls in a protocol of intragastric sensitization and challenge with a peanut allergen administered by gavages (936). In these experiments, only TLR4-deficient animals developed features of allergic inflammation and anaphylactic symptoms. Dr Patrick Holt's group also addressed the question whether oral LPS could serve as a therapy for asthma (937). In the OVA-induced allergic asthma murine model, Strickland et al demonstrated that post-sensitization oral exposures to Gram-negative bacteria-derived lipid A preparations limited allergen-induced airway inflammation and hyperreactivity. This protection against allergy was accompanied by elevated T-reg cell numbers and enhanced suppressive functions (937). In summary, there is compelling evidence that LPS exposure, especially taking place early in life and before allergen sensitization, plays the role of an immunological adjuvant preventing the Th2-type adaptive response to allergens by driving either or both a T-reg- or a Th1-type immune bias.

1.3.4.6. Regulation of LPS responsiveness and TLR4 complex

Before to summarize how allergic disorders may affect LPS responses and the expression of the TLR4 complex, it is worth describing how LPS or microbes also regulate TLR4 functions in the context of microbial infections. Human immunodeficiency virus do not alter TLR4 expression on monocytes (938), but

increases it on circulating T cells (939), likely reflecting that activated T cells expressed elevated TLR4 levels (735). Similarly, helminth infections increase TLR4 on T cells (940), while hepatitis C virus increases TLR4 on B cells (941). Respiratory syncytial virus infections increases TLR4 expression by airway epithelial cells (942), as it also promotes Th2-type inflammation (943) and asthma exacerbations (944). *Mycobacterium tuberculosis* infections induces macrophage death (945), whereas *M. tuberculosis*-derived extracts decreases TLR4 on macrophages (946) and reduce LPS responsiveness (947). Bacterial colonization of patients' BALF is associated with increased IL-8 and neutrophilia, but also with reduced TLR4 mRNA expression by the BAL hematopoietic cells (948). *Staphylococcal* enterotoxins (i.e. superantigen) controversially regulate TLR4 mRNA (949, 950) on macrophages/monocytes. In the human nasal explant model, LPS increased TLR4 immunoreactivity in children tissues regardless of atopic status (736). This increase in TLR4 expression likely reflects LPS-induced proliferation (888). LPS increases its own receptor's expression in intestinal epithelial cells, whereas other TLR ligands unanimously decrease it (951). Conversely, LPS has limited effect on TLR4 mRNA levels in macrophages (952, 953), but greatly reduces their TLR4 surface expression (949, 953).

The LPS-induced decrease in TLR4 surface expression recalls the two phases of LPS recognition, namely the extracellular early-phase signalling followed by TLR4 complex internalization and the late-phase signalling (761).

Such relocation is required for the delayed-phase endosomal LPS/TLR4-MD2 complex-mediated signal transduction and mediator production (700, 775). The reduction in TLR4-MD2 complex surface expression, but not necessarily CD14 (954, 955), significantly limit the ability of the cells to respond to LPS. This relative state of LPS hyporesponsiveness has long been described and was termed 'endotoxin tolerance' (953, 956, 957). This latter is considered as an adaptation to avoid excessive responses that could lead to septic shock. Endotoxin tolerance reflects the transient inability of tolerized cells to release pro-inflammatory mediators upon further LPS challenge. Upon LPS re-stimulation *in vitro*, tolerized cells from sepsis patients display marked decreases in the release of pro-inflammatory mediators including IL-6, IL-8, IL-12 and TNF- α , but not IL-10 and IL-1Ra which were increasingly released (958, 959). The altered phenotype of LPS-tolerized cells may reflect dysregulated signalling pathways (958, 960), but the mechanisms remain unknown. This LPS-induced tolerance to further LPS or bacterial exposure, along with the basal intracellular localization of the TLR4 complex in some cell types including epithelial cells (738, 744, 961), consist of the primary regulatory mechanisms preventing excessive and injurious LPS-induced inflammatory responses.

Cytokines also act as significant regulators of TLR4 complex expression and LPS responsiveness in both immune and structural cells. The Th1-type cytokine IFN- γ increases TLR4 expression and LPS responsiveness (i.e. LPS-

induced mediator production) in monocytes (962, 963) and intestinal epithelial cells (964). Similarly, IL-6 augments TLR4 expression (965), while IL-1 β down-regulates it on monocytes/macrophages (966). However, TNF- α , IL-1 β and IL-6 all increase LPS co-receptor CD14 expression, while IFN- γ has no effect (967). Besides, anti-inflammatory or immuno-suppressive cytokines IL-10 and TGF- β would not alter TLR4 complex expression but would rather inhibit its signalling (968, 969). In contrast with Th1-type cytokines, Th2-type cytokines IL-4 and IL-13 reduces TLR4 complex expression in both monocytes (970) and intestinal epithelial cells (971). In these conditions, it appears that IL-4 induces the activation of the transcription factor STAT6 which, in turn, would play roles in down-regulating TLR4 through specific DNA binding sites in the promoter of the TLR4 gene (972). IL-4 and IL-13 have been described to inhibit LPS-induced mediator production by monocytes and macrophages (973-978). IL-4 reduces LPS-induced TNF- α , IL-1 β , IL-10 and IL-12 (974, 979, 980). IL-4 also reduces CD14 expression as well as LPS responsiveness in monocytes (967, 981, 982). In summary, it appears that Th1-type milieu or anti-microbial responses generally promote TLR4 complex expression and functions. Conversely, Th2-type conditions likely reduce TLR4-mediated immune functions.

Besides addressing the potential role of the LPS/TLR4 axis in the development of allergies through epidemiological cohort studies (630, 983), some reports characterized TLR4 expression and functions in allergic compared with

non-allergic subjects. Lauener et al demonstrated that children of age 10 raised on farms exhibit increased CD14, but not TLR4 expression compared with age-matched children from urban environments (984). Peripheral leukocytes of newborns from parents with positive histories of allergy display lower TLR4 and CD14 mRNA than those from non-allergic parents (985). Similarly, elevated serum levels of soluble CD14, which serves as an LPS co-receptor, were detected in serum of non-atopic subjects compared with atopic patients (986). The exposure of pregnant mothers to environments rich in microbial compounds is also associated with reduced incidence of atopic sensitization and increased expression of CD14 and TLR4 in their babies (987). Other studies also compared TLR4-mediated functions in peripheral immune cells from controls and allergic and/or asthmatics. Peripheral blood mononuclear cells (PBMC) from allergic asthmatic adults produced lower amounts of TNF- α /IFN- γ cytokines in response to LPS compared with cells from controls (734, 988-990). When analyzed separately, the monocytes and macrophages from allergic asthmatics adults produce less IL-10/IL-1 β in response to LPS, compared with cells from non-atopics (990, 991). Monocytes from children of mothers without allergy had elevated TLR4 expression, LPS-induced p38 MAPK phosphorylation and IL-6 production compared with those from children of mothers with allergies (992, 993). Monocytes from adult patients with atopic dermatitis display impaired LPS-induced IL-12 production compared with non-atopic controls (994). The latter

data were mirrored by reduced TLR4 expression by monocytes and CD4 T lymphocytes in allergic asthma patients compared with cells from control subjects (734, 989). This impaired LPS-mediated IL-12 production in asthma was further associated with a previously-mentioned TLR4 polymorphism (881), as well as with a failure to mount Th1-type responses *in vitro* (989). Taken together, these data suggest that exposure to microbes or infections may confer protection against allergies through the activation of the LPS receptor complex. While microbes still have controversial effects on TLR4 expression, it appears that ongoing allergic disease and Th2-type immune bias may reduce TLR4 expression and impair LPS receptor complex-mediated functions.

1.4. Research hypothesis and objectives

1.4.1. Rationale

In the introduction, I discussed the immunological basis of the allergic diseases and more specifically of allergic asthma and rhinitis. The prevalence of these disorders has consistently risen in the past decades and has now reached epidemic proportions. This rapid and marked increase is mainly restricted to populations of westernized countries, supporting the argument for influence of environmental factors rather than a strict genetic drift. The allergy epidemic is also more prominently detected in paediatric populations, suggesting that childhood may be a window of opportunity for studying the differential maturation of immune systems of individuals manifesting atopic phenotypes. Despite

extensive research efforts, the aetiology of the allergy epidemic remains a matter of debate. Aside from inherent genetic factors which predispose individuals to become atopic and develop allergic diseases, the hygiene hypothesis is still considered as one of the most plausible explanations to the modern and localized increase in the prevalence of allergies and autoimmune disorders (595). The hygiene hypothesis states that increased exposure to microbes and infections, particularly early in life, may be protective against the development of allergies as they modulate the developing immune system and promote the mounting of tolerant responses to innocuous allergens later in life. In contrast, the increased hygiene and reduced exposure to microbes taking place in our Western societies may under-stimulate the developing immune system of children, therefore rendering it more likely to mount Type I hypersensitivity reactions to environmental allergens.

Lipopolysaccharide (LPS) is a component of cell walls ubiquitously found in Gram-negative bacterial strains. It is universally present in our environment and is of the most extensively studied microbe- or pathogen-associated molecular patterns (PAMPs). Numerous cohort studies reported that a lack of exposure to LPS in early childhood is associated with elevated rates of allergic sensitization, and subsequent Th2-mediated allergic disorders (631). Despite the fact that mechanisms underlying the hygiene hypothesis have not yet been fully characterized, it has been proposed that exposure to environmental LPS doses

early in life trigger Th1-type immune bias and/or favour the development of immuno-suppressive regulatory T cells. These responses may counter-balance and inhibit pro-allergic and Th2-type immune responses to allergens (927, 995). In contrast, exposure to high LPS doses, either systemically or by inhalation, is associated with heavy Th1-type, neutrophil-mediated inflammation and impaired lung function. Furthermore, LPS exposure in individuals that suffer from airway inflammatory diseases (including allergic asthma) appears to worsen inflammation and exacerbate their symptoms (631, 859, 996). Our immune system senses LPS through innate immunity receptor complex composed of Toll-like receptor 4 (TLR4), the adaptor molecule MD2 and the co-receptor CD14 (700). Upon LPS binding, the TLR4 complex mediates signal transduction by activating a variety of pathways culminating primarily with a pro-inflammatory/anti-microbial immune response, and followed by an anti-inflammatory response or negative feedback loop resolving inflammation. The first cells encountering LPS are primarily monocytes and endothelial cells in the periphery, and both macrophages and epithelial cells in the lung (749).

In our laboratory, Dr. Meri Tulic previously implemented a nasal mucosa explant model to study the effect of LPS on allergen-induced Th2-type inflammation using tissues from atopic and non-atopic children and adults (736). These studies demonstrated that LPS may limit the allergen-induced Th2-type inflammation in tissues from atopic children, but not in those from atopic adults.

Interestingly, the absence of LPS-mediated protection against an allergic reaction was accompanied by reduced TLR4 expression in adults compared with children, and in atopic adults compared with age-matched non-atopic controls (736). In parallel, Dr. Pierre-Olivier Fiset demonstrated that IL-4-induced activation of Th2-type transcription factor STAT6 contributes to the down-regulation of TLR4 using a human monocytic cell line (972). Taken together, these results suggested that the LPS/TLR4-mediated protective effects against allergies may be impaired due to the subject age and to Th2-type conditions and transcription factors.

These studies enabled us to identify several areas in need for research, and drove the generation of new research hypotheses. Importantly, little is known about the regulation *in vivo* of TLR4 in the different peripheral leukocytes populations, in both atopy and under allergic conditions. We also lack an understanding of how TLR4 expression varies with atopy and as a function of the subject's age through a cross-sectional study. On the other hand, studies looking at the LPS-induced production of both pro-inflammatory and anti-inflammatory mediators suggested impaired innate immune functioning in atopic or allergic individuals compared with non-atopic controls (734, 988-990). Whether such dysregulated LPS-mediated responses are due to impaired TLR4 expression and/or complex signalling has not been clearly established. Furthermore, numerous pro-allergic (Th2-type) transcription factors are increasingly activated and are displayed at elevated expression levels in the context of allergic

disorders (131, 174, 175, 208). The best-characterized Th2-type transcription factors are STAT6 and GATA-3. It is well known that IL-4/IL-13 signalling transiently activates STAT6, but also induces the expression of GATA-3, the master regulator of Th2 differentiation in lymphocytes. While our laboratory demonstrated that STAT6 participates in the regulation of TLR4, it is currently unclear whether GATA-3 could also play a role in this matter. Finally, LPS exposure is a potent immuno-regulatory molecule. However, it was controversially reported to limit the development of atopy and allergic disease during childhood, while it would exacerbate on-going allergic diseases. Understanding the regulation of TLR4 expression and function in children and adults with and without atopic histories and in on-going disease may help to explain this paradox.

1.4.2. Research Hypotheses

In this thesis project, we hypothesized that (1) atopic status or a history of allergy (and Th2-type conditions), could affect the expression of TLR4 during childhood but also the LPS recognition and signalling of the TLR4 complex on peripheral leukocytes. We also hypothesized that (2) on-going allergic diseases (asthma and rhinitis) in adults, and the associated Th2-type inflammatory conditions, are associated with decreased TLR4 expression in epithelial cells lining the airways and that the expression of GATA-3, a Th2-type transcription factor, plays a role in the regulation of TLR4 expression.

1.4.3. General objectives

The general objective of this thesis is to characterize the counter-regulation of TLR4 expression, LPS recognition and signalling caused by atopy and the allergic (Th2-type) microenvironment. These effects of atopy will be elucidated using cells that respond to LPS and contribute to allergy-protective immune-regulatory functions under normal conditions. We addressed this main objective through the use of two different *ex vivo* human tissue models. In the first model, peripheral blood mononuclear cells were obtained from a paediatric cross-sectional cohort. These cells were characterized for their ability to recognize and respond to LPS. In the second model, we used airway mucosal tissues excised from adult subjects with either established allergic diseases such as rhinitis and asthma or controls in order to characterize the expression and regulation of TLR4 on airway epithelial cells. These observations were complemented by *in vitro* experiments in which cultured epithelial cells exposed to Th2-type conditions were used to study the potential role of GATA-3 transcription factor in TLR4 regulation.

1.4.4. Specific objectives

(1a) To determine the expression of TLR4 on circulating T 'helper' lymphocytes and monocytes in a cross-sectional cohort of children with or without histories of atopic disease.

(1b) To investigate LPS recognition and LPS-induced signalling by circulating T 'helper' lymphocytes and monocytes in children with or without histories of atopic disease.

(2a) To assess the expression of TLR4 in airway epithelial cells in subjects with allergic asthma and rhinitis, and in Th2-type cytokine-stimulated cultured airway epithelial cells.

(2b) To determine whether the Th2-type transcription factor GATA-3 contributes to the regulation of epithelial cell TLR4 expression.

Chapter 2: Materials and Methods

2.1. Summary of experimental design

To address the research hypotheses and objectives, we conducted two main studies. We addressed the first hypothesis and two first objectives by conducting a cross-sectional study on TLR4 expression and the differential LPS-induced signalling in peripheral leukocytes of children with/without history of atopic disorders. In this study, children (from 2 to 18 years of age) and adults (>18 years-old) were recruited and we documented their history of atopic diseases by means of a questionnaire. All participating subjects provided a blood sample which was used to isolate peripheral blood mononuclear cells (PBMC). The cells were cultured *in vitro* in the presence or absence of the pro-allergic Th2-type cytokine IL-4; the expression of TLR4 was assessed for different cell populations (T 'helper' lymphocytes, B lymphocytes and monocytes). The characterization of TLR4 expression was mainly performed by means of flow cytometry, considering both the age and the atopic histories of the subjects. Some of PBMC were also stimulated with LPS *in vitro* for short periods. The ability of the peripheral leukocytes to recognize LPS and trigger signal transduction pathways through TLR4 was measured by assessing the surface receptor expression and the phosphorylation of different mitogen-activated protein kinases (MAPK) downstream of the TLR4 receptor complex. The

response of leukocytes from atopic and non-atopic patients to LPS was also compared on the basis of the expression of a well-documented LPS-induced immuno-regulatory gene, IL-10.

We addressed the second research hypothesis and the two last objectives by performing a study of the regulation of TLR4 in airway epithelial cells from subjects with allergic asthma and rhinitis. We also evaluated the effect of Th2-type inflammatory conditions on the expression of TLR4 in epithelial cells cultured *in vitro*. We investigated the potential effect of epithelial GATA-3 transcription factor expression on TLR4. GATA-3 has well-documented Th2-promoting functions in T 'helper' lymphocytes but its functional role has never been studied in epithelial cells. To assess the expression of TLR4 in on-going allergic disease, we recruited adult subjects with and without diagnosed allergic rhinitis undergoing nasal surgery, and obtained nasal mucosa tissue specimens that were separated into two samples. Tissues from the first sample were processed and sectioned for further histological and immunocytochemical analyses, while frozen tissues from the second served for gene expression profiling by real-time PCR. These tissues served as an *ex vivo* upper airway model of Th2-type allergic disease to study TLR4 expression. We obtained primary bronchial epithelial cells from patients with diagnosed asthma and from normal controls. These cells served as a model of allergic disease in the lower airways. We also used a bronchial epithelial cell line as well as normal human bronchial epithelial cells to perform a series of

stimulations mimicking Th2-type conditions encountered in asthma. The cultured cells were assessed for their expression of TLR4 and GATA-3 through various techniques, including real-time PCR, fluorescent immunostaining, immunoblotting and flow cytometry. Finally, we used transfection techniques to respectively silence endogenous GATA-3 expression (by small interfering RNA, siRNA) or by over-expressing GATA-3 (transient transfection with a human GATA-3 gene-containing plasmid), and then evaluated their effects on TLR4 and GATA-3 expression *in vitro*. The commercial and non-commercial sources of materials used in this thesis are integrated to the methods description that follows.

2.2. Cross-sectional study on TLR4 expression and signalling in peripheral leukocytes of children with/without atopic histories

2.2.1. Subject recruitment and blood sampling

Subjects were recruited from the Paediatric Test Center of the Montreal Children's Hospital. The study protocol was approved by the Research Ethics Board of the Montreal Children's Hospital, McGill University Health Center (section on Research compliance certificates and permissions). A total of 376 patients (ages 2 to 45) were recruited in the course of the study. Written consent was obtained from each of the enrolled subjects, while accompanying parents gave additional written consent in the case of subjects younger than 18 years of age. All of the enrolled subject (and parents) answered a short screening questionnaire to ascertain the presence of a personal or family history of allergic

conditions (including asthma, atopic dermatitis, food allergies, aeroallergen reactivity and rhinitis), based on ISAAC questionnaires (500). Subjects were classified on the basis of their age (2 to 6, 7 to 12, 13 to 18 and >18 years of age) and their status of atopic histories ('atopic' vs. 'non-atopic'). Subjects that were taking anti-inflammatory medication regularly or that reported any viral or bacterial infections in the last month, or any known blood or genetic disorders were excluded from the study. All of the enrolled had 2-5 cc of intravenous whole blood procured in heparinized tubes (BD Biosciences, Franklin Lakes, NJ) in the context of other clinical testing. Blood draws were performed by the nurses of the Paediatric Test Center, and the blood tubes were kept at room temperature until the blood was processed at the Meakins-Christie Laboratories.

2.2.2. Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation. Ficoll columns serve to separate blood in its constituents. From bottom to top of the column, the pellets of erythrocytes and granulocytes, the Ficoll-Paque solution, the leukocytes (lymphocytes, monocytes, etc.) or PBMC buffy coat, and the plasma are normally visible. Briefly, the blood from heparinised tubes was drawn out and resuspended in at least twice the volume of room temperature phosphate-buffered saline (PBS) (pH 7.2) to a volume up to 30 ml in 50 ml tubes. Ten millilitres of Ficoll-Paque PLUS (Pharmacia, Uppsala, Sweden) were aliquoted into 50 ml tubes. The resuspended blood volume was

carefully layered over the Ficoll volume into two tubes. The samples were centrifuged for 30 minutes at room temperature at 1,500 rpm without using the brakes not to disturb the density gradient layers. The plasma layer was drawn with a sterile Paster pipette without disturbing the buffy coat of underlying PBMC. The PBMC layer was collected carefully without touching the Ficoll layer. The PBMC were then washed twice with 15 ml of PBS by centrifuging the pellet for 10 minutes at 1,500 rpm and discarding the supernatant. The PBMC pellet was resuspended into less than 5 ml of complete culture medium (section 2.2.3), before duplicated cell count was performed using the Beckman Coulter Act Diff cell counter (Beckman Coulte, Wesley Hills NY) or the Countess Automated Cell Counter (Invitrogen). Cell viability uniformly remained above 90% in all experiments as assessed by Trypan blue exclusion assay. For all experiments, the cells were resuspended in culture medium at 1.0×10^6 cells/ml and dispensed in 6 or 12-well culture dishes for further stimulations.

2.2.3. Cell culture and stimulations

The PBMC were maintained in complete culture medium which refers to RPMI 1640 (Invitrogen, San Diego, CA) supplemented with 10% v/v heat-inactivated fetal-bovine serum (FBS; HyClone, Logan, Utah), 4 mM L-glutamine (Invitrogen), 50 U/mL penicillin G, 50 µg/mL streptomycin (Invitrogen), 1 mmol/L sodium pyruvate (Invitrogen), and 1.5% of 1M HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) buffer (Invitrogen), incubated at 37°C and 5%

CO₂. Prior to perform any other experiments or analysis, the PBMC were incubated with or without recombinant human IL-4 (Medicorp, Montreal, QC, Canada) at a concentration of 13.5 ng/ml, or with or without LPS derived from *Salmonella minnesota* R595 (from 0.05 to 5 µg/ml; Alexis Biochemicals, San Diego, CA) for up to 24 hours. Cell viability uniformly remained above 90% in all experiments, as assessed by the Trypan blue exclusion assay using Countess Automated Cell Counter (Invitrogen).

2.2.4. Flow cytometry staining of PBMC

At the end of the stimulation, PBMC were collected from the culture dish and washed twice with 5 ml of cold PBS by centrifuging the pellet for 10 minutes at 1,500 rpm and discarding the supernatant. The cell pellets were resuspended at a concentration of 0.5×10^6 cells/ml and 1 ml aliquots were further dispensed to serve as a sample for flow cytometry experiments. Prior to staining, PBMC were pre-incubated with 20 µg/ml human intravenous immune globulin (IVIG, 10%, Bayer Corporation, Elkhart, IN) to diminish non-specific Fc receptor binding of antibodies, for 30 min at 4°C. The cells were then centrifuged and the supernatant discarded before to resuspend the pellet within 100 µl sterile PBS containing the first fluorophore-conjugated primary antibody at concentrations detailed in Table 5, or corresponding isotype control antibodies. Incubations of given samples with multiple antibodies were performed consecutively, with two

Antibody target	Clone	Antibody clone and/or conjugate	Supplier and location	Working dilution (µl of Ab:µl PBS)
Primary antibody (Ab)				
CD3	UCHT1	Fluorescein isothiocyanate (FITC)-conjugated mouse IgG ₁	Becton Dickinson, Pharmingen, Toronto, ON	4:100
CD4	RPA-T4	Allophycocyanin-mouse IgG ₁	BioLegend, San Diego, CA	4:100
CD14	M5E2	Phycoerythrin (PE)- mouse IgG _{2a}	BioLegend	2:100
Phosphorylated p42/44ERK (Thr202/Tyr204)	N/A	Rabbit anti-human IgG	Cell Signaling Technology, Boston, MA	1:100
Phospho-p38 MAPK (Thr180/Tyr182)	N/A	Rabbit anti-human IgG	Cell Signaling Technology	1:100
TLR4	HTA125	AlexaFluor 488-mouse anti-human IgG _{2a}	Serotec, Cedarlane, Burlington ON	4:100
Secondary antibody				
Rabbit IgG	N/A	FITC- goat anti-rabbit IgG (H+L)	Jackson ImmunoResearch, West Grove, PA	1:167

Table 5: List and characteristics of antibodies used for flow cytometry experiments

washing steps in PBS (i.e. centrifugation and removal of the supernatant) between each staining. Incubation with primary antibody was performed in the dark, for 30 min at 4°C. Cells were finally washed twice with 5 ml of cold PBS and resuspended in 0.35 ml of PBS stored in the dark at 4°C until data acquisition by flow cytometry within the next hour. The data were obtained by acquiring a minimum of 10,000 events using FACS Calibur flow cytometer (BD-Biosciences, Toronto, ON), and subsequently analyzed using the CellQuest™ Pro software version 4.0.2 (BD-Biosciences). When the suspension of individual cells is vacuumed in and passes through the flow-cell, fluorophore labels coupled to the antibodies bound to their target on the cells are stimulated by the laser light. The emitted fluorescent light from the different fluorophores (i.e. at different wavelengths/colours) is collected by detectors (photomultipliers) and the software eventually presents fluorescence data graphically as a dot plot (in the case of double-colour staining) or histogram (single-colour staining). Light detectors also collect light scattering as it hits the cells, therefore providing with information on the size or volume of the cells i.e. Forward Scatter (FSC) and complexity of the cells i.e. the so-called sideways scatter (SSC). As opposed to the FSC values which depend on the cell size, the SSC data also consider the cell's granularity, the structure of the nucleus and the presence of vesicles since the SSC detector collects perpendicular light diffraction rather than collecting light in a flat angle. In all experiments, negative controls consisting of unstained cells as well as cells

incubated with isotype antibodies of the target-specific antibodies were concomitantly prepared and served to discriminate non-fluorescent cells and set the analysis gates. Data were presented as percentages of stained cells within gated cell populations for different markers. In all cases, the percentage of cells stained with isotype-control antibodies was subtracted from that of antigen-specific antibodies.

2.2.5. Isolation of CD4 T 'helper' lymphocytes

Isolation of CD4-expressing leukocytes from PBMC was achieved through immunomagnetic bead-based cell separation using the MACS[®] CD4 Microbeads (Miltenyi Biotec, Auburn, CA) as per manufacturer's instructions. The PBMC sample stained using MACS[®] CD4 Microbeads are pipetted into a MACS[®] MS-type column, placed in a MACS[®] Separator consisting of a strong permanent magnet. Cells loaded on the column pass through it but only cells that have cell surface human CD4 targeting-MACS[®] CD4 Microbeads are retained in the column by the magnetic field. This process refers to the principle of positive selection, i.e. only the cells targeted by the MACS[®] CD4 Microbeads are retained by the column, whereas untouched CD4 non-expressing cells are eluted through it. Cells retained by the column are further purified through consecutive passive washings, and CD4-expressing cells are collected by elution after removal of the column from the magnet.

Briefly, freshly isolated PBMC were first incubated for 24h with or without human recombinant cytokine IL-4 (13.5 ng/ml). After stimulation, the PBMC were recovered into 15-ml tubes and rinsed with 8 ml of staining buffer and centrifuged for 5 min at 1,500 rpm. The staining buffer solution composed of PBS pH 7.2, 0.5 % bovine serum albumin (BSA, Sigma-Aldrich Canada, Oakville, ON) and 2 mM of ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich Canada, M_w : 372.27 g/mole). The staining buffer solution was sterilized by passing through a filtering unit (i.e. 0.22 μ m porous membrane, Corning, Corning, NY) and allowed to degas for at least 2 hours prior to use. After the cells were washed with the staining buffer solution, the cell pellet was resuspended within 80 μ l of staining buffer solution to a maximum of 10^7 PBMC, to which we added 20 μ l of MACS[®] CD4 Microbeads. Cells and beads were mixed gently by pipetting or inverting the tube and incubated for 15 min at 4°C. After the staining, the cells were resuspended in PBS by adding 2 ml of staining buffer and centrifuged at 1,500 rpm for 10 min. Supernatant was discarded to remove exceeding and unbound MACS[®] CD4 Microbeads and the cell pellet (up to 10^7 cells) was resuspended within 0.5 ml of staining buffer solution prior to magnetic separation.

The cell suspension stained with MACS[®] CD4 Microbeads are further deposited in the reservoir of a MACS[®] MS-type column placed in the MACS[®] Separator magnet. Unlabelled cells which pass through the column are collected in a tube. After the 0.5 ml volume of cell suspension passed through the column,

the latter is rinsed 3 times with 0.5 ml of staining buffer solution, each time after the column reservoir emptied completely. Finally, the washed MACS® MS-type column is removed from the magnetic MACS® Separator and placed in a 15 ml tube. The CD4-expressing cells retained into the column are then flushed out by pipetting 1 ml of staining buffer and firmly applying the plunger supplied with the column. The recovered cell fractions enriched in CD4⁺ cells were periodically tested for CD4-positive cell purity assessed by flow cytometry using antibodies against the human CD4 molecule. Purified CD4⁺ T lymphocytes were typically washed twice with sterile PBS by centrifuging at 1,500 rpm for 5 min, before proceeding to total RNA extraction. The purity of CD4⁺ cell fraction was assessed by flow cytometry using the anti-human CD4 antibodies as described earlier (section 2.2.4).

2.2.6. RNA extraction and quality assessment

After the incubations of PBMC and/or the isolation of cell fractions, total RNA was isolated using the RNeasy mini kit extraction columns (Qiagen, Valencia, CA). Briefly, the washed cell pellets were lysed with 0.3 ml of β -mercaptoethanol (1% v/v)-supplemented RNA lysis buffer, slightly vortexed and frozen at -80°C prior to RNA extraction. The RLT buffer contains highly denaturing guanidine-thiocyanate which inactivates RNases. On the day of extraction, RNA cell lysates were thawed at room temperature and mixed with 70% ethanol (Commercial Alcohols, Brampton, ON) before the sample were

applied onto RNeasy Mini spin column. The ethanol ameliorates the affinity of the RNA with the columns containing a silica membrane that captures and holds RNA. Contaminants were efficiently washed away through a series of rinsing steps, and RNA is finally eluted within 35 µl of RNase-free distilled water. RNA quantification and quality assessment were performed by spectrophotometry for all samples (A_{260}/A_{280} ratios greater than 1.9 were used).

2.2.7. Reverse transcriptase PCR (RT-PCR)

Complementary DNA (cDNA) was prepared through reverse transcription (RT) of purified RNA samples. For each reaction, 500 ng of isolated RNA was used as a template for polymerase chain reaction-based amplification of messenger RNA (mRNA). This was achieved through the use of synthetic primers composed multiple (12 to 18) thymidines (i.e. oligo[dT] 12-18 primers) which are complementary to the non-coding poly-adenosine tails (poly A tails) of mRNA. The elution volume containing 0.5 µg of RNA is mixed with oligo[dT] 12-18 primers and heated at 65°C for 5 min, a step that delinearizes the RNA molecules and allows primers to anneal to poly A tails. The denatured RNA is further chilled on ice before 20 µl of RT-PCR master mix was added to each reaction. The RT-PCR master mix RNase-free water, reverse transcription buffer (250 mM Tris-Cl, 375 KCl, and 15 mM $MgCl_2$), 0.1 M dithiothreitol (DTT), dNTP mix (dATP, dTTP, dGTP, dCTP, all at 25 mM), 40 units of RNAGuard ribonuclease inhibitor (40 U/µl, Amersham-Pharmacia, GE Healthcare, Baie

d'Urfe, QC), and 200 units of Superscript™ II reverse transcriptase enzyme (200 U/μl, Invitrogen). This final solution is incubated at 42°C for 1 hour to allow reverse transcription to take place. Finally, the RT enzyme of the mix is inactivated by heating the solution for 15 min at 70°C. The cDNA is stored at -20°C until it was used as a template for further PCR.

2.2.8. Quantitative real-time PCR (qPCR)

This technique serves to amplify specific DNA sequences and quantify the expression of genes of interest at the mRNA level. This is achieved through the use of DNA probes or primers that specifically anneal to sequences from specific mRNA transcripts that were reverse-transcribed into cDNA template by RT-PCR. Quantification of mRNA levels was performed using the Roche LightCycler real-time PCR system (version 1.2, Roche Diagnostics, Laval, QC, Canada) and QuantiTect SYBRGreen PCR master mix (Qiagen). This system enables the continuous quantification of PCR reactions i.e. the generation of newly synthesized double-stranded DNA strands i.e. amplicons from the interaction between specific primers on complementary sequence of the cDNA template. This real-time quantification is based on the detection of the SYBR Green fluorescent dye that intercalates in the amplicons. Each qPCR reaction consists of 0.5 μl of cDNA generated by RT-PCR and 10 μl of master mix containing 3.5 μl of water, 0.5 μl of 5 μM sense (5') and anti-sense (3') primers targeting specific mRNA sequences, and 5 μl of QuantiTect SYBRGreen PCR master mix. This

master mix contains HotStartTaq DNA polymerase, PCR buffer, dNTPs and the SYBR Green dye. The PCR reaction takes place in a glass capillary allowing rapid temperature variations. The LightCycler real-time PCR system uses a diode that excites the SYBR Green dye within the capillary. The fluorescence detector quantifies the SYBR Green emitted light as a correlate of the amount of amplicon generated by the PCR reaction. The qPCR reaction starts with a 15 min polymerase activation step at 95°C, during which double-stranded DNA strands are denaturated. It is followed by about 35 cycles of amplification, each consisting of a quick 15 s denaturation at 94°C, 30 s-long primer annealing step between 55 and 60°C, and a polymerisation step of 20 s at 72°C.

The primers used in this thesis, except those encoding the GATA-3 mRNA transcripts (purchased from Qiagen: QuantiTect® Primer Assay), were all designed using the online Primer3 software and purchased from Invitrogen (San Diego, CA). A list and characteristics of all primers used for qPCR experiments in this thesis can be found as Table 6 Each primer set was designed to span one intron, in order to prevent co-amplification of genomic DNA. Relative quantification of gene-specific qPCR amplicons was achieved using a standard curve obtained by simultaneously amplifying samples with serial dilutions of the amplicon purified from a previous experiment. Briefly, the amplicons generated from all primer sets were all purified using the QIAquick PCR purification kit and the specificity of the products was confirmed by gel electrophoresis. The purified

Gene name	Amplicon length (bp)	Primer sequences (5' to 3')		Annealing temperature (°C)
		Sense	Antisense	
<i>Target genes</i>				
CD14	202	GCCGCTGTGTAGGAAGAAG	GCTGAGGTTCCGAGAA GTTG	55
GATA-3	110	Purchased from Qiagen		55
IFN-γ	118	AGCTCTGCATCGTTTTGGGT	GTTCCATTATCCGCTACATCTGAA	55
IL-4	152	TGCCTCCAAGAACACAACTG	GTTTCAGGAATCGGATCAGC	55
IL-5	129	TCAGGGAATAGGCACACTGG	CTCCGTC TTTCTTCTCCACAC	55
IL-10	200	CCTGCCCTAACATGCTTCGAG	GGTCTTGGTTCTCAGCTTGG	55
MD2	169	GAATCTTCCAAAGCGCAAAG	GCTCCCAGAAATAGCTTCAAC	55
MyD88	139	GATGGTGGTGGTTGTCTCTG	GAACTCTTTCTTCATTGCCTTG	55
STAT4	181	TACCTAATGCTTGGGCATCC	TCTGCCAGCATATGGAGTTG	55
STAT6	222	ACCCACCACACTCTCACTCC	TGGTCACATCTGAGCAGAGC	55
TLR4	151	CAACCAAGAACCTGGACCTG	GAGAGTGGCTTAGGCTCTG	55

Table 6: List of all primers used for real-time quantitative PCR experiments

Gene name	Amplicon length (bp)	Primer sequences (5' to 3')		Annealing temperature (°C)
		Sense	Antisense	
<i>Reference genes</i>				
GAPDH	137	AGCAATGCCTCCTGCACCACC	CCGGAGGGGCCATCCACAGTC	58
S9	307	TGCTGACGCTTGATGAGAAG	CGCAGAGAGAAATCGATGTG	58

Table 6: List of all primers used for real-time quantitative PCR experiments (continued)

amplicons elution was quantified by spectrophotometry. Ten-fold dilutions were prepared in Tris-HCl pH 8.0 (dilutions ranging from 10^{-1} to 10^{-10} ng/ μ l), and were used as a standard curve to quantify gene expression of unknown samples. Results were analyzed with LightCycler software version 3.5.3 (Roche Diagnostics). This software converts the crossing point (Ct) values of a unknown sample into a known amplicon concentration based on interpolation from the Ct values and concentrations of the standard curve. Amplification specificity was also assessed by melting curve analysis. Relative expression data were always normalized to that of endogenously and stably expressed reference (formerly known as 'housekeeping') gene. In this thesis, relative quantification of target genes was normalized to that of 40S ribosomal protein S9 (S9) and/or glyceraldehydes-3-phosphate dehydrogenase (GAPDH), which are both ranked in the top 150 best reference genes (997). Quantification of mRNA for all target genes was expressed as a ratio i.e. fold-change of relative target gene/reference gene expression to a reference sample from the same experiment (e.g. baseline expression in untreated cells).

2.2.9. LPS signalling experiments

Peripheral blood mononuclear cells from age-matched children with or without atopic histories were dispensed in 12-well culture plate and incubated for 1 hour in FBS-depleted culture medium, in the presence or absence of human recombinant IL-4 (13.5 ng/ml), or human TLR4 neutralizing antibody (5 μ g/ml,

mouse anti-human IgG_{2a}, clone HTA125, eBioscience, San Diego, CA) or human IL-4 neutralizing antibody (68 ng/ml, mouse IgG₁, clone AVU02, R&D Systems, Minneapolis, MN) or corresponding isotype control antibodies, at 37°C. Cells were then either left unstimulated or stimulated by adding LPS (0.25 µg/ml) for 1 to 60 min. The LPS-pulsed cells were resuspended in 10 ml PBS to stop reaction and the pellet was recovered by centrifugation for 5 min at 1,500 rpm. Cells were then fixed in 4% methanol-free formaldehyde (from 10% stock solution, Polysciences Inc., Warrington, PA) for 10 min at 37°C. The cells were washed and processed for IVIG blocking and CD4 surface staining as mentioned previously. Cells were further permeabilized by simultaneously adding 1 ml of pure frozen methanol (-20°C) and slightly vortexing for 5 s. The cells were chilled on ice for 30 min and washed twice with PBS prior to incubate them with antibodies rabbit against human phosphorylated p^{42/44}ERK (Thr202/Tyr204) or phospho-p38 MAPK (Thr180/Tyr182) for 15 min at room temperature, in the dark. Cells were rinsed again in PBS and stained with FITC-conjugated goat anti-rabbit IgG for 15 min at 4°C. The cells were finally rinsed with PBS and resuspended in 0.35 ml of PBS and kept in the dark prior to acquire two-colour flow cytometry data on the FACS Calibur. Data are presented as median fluorescence intensity (MFI) values for given CD4-positive cell populations. The LPS-stimulated cells were compared to a negative control made of unstimulated cells along with the isotype control reflecting non-phosphorylated targets.

2.2.10. Statistical analyses

In this study, appropriate parametric or non-parametric one-way analyses of variance (ANOVAs; with Bonferroni's or Kruskal-Wallis tests, respectively), followed by post hoc tests (Tukey's or Dunn's multiple comparison tests, respectively) were used to analyze all phenotyping data sets. For analyzing LPS-induced-signalling data, non-parametric one-way ANOVAs/Friedman tests followed by Dunn's tests were performed. Data are representative of the indicated number of subjects (n). Appropriate t-tests were used when comparing two groups. Spearman's rank tests were conducted for correlation analyses. For qPCR data analyses, appropriate parametric or non-parametric, paired or non-paired Student's t-tests were performed to compare gene expression means. Values are presented as means $\pm$ SEM. p-values < 0.05 (*) were considered significant. All statistical analyses in this thesis were performed using the GraphPad Prism version 5.0 (GraphPad Software Inc., La Jolla, CA).

2.3. Regulation of TLR4 in airway epithelial cells in asthma and rhinitis: the effect of Th2-type conditions and potential role for epithelial GATA-3 transcription factor

2.3.1. Subject recruitment: nasal mucosa biopsy specimens

Nasal mucosa biopsies were obtained from the inferior turbinate of a total of 17 adults with/without allergic rhinitis undergoing endoscopic nasal septal surgery. Subjects were recruited at the Department of Otolaryngology from both the Notre-

Dame Hospital and the Jewish General Hospital-Sir Mortimer B. Davis (Montreal, QC). The study was approved by the ethics committee Board of the McGill University Health Centre and Research Centre (see section on Research compliance certificates and permissions). Informed consent was obtained from participants before the procedure. Diagnosis of allergic rhinitis was based on positive history of allergic rhinitis for at least 2 years and a positive skin prick test to a panel of common aeroallergens. Subjects were non-smokers, and had no recent history of upper airway infection. Subjects were also free of medication for 2 weeks before the surgery. Finally, biopsies were taken outside of the pollen season.

2.3.2. Tissue processing: histology and tissue homogenization

During intranasal surgery, each subjects had two 3-mm³ nasal mucosa specimens were collected. The tissue specimens were immediately placed in a 3-ml volume of RNA later[®] (Sigma-Aldrich, St. Louis, MO) and in 3-ml of 4% formalin i.e. formaldehyde, respectively. RNA later[®] tubes were frozen at -80°C until tissue homogenization and RNA extraction, whereas tissues in formalin were left on the bench for fixation, overnight and at room temperature. The next day, formalin-fixed tissues were processed for 12 hours using the Sem-enclosed Benchtop Tissue Processor Leica TP1020 (Leica Microsystems, Richmond Hill, ON). Finally, the fixed and processed tissues were embedded in paraffin before 5

µm-thick sections were cut on a microtome. Slides were numbered and kept at room temperature.

2.3.3. Histology and immunocytochemistry

Formalin-fixed tissue sections were deparaffinated and re-hydrated in a graded alcohol series. Some sections (1 per subject) stained using haematoxylin & eosin (H&E), while most sections were processed for staining by immunocytochemistry (ICC) using the avidin-biotin-peroxidase method, as previously described (998). Tissue sections were processed for heat-induced antigen retrieval through boiling in 0.01 M EDTA buffer (pH 8.0) for 2 min. Sections were permeabilized with Triton 0.2% and endogenous peroxidase inactivated within 5% hydrogen peroxide. Unspecific antibody binding was reduced using Universal Blocking Solution (DakoCytomation, Mississauga, Ontario, Canada) for 30 min. Tissue sections were incubated overnight with biotinylated-LPS (ultrapure *E. coli* O111:B4, Invivogen, San Diego, CA, 1/40), with rabbit IgG anti-human TLR4 (Abcam, Cambridge, MA, 0.83 µg/ml), with mouse IgG1 anti-eosinophil cationic protein (ECP) antibody (EG2, Diagnostics Development, 11.1 µg/ml), with mouse IgG1 anti-human CD4 (clone 4-B12, Vector Laboratories, Burlingame, CA, 1/50), with mouse IgG1 anti-human CD68 (clone KP1, DakoCytomation, 1/60) or with mouse IgG1 anti-human GATA-3 (clone HG3-31, Santa Cruz Biotechnology Inc., 10 µg/ml). Appropriate biotinylated-secondary antibodies (DakoCytomation) raised against the primary

antibodies were applied for 1 h (concentrations of 1:100) but were omitted when biotinylated-LPS was used. Stainings were developed using the streptavidin-conjugated horseradish peroxidase (HRP) substrate and 0.5 mg/ml diaminobenzidine tetrahydrochloride in Tris buffer (pH 7.6) i.e. DAB chromogen as per manufacturer's instructions (DakoCytomation) for 1 min. Sections were counterstained with hematoxylin, de-hydrated and mounted with Cytoseal (ThermoScientific, Waltham, MA). Photomicrographs were taken using a BX51 Olympus microscope attached to a CoolSNAP-Pro color digital camera (Cارسen Group, Markham, Ontario, Canada) using the Image Pro-plus 4.0 system (Media Cybernetics, Silver Spring, MD). Numbers of EG2+ cells were scored blindly and expressed as the number of positive cells per mm² of submucosal surface (excluding areas covered by blood vessels and mucous glands). The intensity of epithelial TLR4 immunoreactivity was blindly graded based on the percentage of stained epithelial cells: isotype negative control (0), 20% (2), 40% (4), 60% (6), 80% (8), 87.5% (7), and 100% (10).

2.3.4. Isolation of bronchial epithelial cells

Nine adult age-matched volunteers were recruited from the Asthma Clinic at Hôpital Laval (Quebec, QC, Canada). The ethics committee of Hôpital Laval approved the study and all subjects gave written informed consent. Five asthmatic subjects (aged 24.0±2.5) were diagnosed based on the American Thoracic Society criteria (39): mean FEV₁=96.3±6.3% / mean PC₂₀=3.6±5.3

mg/ml. All asthmatics were non-smokers, atopics, and had skin test positive for house dust. The asthmatic patients used inhaled β 2-agonists on demand but none used inhaled or systemic corticosteroids. Normal subjects (n=4; aged 23.2 ± 2.8) were non-atopics and non-smokers had no history of asthma or systemic diseases: mean $FEV_1 = 105.6 \pm 4.3\%$ / mean $PC_{20} = 115.2 \pm 28.6$ mg/ml. Epithelial cells isolated from bronchial biopsies obtained by bronchoscopy were characterized by immunofluorescence and flow cytometry using anti-cytokeratin antibody (Calbiochem, San Diego, CA) before they were cultured in a 3:1 combination of Dulbecco's modified Eagle medium (DMEM) with Ham's F12 with supplements (Invitrogen, San Diego, CA), as previously described (999). Near confluent cell cultures were lysed for total RNA extraction.

2.3.5. Cell culture and stimulations

The BEAS-2B cell line (CRL-9609™) (1000) was obtained from the American Type Culture Collection (Manassas, VA). Primary Normal Human Bronchial Epithelial (NHBE) cells were purchased from Cambrex, Lonza Walkersville Inc. (Walkersville, MD). All cells were grown at 37°C and 5% CO₂ atmosphere. BEAS-2B cells below the 30th passage were cultured in DMEM containing 10% FBS (HyClone Laboratories, Logan, UT), 1% L-glutamine, 100 U/ml penicillin and 100 µl/ml streptomycin (all from Invitrogen). NHBE cells below passage 6 were grown in Bronchial Epithelium Basal Medium (BEBM) supplemented with Bronchial Epithelium Growth Medium SingleQuots® (Lonza

Walkersville Inc.). Cell viability was determined by morphology and trypan blue exclusion test. Cells were subcultured using 0.5% trypsin and 1 mM EDTA once confluence was reached. NHBE cells were grown onto rat collagen type 1-coated plastic surfaces (0.5 mg/mL; Trevigen Incorporated, Gaithersburg, MD) or Lab-Tek® glass slides (Nalge Nunc Int. Corp., Thermo Fisher, Rochester, NY). Cells were starved in serum-free DMEM (BEAS-2B) and 1% supplemented BEBM medium (NHBE) for 24h prior to stimulations with recombinant cytokines (all from R&D, Minneapolis, MN: IL-4, IL-13 or IFN- γ , ranging from 1 to 100 ng/ml starving medium) for up to 48h. Cells were rinsed with sterile PBS before they were lysed with 0.3 ml of β -mercaptoethanol (1% v/v)-supplemented RNA lysis buffer.

2.3.6. Immunofluorescence

Normal bronchial epithelial cells grown in wells of rat collagen I-coated Lab-Tek® slides were stimulated for 24 h without or with 10 ng/ml recombinant IL-4 before they were fixed and stained by fluorescent immunostaining. After stimulations, cells were successively rinsed in sterile PBS, fixed in 4% paraformaldehyde, and air dried prior to storage at -80°C. On the day immunostaining, the Lab-Tek® slides were thawed at room temperature and rehydrated in PBS for a few minutes. Lab-Tek® slides were then immersed in 50 mM ammonium chloride (NH₄Cl) to reduce endogenous fluorescence. Slides were blocked with 20% normal goat serum before a permeabilization in Triton-X 0.02% for 12 min. Cells were then incubated with mouse antibodies raised

against human GATA-3 or an isotype control antibodies (1 µg/ml overnight at 4°C; both from Santa Cruz) followed by secondary antibody staining with Alexa Fluor 555-conjugated goat anti-mouse IgG (Molecular Probes®, Invitrogen). Cells were counterstained with Hoechst 33342 (5 µg/ml) and mounted with PermaFluor (Immunon, ThermoShandon, Pittsburgh, PA) prior to microscope viewing. Fluorescent pictures from an Olympus inverted-phase microscope (IX-81), using a digital camera (CoolSnapPro CF monochrome) equipped with a CRI Micro Color filter kit. The images were analyzed and overlaid using ImagePro Plus software (Media Cybernetics, Inc., Bethesda, MD).

2.3.7. RNA extraction and quality assessment

The purification of total RNA from homogenized nasal mucosa specimens and from epithelial cell cultures were performed using the RNeasy mini kit extraction columns (Qiagen), as previously described. Furthermore, RNA samples were periodically processed for RNA quantification confirmation and integrity assessment using the Experion™ System and RNA StdSens analysis kit (Bio-Rad Laboratories, Mississauga, ON). This additional RNA quality assessment validated the protocol for processing nasal mucosa specimens. The Experion™ System uses an automated agarose gel electrophoresis station and enables RNA quality (degradation) assessment based on ratios of two large ribosomal RNA (rRNA) molecules, 28S and 18S rRNAs, as well as RNA quantification (Diagram 11). The 28S to 18S rRNA ratio should be ratio above 1

Electrophoretic

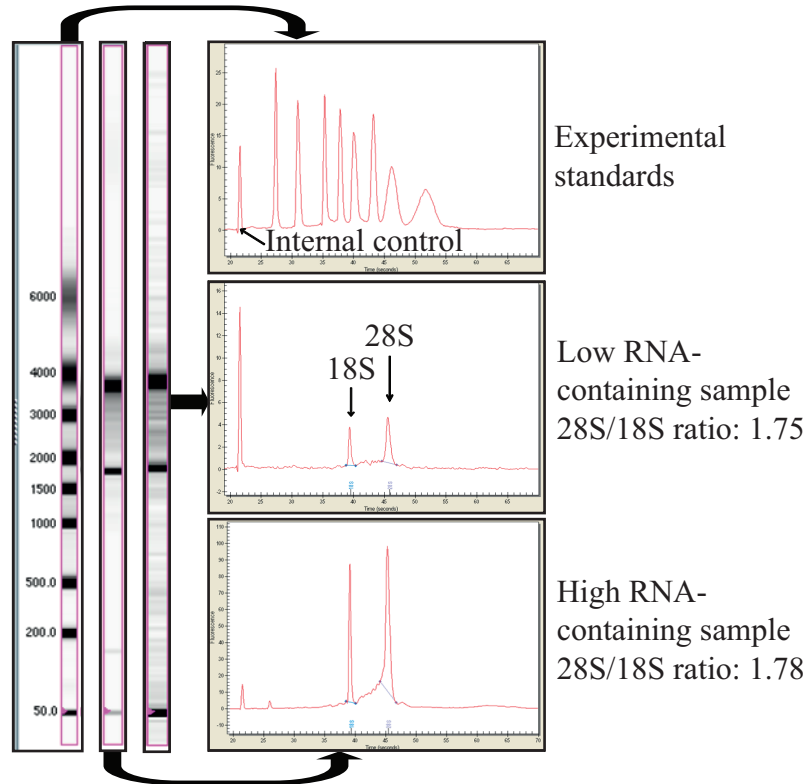


Diagram 11. Assessment of RNA quality with the Experion System. Coupled to the RNA StdSens analysis kit, this automated electrophoresis system (from Biorad) enables determining both the quality and quantity of RNA in gene expression analysis experiments. Representative experimental standards, low RNA- and high RNA-containing samples are displayed from left to right on the electrophoretic diagram and from top to bottom on the fluorescence-based histograms, respectively.

or 1.5 for biopsy tissue specimen- or cell culture-derived RNA samples. Tested samples displaying smears between 28S and 18S peaks reflect degraded RNA and were excluded from the experiments.

2.3.8. Reverse transcriptase and quantitative real-time PCR

These techniques were performed as previously described in an earlier section (sections 2.2.7 and 2.2.8).

2.3.9. Flow cytometry on epithelial cells

Human bronchial epithelial BEAS-2B cells cultured in 25 or 75 cm² were detached from plastic by incubating them with sterile 10 mM EDTA PBS solution at 37°C for 10 min. Half-a-million cell pellet were dispensed in flow cytometry plastic tubes. Unspecific antibody binding was avoided by incubating the cells with 20 µg/ml intravenous immune globulin (IVIG). Cells were fixed in 4% paraformaldehyde and permeabilized in -20°C methanol for 20 min prior to staining. Cells were incubated with antibodies against GATA-3 (2 µg/mL; Santa Cruz Biotechnology Inc.) or TLR4 (5 µg/mL; Abcam) or isotype controls for 30 min at 4°C. Rinsed cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse or goat anti-rabbit polyclonal IgG antibody for detection by flow cytometry using FACSCalibur (BD-Bioscience, Toronto, Ontario). Data from at least 10,000 events were analyzed using CellQuest™ Pro software version 4.0.2 (BD-Biosciences) and expressed as median fluorescence intensity (MFI).

2.3.10. Western blotting

Cultured cells were lysed in ice-cold buffer A (50mM Tris–Cl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% (v/v) Triton x-100, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 0.27 M sucrose, Complete Mini protease inhibitor cocktail [Roche Diagnostics] and 2 mM DTT). Protein concentrations were determined using the Bradford assay. Twenty-five micrograms of protein was resolved by 10-12.5% SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were incubated in LI-COR blocking buffer (LI-COR Biosciences, Cambridge, UK) for 1h at room temperature before being incubated with specified antibodies: mouse IgM anti-human GAPDH (1.0µg/ml, Affinity BioReagents), goat IgG anti-human GATA-3 (0.2µg/ml, R&D), rabbit IgG anti-human TLR4 (clone H80, Santa Cruz Biotechnology Inc., 0.2µg/ml) for 1h at room temperature. After rinsing, membranes were incubated with IRDye 800-conjugated goat anti-rabbit IgG or IRDye 800-conjugated donkey anti-goat IgG and/or Alexa Fluor 680-conjugated goat anti-mouse IgG (diluted 1:15,000) in LI-COR blocking buffer. When necessary, membranes were stripped using Re-Blot Plus antibody stripping solution (Millipore, Billerica, MA). After washing, immunoblotting signals were visualized and analyzed by the Odyssey IR imaging system (LI-COR Biosciences).

2.3.11. Plasmid constructs and over-expression experiments

A 1.332 kb fragment representing the open reading frame of the human GATA-3 gene was amplified from Jurkat T cell cDNA using high-fidelity LongAmp *Taq* DNA polymerase (New England Biolabs, Ipswich, MA) and the following primers 5'-CCGAATTCATGGAGGTGACGGCGGA-CCAGCCG-3' and 5'-GCTCTAGACTAACCCATGGCGGTGACCATGCT-3', which respectively contain *EcoRI* and *XbaI* restriction sites at their 5' end. Digested fragments were then ligated into identically cleaved sites in the linear p cytomegalovirus (CMV) 5 (pCMV5) expression vector to yield pCMV5-GATA3 (1001). Recombinant plasmids transformed into DH5 α competent cells were further purified using Qiagen EndoFree Plasmid Purification Maxi Kit (Qiagen). Recombinant plasmids were verified by restriction enzyme analysis and sequencing at the Genome Quebec Innovation Centre (McGill University). GATA-3 over-expression was achieved by transfecting >75% confluent NHBE and BEAS-2B cells grown into 6-well plates using 1.5 μ l of Superfect reagent (Qiagen) and 0.375 μ g pDNA per well of a 6-wellplate (e.g. pCMV5-GATA3 vectors or pCMV5-empty vectors) within 600 μ l SingleQuots-free BEBM for 3h followed by addition of SingleQuots-supplemented BEBM medium, as per manufacturer's instructions. Transfected cells were re-incubated for an additional 1 or 21 h (total of 4 or 24 h).

2.3.12. RNA interference experiments

GATA-3 gene silencing was achieved by transfecting >75% confluent NHBE and BEAS-2B cells with GATA-3-specific or negative control small

interfering RNA (siRNA): HP GenomeWide siRNA 1027400, and 1022076, respectively, and the HiPerfect Transfection Reagent (all from Qiagen, Valencia, CA) as per manufacturer's instructions. BEAS-2B and NHBE cells were previously cultured to a near confluent state in 6-well culture plates, in complete culture medium. Transfection complexes were prepared in 2.3 ml of serum-free medium by adding 5 nM of siRNA vectors and 1.5 μ l of HiPerfect Transfection Reagent (Qiagen). After 8 min of incubation at room temperature, the transfection complexes were then added to the cells. The culture plates were then incubated for 3h prior addition of complete DMEM or BEBM, and incubation for a total of 72h before cell lysis for RNA extraction.

2.3.13. Statistical analyses

Parametric or non-parametric tests were selected based on D'Agostino and Pearson omnibus normality tests. Data from two or more groups were analyzed using two-tailed Student t-tests or ANOVAs followed by Bonferroni's multiple comparison tests, considering 'n' independent experiments as detailed in the Figure legends. Data across this study were considered statistically significant when p values were inferior to 0.05 ($p < 0.05$).

Chapter 3: Results

3.1. Cross-sectional study on TLR4 expression and signalling in peripheral leukocytes of children with/without atopic histories

3.1.1. Introduction

The hygiene hypothesis has been used to explain the increasing prevalence of allergic disorders. Extensive epidemiologic data from multiple European and North American studies suggests that exposure to microbial products modifies immune responses in children against developing allergic diseases (592). Decreased atopy in rural areas has been correlated with exposure to farm animals (629, 630, 636, 637, 1002), unpasteurized milk products (635), multiple household pets (1003) and bacterial endotoxin or lipopolysaccharide (LPS) (637, 866, 870, 871). How bacterial endotoxin modifies immune responses in childhood is an area of intense investigation. Few studies have investigated how allergic diseases and Th2-type microenvironment affect immune cells' ability to respond to endotoxin.

Recognition of bacterial endotoxins is largely mediated via Toll-like receptors (TLRs). TLRs are hallmarks of the innate immune system, facilitating rapid recognition of common infectious patterns encountered by higher organisms (via Pattern Associated Molecular Patterns or PAMP) (700, 773). Toll-Like receptor 4 (TLR4) recognizes LPS, and is part of a key signalling complex

comprising CD14, MD-2 and intracellular components which initiate protective immune responses against bacterial invaders (711, 1004). In mucosal tissues, LPS' targets are assumed to be mainly innate immune system cells such as monocytes/macrophages, dendritic cells and epithelial cells. However, TLRs are also expressed by structural cells, and on cells of the adaptive immune system, including B- and T-lymphocytes (246, 735). In the periphery, monocytes are commonly used to study LPS effects as they constitutively express the TLR4 complex (728, 1005). Upon binding, LPS and TLR4 complexes are quickly internalized (759-761) and activate cells through a variety of signal transduction pathways including NF- κ B, mitogen-activated protein kinase (MAPK) p38 and extracellular signal-regulated kinase (ERK) 1/2 (p42/p44ERK) (778, 1004). LPS was shown to sequentially induce monocyte production of pro-inflammatory (TNF- α , IL-1 β , IL-6, IL-8, etc.) (792, 1006) and anti-inflammatory mediators (IL-1Ra, IL-10, etc.) (831, 833, 836). While exposure to high concentrations of bacterial LPS induces detrimental levels of pro-inflammatory mediators, exposure to low or environmental LPS levels, especially early in life, may provide immune-regulatory effects (1007).

Besides epidemiological evidence supporting the hygiene hypothesis, the role of LPS in the development of allergic diseases remains a matter of controversy. While exposure of newborn mice to low levels of LPS abolished Th2 responses and bronchial hyper-responsiveness to inhaled allergens in an asthma

model (920, 921), studies in mature mice yielded conflicting results (899-905, 911, 919). Previous work conducted in our laboratory demonstrated that LPS down-regulated allergen-induced Th2-type responses in explanted nasal mucosa from allergen-sensitized children stimulated *in vitro*, but not in atopic adults (736), likely reflecting that age affects LPS responsiveness (736, 1008). This protective effect of LPS was reflected by an immune bias toward developing Th1-type instead of Th2-type responses seen in tissues stimulated with allergens alone. The LPS-induced neutralization of allergen-specific Th2-type inflammation was indeed abolished by adding antibodies blocking the effects of the Th1-promoting IL-12, or immuno-suppressive IL-10 cytokines. Furthermore, LPS exposure in this model increases the numbers of CD3+ CD4+ T helper lymphocytes expressing both TLR4 and IL-10 (736, 888). It appeared that TLR4 expression was reduced in adults compared with children, and that atopy especially in adults further decreased TLR4 expression *in situ* (736). These data documented LPS-mediated protection against allergic inflammation, but suggested that atopy may affect TLR4 expression in adults. Studies also reported differences in the LPS-induced cytokine production by blood mononuclear cells based on maternal atopy (981, 992, 1009) and atopy, and by PBMC from asthmatic and non-asthmatic adults (734, 988). Finally, our laboratory demonstrated that the Th2-type cytokine IL-4 contributes to the down-regulate TLR4 and LPS-induced NF- κ B signalling pathway activation in a monocytic cell line (972).

Although difference in LPS-mediated responses may reflect differential TLR4 complex expression/function in atopy, few studies have investigated whether atopic status and/or Th2-type microenvironment affects the way LPS activates TLR4-expressing leukocytes of children. In light of previous data generated from our laboratory regarding the possible negative feedback of allergies on the numbers of TLR4-expressing cells *in situ*, we employed a more systemic approach and used peripheral blood mononuclear cells to characterize the expression of TLR4 and LPS responsiveness in a cross-sectional cohort of atopic and non-atopic children and adults (n=376). Given the importance of IL-10, IL-12 as well as of TLR4-expressing CD4+ cells in the LPS-mediated suppression of allergic inflammation, we decided to address the question whether a history of atopic disease affects the ability of CD4-expressing peripheral blood mononuclear cells, namely T 'helper' lymphocytes and monocytes (251) to respond to LPS.

3.1.2. Results

3.1.2.1. Discriminating between peripheral CD4-expressing monocytes and T 'helper' lymphocytes

In order to study the TLR4 expression and LPS responsiveness of circulating CD4+ leukocytes, we studied a sample of children ages 2-18 and adult volunteers (18-45). We obtained blood samples from a total of 376 subjects. The cohort demographics and histories of atopic disease are presented in Table 7.

The isolated PBMC were analyzed by flow cytometry to discriminate the different CD4-expressing cell populations in the peripheral blood. From the global FSC/SSC dot plot we obtain for a representative PBMC sample, we easily discriminate two main leukocyte populations, as referred to as FSC/SSC small and the FSC/SSC large cells (Figure 1a). Both of these gates represented about 90% of PBMC in all experiments. The FSC/SSC large population incorporated a majority of cells expressing low levels of CD4, termed CD4^{Low} (Figure 1b). In comparison with the FSC/SSC large population, 40 to 55% of the FSC/SSC small population leukocytes (Figure 1a) consisted of CD4^{High}-expressing T 'helper' lymphocytes as they co-expressed high levels of both CD4 (Figure 1c). These CD4^{High} T 'helper' lymphocytes lack the monocytic cell markers and LPS co-receptor CD14 (Figure 1d), while they uniformly expressed the T lymphocyte surface marker CD3 (Figure 1e). In contrast, the CD4^{Low} cells incorporated the greatest proportion of CD14⁺ cells or monocytes (Figure 1d), as they included few CD3⁺ T lymphocytes (Figure 1e). CD14-expressing cells also uniformly expressed TLR4 (Figure 1f and 1g) and were predominantly (>95%) detected in the CD4^{Low} subset (Figure 1d). We also detected approximately 5% of the large FSC/SSC cells that co-expressed CD4^{High} (Figure 1b); these cells also co-expressed CD3 and were analyzed as T 'helper' lymphocytes (Figure 1b and c, arrow). Less than 5% of CD4^{High}-expressing T 'helper' lymphocytes express TLR4 (Figure 1f and h). In contrast with monocytes, only small percentage (< 1%)

Atopic status	Atopic subjects				Non-atopic subjects			
Age groups	2-6	7-12	13-18	>18	2-6	7-12	13-18	>18
Mean age	4.0	10.0	15.1	24.9	4.1	10.0	15.3	24.2
($\pm$ SD)	(1.4)	(1.7)	(1.4)	(10.3)	(1.6)	(1.8)	(1.3)	(9.2)
Number of subjects	25	49	77	23	39	63	86	14
History of allergies (% of subjects with positive history of allergic diseases)								
Asthma	28	39	47	44	0	0	0	0
Eczema	28	16	25	26	8	16	14	0
Food allergies	52	33	26	35	0	0	0	0
Aeroallergen reactivity	28	55	60	70	0	0	0	0
Multiple allergies	28	41	34	52	0	0	0	0

Table 7: Characterization of the subject's atopic status and demographics.

of the CD3+CD4^{High} T 'helper' lymphocytes co-expressed CD14 and TLR4 and approximately 1% of CD4-expressing cells were CD14-positive.

3.1.2.2. Cross-sectional survey of TLR4 expression by CD4-expressing peripheral leukocytes

Previous studies conducted in our laboratory (736) and by others (1008) suggested that atopy and age may both contribute to reduce TLR4 expression and limit endotoxin responsiveness or LPS-mediated protection against allergen-

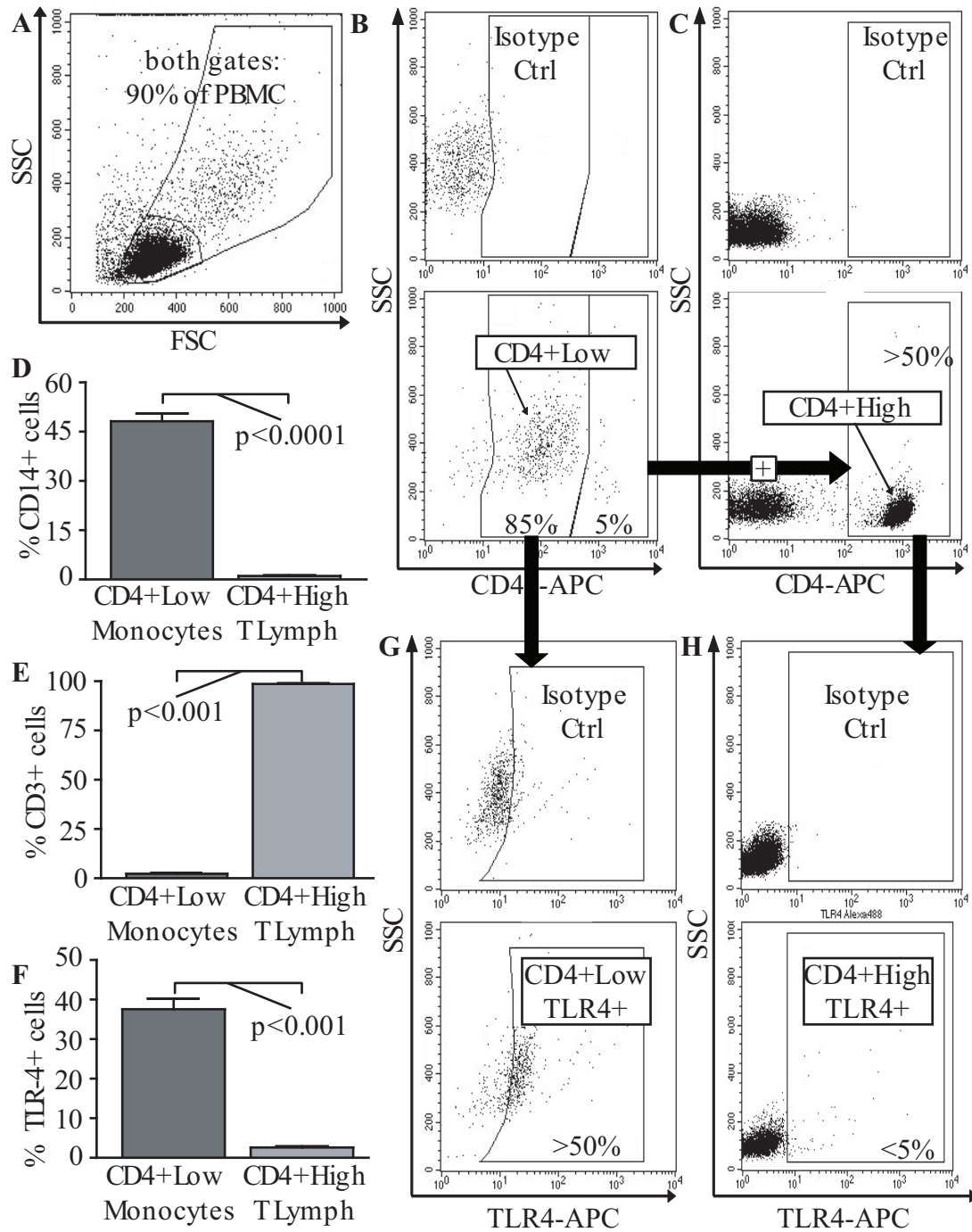


Figure 1. Characterization of circulating TLR4⁺ CD4-expressing cell populations: monocytes and T ‘helper’ lymphocytes. PBMC were analyzed by flow cytometry for surface expression of CD4, CD14, CD3 and TLR4. Representative FSC/SSC dot plot for PBMC, with gates around the two populations (A). CD4/SSC dot plot of monocytes (B) and lymphocytes (C) identified as CD4+Low and CD4+High cells, respectively. (D) Relative percentage of CD14⁺ cells (n=22) and (E) CD3⁺ (n=4). (F) The expression of TLR4⁺ (n=61) in monocytes and CD4⁺ T lymphocytes, along with representative dot plots (G and H, respectively).

induced immune responses. In order to test this hypothesis, we used the above-described cross-sectional cohort and evaluated the percentages of TLR4-expressing circulating T 'helper' lymphocytes and monocytes, based on the flow cytometric approach described in Figure 1. As depicted in Figure 2a, the percentages of TLR4-expressing CD4⁺High T 'helper' lymphocytes in non-atopic control children (<18 years-old) remained greater than in atopic children, although these differences were not statistically significant based on appropriate statistical analysis (ANOVA/Kruskal-Wallis tests, with Dunns post-hoc tests). In contrast, the percentages of TLR4+ T 'helper' lymphocytes increased from the atopic subjects between 2 to 6 and 13 to 18 years of age ($p < 0.05$). Regardless of the age factor, the percentages of TLR4+ T 'helper' lymphocytes was higher in atopic than in non-atopic adults ($p < 0.05$; data not shown, $n = 72$ vs. 84).

As presented in Figure 2b, the percentages of TLR4+ monocytes varied minimally with age in non-atopic subjects whereas we observed a trend toward age-dependent decrease in the percentage of TLR4+ monocytes in atopic subjects. Again, these differences did not reach statistical significance. The lack of statistical significance characterizing these data sets likely reflects both the high inter-individual variability in TLR4 expression as well as the stringency of the performed statistical analyses. Interestingly, correlation analyses of the age effect on the percentages of TLR4+ cells either among T 'helper' lymphocytes and monocytes enabled the identification of significant effects. As presented in Figure

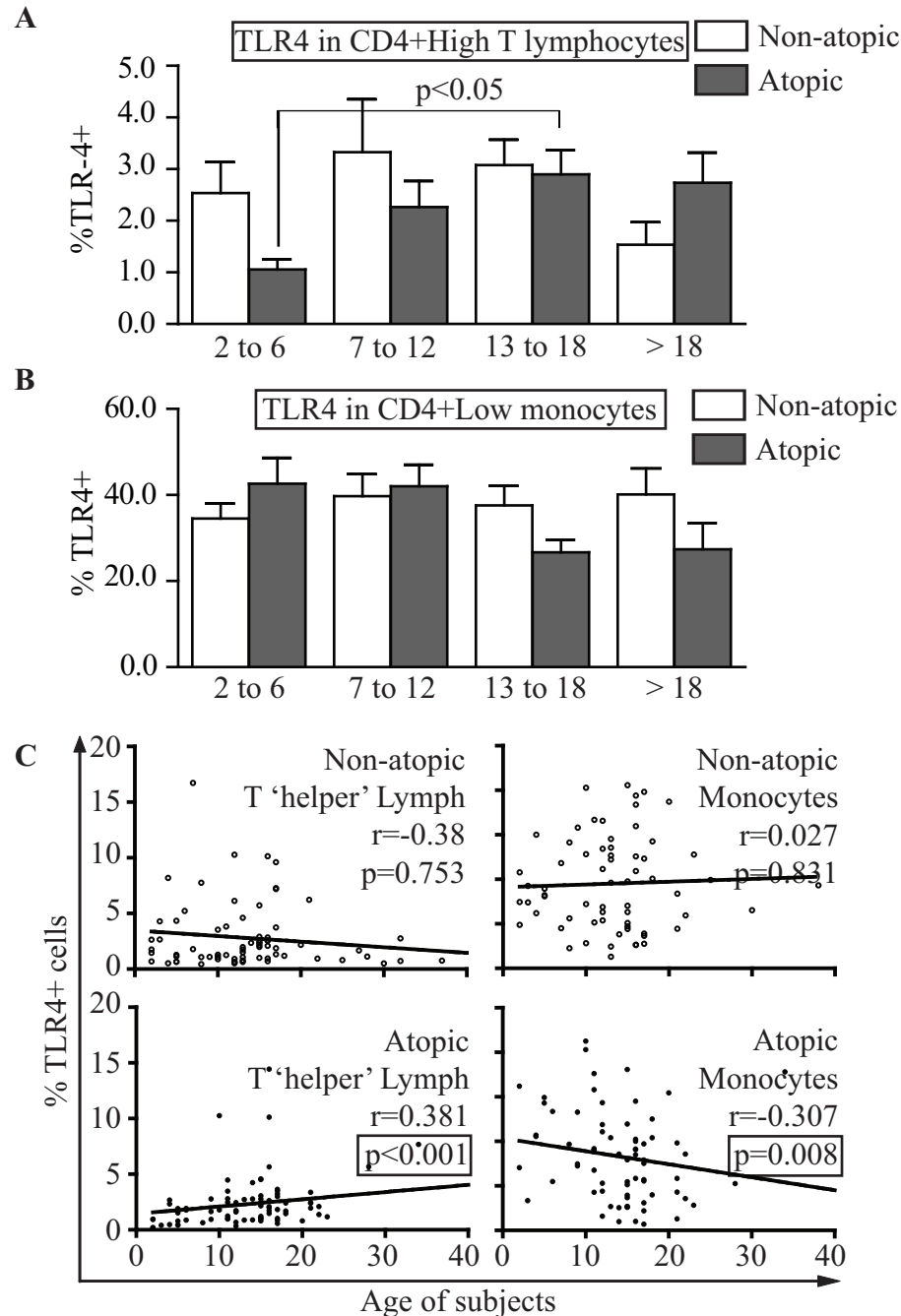


Figure 2. Age- and atopy-related variations in TLR4 surface expression by CD4-expressing cell populations: T 'helper' lymphocytes and monocytes. PBMC were analyzed by flow cytometry for surface CD4 and TLR4. Data are presented as relative percentages of TLR4+ cells for subjects without/with atopy, respectively: 2 to 6 (n=14/17), 7 to 12 (18/19), 13 to 18 (28/34), and >18 (12/13). Data for lymphocytes (A) and monocytes (B) were analyzed by non-parametric ANOVAs. Correlation analyses illustrates age-dependent variations in TLR4 expression based atopic status, both in T 'helper' cells and monocytes (C). Spearman's rank correlation coefficients appear as 'r'. p-values<0.05 were considered significant.

2c, the upper left graphs depict the absence of significant association or age effect on the variation in the percentage of TLR4+ cells among non-atopic T 'helper' lymphocytes, while a significant trend toward increasing TLR4 expression with age in atopic patients (bottom left graph). On the other hand, the right graphs demonstrate that the expression of TLR4 on monocytes inversely correlated with age in subjects with atopic disease histories. Conversely, there was no association between age and TLR4 expression on non-atopic monocytes as their percentages remains stable throughout childhood. One should note that these opposite and atopy-specific effects of subject age on TLR4 expression were not due to significant variations in the numbers of CD4^{High} T 'helper' lymphocytes or CD4^{Low} monocytes, either due to age nor atopy (data not shown). Taken together, these data suggest that in atopic subjects, the expression of TLR4 decreases with age on monocytes but increases with age on T 'helper' lymphocytes. In contrast, less age-dependent variations are observed in TLR4 expression on cells from non-atopic subjects.

3.1.2.3. Effect of IL-4 stimulation on TLR4 expression by CD4-expressing leukocytes

We pursued our investigations on the potential role of atopy on TLR4 expression and mimicked allergic-like *in vitro* conditions by adding IL-4 to PBMC cultures for 24h prior to analyze TLR4 expression on peripheral leukocyte sub-populations. As presented in Figure 3a for T 'helper' lymphocytes, and in Figure

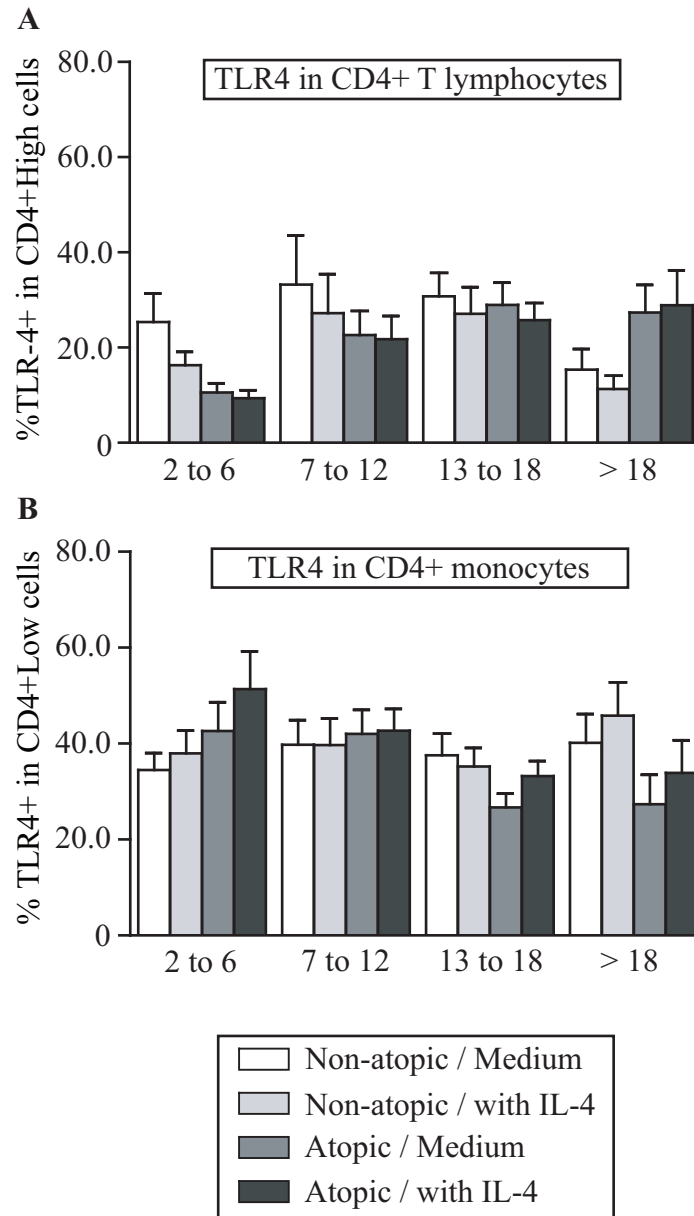


Figure 3. Cross-sectional survey of TLR4 expression by circulating leukocyte populations: combination of age, atopy and IL-4 stimulation effects. PBMC were stimulated with/without recombinant IL-4 and analyzed by flow cytometry for surface expression of CD4 and TLR4. Data for Medium and IL-4-stimulated cells are presented as relative percentages of TLR4+ cells in the different age groups, and for subjects without/with atopy, respectively: 2 to 6 (n=14/17), 7 to 12 (18/19), 13 to 18 (28/34), and >18 (12/13). Data were analyzed by non-parametric ANOVAs and p-values<0.05 were considered significant.

3b for monocytes, IL-4 failed to alter TLR4 expression on these cells in a statistically significant manner, for all of the subject's age groups and regardless of the subject's atopic histories. We then used a subgroup of age-matched subjects to test the atopy and/or IL-4 effect on the expression of the LPS co-receptor CD14, the latter which is also necessary for proper LPS recognition and TLR4-mediated signal transduction. The CD14 expression was significantly reduced in T 'helper' lymphocytes compared with monocytes (Figure 4a and b). Similarly to what was found for TLR4, there was no difference between the expression of CD14 at baseline, on either monocytes or T 'helper' lymphocytes from atopic and non-atopic subjects (Figure 4). However, IL-4 significantly reduced CD14 expression both on monocytes (Figure 4a) and T 'helper' lymphocytes, regardless of atopic status (Figure 4b). The atopic/non-atopic subjects analyzed for CD14 expression were age-matched (age: $13.8 \pm 2.8 / 12.1 \pm 4.7$). As expected, the percentage of CD14-expressing monocytes were, in all conditions (with/without IL-4), significantly greater than the % CD14+ T 'helper' lymphocytes ($p < 0.05$) (data not shown). In summary, our cross-sectional survey of the expression of TLR4 enables us to conclude that a positive history of atopic diseases in children does not translate into significant changes in TLR4 receptor expression on peripheral CD4-expressing leukocytes. Nonetheless, our results stress the effect of age which increases TLR4 expression on T 'helper' lymphocytes and decrease it on monocytes, while no

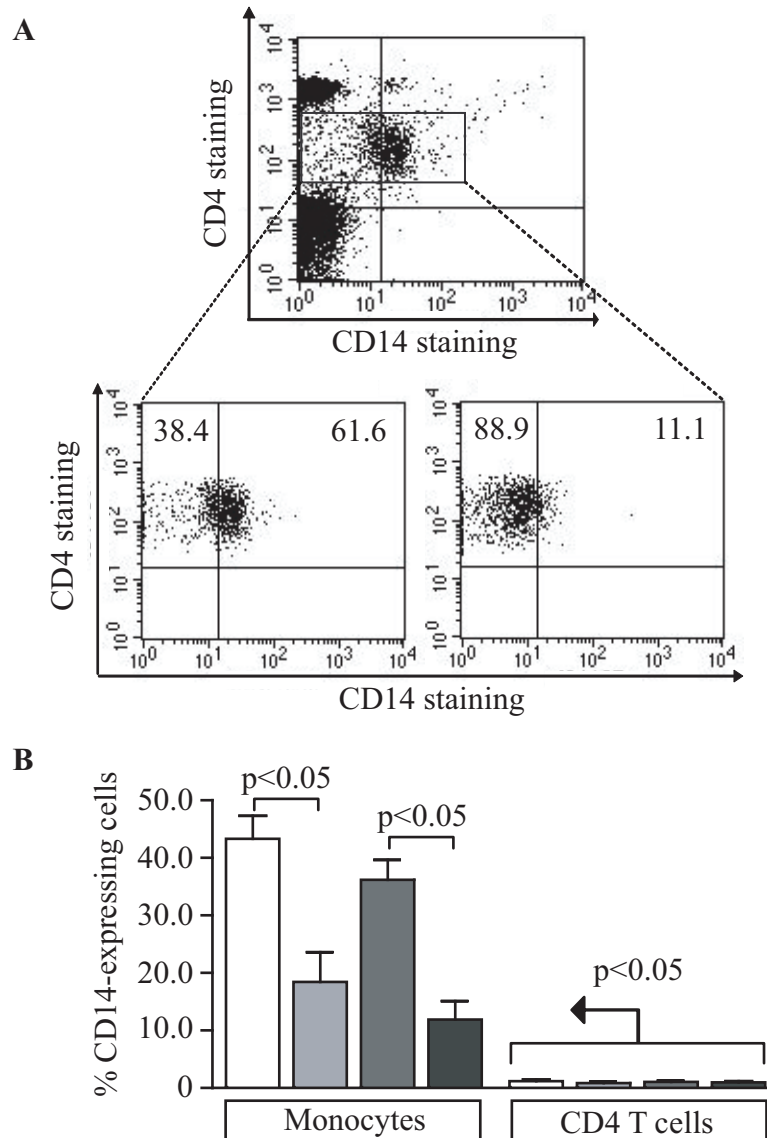


Figure 4. CD14 expression on monocytes is decreased by IL-4 but its baseline expression is not affected by a history of atopic diseases. In A, PBMC were analyzed for surface CD14 on monocytes following 24 h culture without (left graph) or with (right graph) IL-4 (13.5 ng/ml). Analysis of the percentages of CD14 expression on CD4+Low monocytes (left) and CD4+High T 'helper' lymphocytes (right) from age-matched non-atopic/non-atopic children is presented in B (n=10/13). Data were analyzed by ANOVA and p-values<0.05 were considered significant.

such age-mediated variation was seen in cells from non-atopic subjects. Finally, pro-allergic stimuli (i.e. IL-4 stimulation) do not modify TLR4 but reduces CD14 in both T 'helper' lymphocytes and monocytes.

3.1.2.4. IL-4-mediated regulation of TLR4 complex gene mRNAs in CD4+ leukocytes

Despite we did not observe IL-4-induced variations in the expression of TLR4 at the surface of cells after *in vitro* stimulation experiments, we investigated whether IL-4 affected TLR4-complex mRNA. Previous studies reported that IL-4 decreases, but not abolishes TLR4 mRNA expression in peripheral monocytes and in a monocytic cell line (963, 972). On the other hand, studies led in our laboratory documented that nasal tissues from atopic children exposed to LPS were protected against allergen-induced allergic inflammation by increasing the numbers of TLR4-expressing CD3+ and CD4+ cells (736). In contrast, LPS failed to increase TLR4 expression in tissues from atopic adults, and failed to protect them against *ex vivo* allergen challenge-induced Th2-type inflammation. These data suggested that conditions found in prolonged atopy, including Th2-type environment e.g. IL-4, may alter TLR4 expression on T 'helper' lymphocytes themselves. We therefore prepared CD4+ cells fractions from medium PBMC cultures using immunomagnetic cell separation (MACS® CD4 Microbeads, Miltenyi Biotec). As depicted in Figure 5a, immunomagnetic purification of CD4 cells enabled us to successfully isolate >95% pure CD4+High T 'helper'

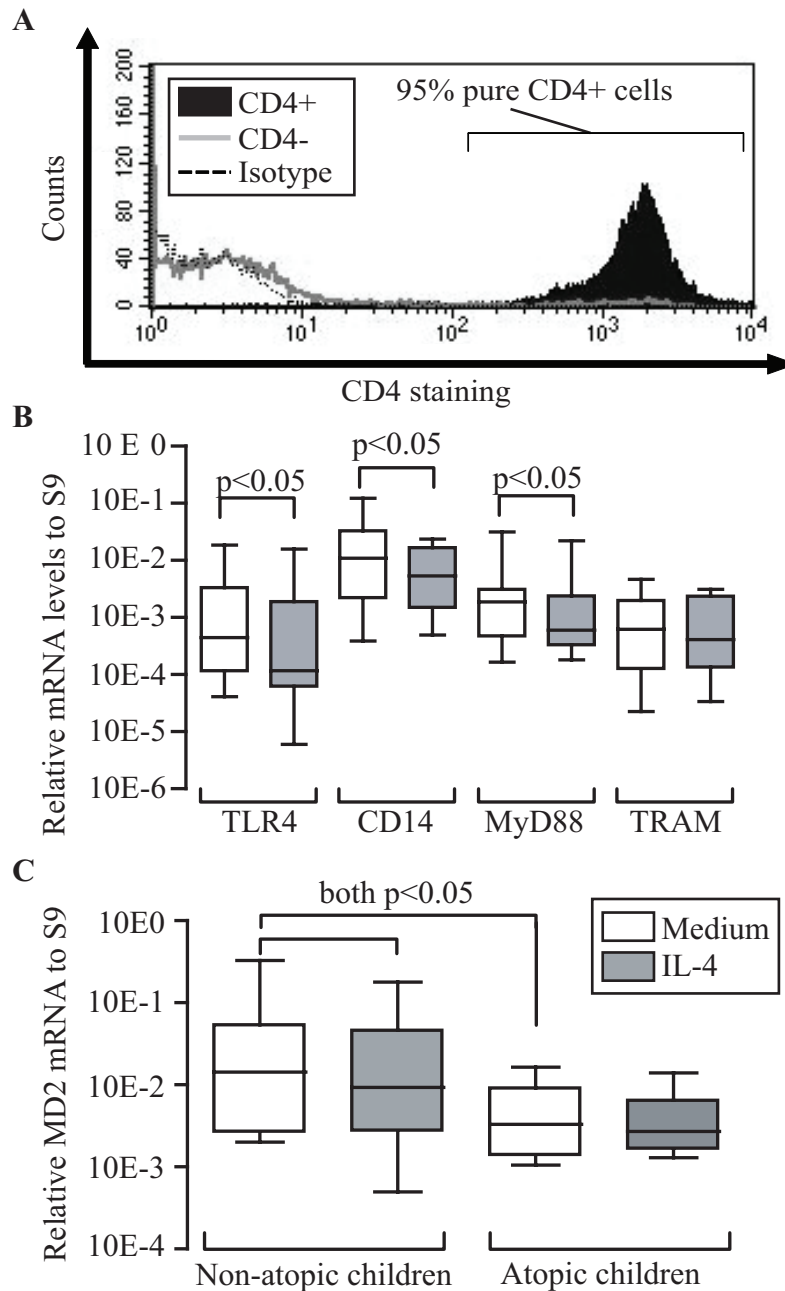


Figure 5. IL-4 reduces mRNA level of TLR4 complex molecules in CD4+ cells. CD4+ cell fraction was isolated from PBMCs of atopic/non-atopic subjects cultured for 24h with/without IL-4 (13.5 ng/ml), by immunomagnetic bead sorting. Purity of CD4+ cell fractions was assessed by flow cytometry (A). Total RNA was extracted and RT-qPCR served to assess mRNA levels for TLR4, CD14, MyD88 and TRAM ($n > 12$ per targets) in CD4+High T 'helper' lymphocytes from non-atopic children (B). IL-4 reduced MD2 adaptor molecule mRNA l in CD4+ from non-atopic children ($n = 15$), and atopic children ($n = 7$) have lower baseline MD2 expression than controls. Presented data are medians normalized to S9 gene transcripts and p -values < 0.05 were considered significant.

lymphocytes, as assessed by flow cytometry. Using subsequent RT-PCR and real-time qPCR, mRNA levels for all the tested components of the TLR4 complex were quantified in purified CD4⁺ cell fractions. Compared with cells maintained in complete culture medium, the addition of IL-4 for 24 hours decreased the mRNA levels encoding most genes of the TLR4 complex in cells from non-atopic subjects, namely TLR4, CD14, the myeloid differentiation primary response gene 88 (MyD88), the myeloid differentiation factor 2 (MD2), but not the Toll-IL-1R domain-containing adaptor inducing IFN- β -related adaptor molecule (TRAM) gene (Figure 5b and c). As described earlier, these genes encode critical molecules part of the LPS/TLR4-mediated signal transduction pathways (section 1.3.4.3, and Diagrams 9 and 10). Preliminary data suggested that IL-4 stimulation similarly diminished the mRNA expression of LPS receptor complex-related genes in CD4⁺ cells from atopic children as well but the data did not reach statistical significance ($n < 10$) (data not shown). The baseline gene expression levels of members of the LPS receptor complex were also lower in CD4⁺ cells from atopic children than those observed in cells from the non-atopic children, although only MD2 mRNA levels reached statistical significance (Figure 5c). These data suggest that IL-4 (and atopy) may contribute to limit LPS responsiveness of T 'helper' cells by limiting the transcription of most of the TLR4 complex-associated molecules.

3.1.2.5. A history of atopy in children impairs LPS-induced signal transduction in CD4-expressing cells

Since our experiments regarding the effects of atopic status and IL-4 on the expression of the LPS receptor complex did not demonstrate drastic changes in the expression of its associated molecules, we sought to determine whether these changes had biological significance and if atopy reflected any change in LPS responsiveness. We therefore assessed whether atopy and IL-4 can affect the ability of CD4-expressing leukocytes to respond to LPS and activate specific signalling pathways. Using flow cytometry approach, we sought to detect intracellularly phosphorylated signalling proteins in LPS-stimulated cells, and focussed specifically on mitogen-activated protein kinase (MAPK) p38 and ERK 1 and 2 (p42/p44). Data on the LPS-induced phosphorylation of p42/44ERK and p38 MAPK are presented as Figure 6 and 7, respectively.

Stimulation with LPS induced rapid and consistent increases in phosphorylated p38 MAPK in CD4⁺Low monocytes of children with no history of atopic disorders (Figure 6a, left graph). Significantly elevated levels of phosphorylated p38 MAPK were observed within 5 min after LPS exposure. Surprisingly, there was a clear difference in the LPS-induced p38 MAPK phosphorylation in cells from children with histories of atopic conditions compared with those from non-atopic children (Figure 6a, right vs. left graphs, respectively). In contrast, limited or no consistent phosphorylation was seen among the

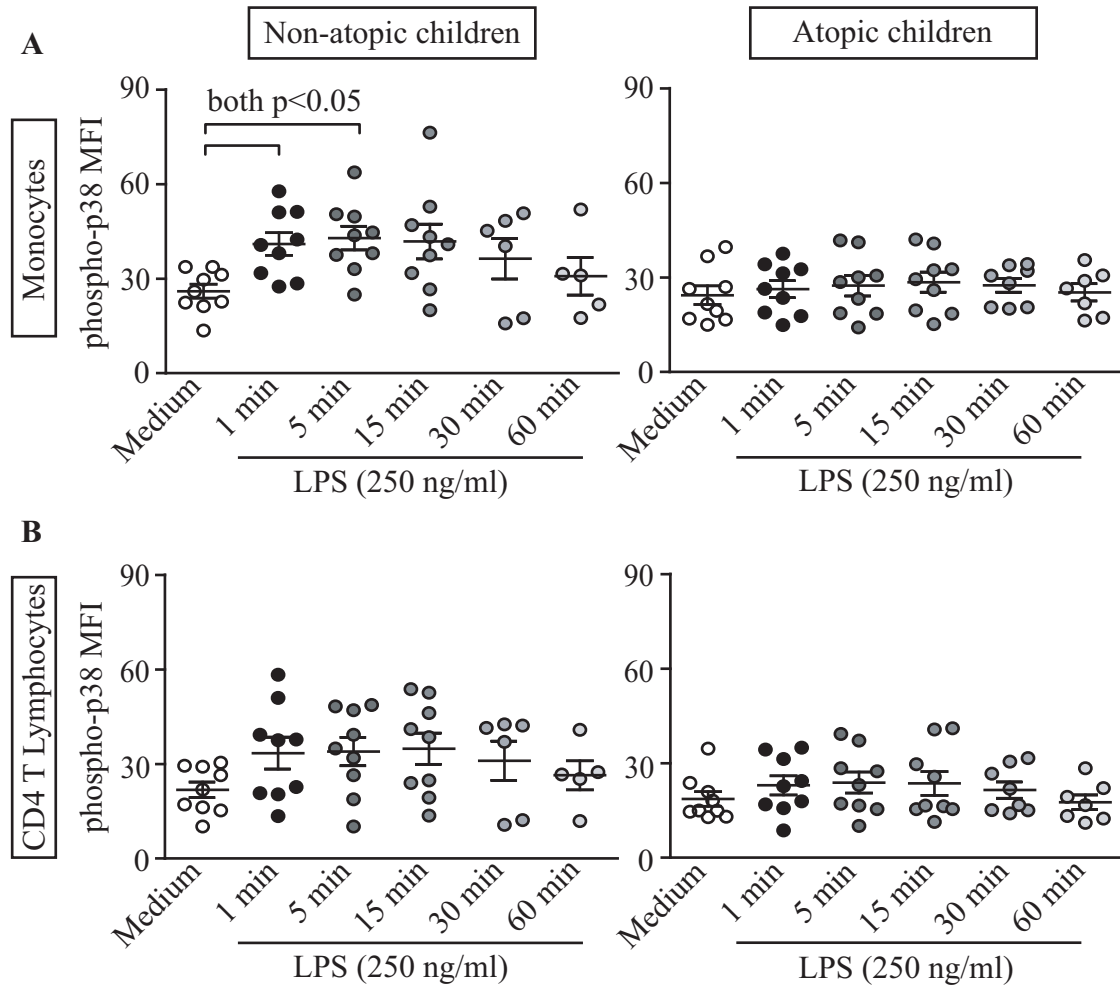


Figure 6. LPS-induced p38 MAPK phosphorylation is impaired in CD4+cells from children with histories of atopic diseases. Cultured PBMC were stimulated with 250 ng/ml LPS for 1 to 60 min, and then stained for CD4 and intracellular phospho-p38 MAPK and analyzed by flow cytometry. Data are presented as median fluorescence intensity (MFI) of phospho-p38 MAPK detected in (A) CD4+Low monocytes and (B) CD4+High T ‘helper’ lymphocytes from non-atopic (left graphs; $n = 9$; age: 14.0 ± 5.5) and age-matched children with atopic histories (right graphs; $n = 9$, age: 13.9 ± 9.5).

CD4⁺High T 'helper' cells, regardless of the atopic status of subjects (Figure 6c, bottom graphs). In conclusion, CD4⁺Low monocytes from children with atopic histories appeared relatively insensitive to the effects of LPS with respect to p38 activation, compared cells from age-matched non-atopic controls.

We then sought to verify the relative LPS unresponsiveness of atopic monocytes compared to non-atopic cells by testing the activation of another signalling pathway. The difference in LPS-induced p38 MAPK phosphorylation between monocytes of non-atopic and atopic children was then confirmed through the assessment of p^{42/44}ERK phosphorylation (Figure 7). The CD4⁺Low monocytes from non-atopic children exhibited significantly increased intracellular phosphorylated p^{42/44}ERK from 1-5 min after LPS exposure (Figure 7a, left graph). Again, these results contrasted with data obtained with monocytes from individuals with atopic histories (Figure 7a, right graph). In line with the p38 signalling data (Figure 6b), LPS also failed to increase the phosphorylation of p^{42/44}ERK in CD4⁺High T lymphocytes, regardless of atopic status (Figure 7b). Interestingly, culturing PBMC from non-atopic children for 1h with IL-4-supplemented medium markedly attenuated LPS-induced p^{42/44}ERK phosphorylation (Figure 7c). This effect was specific since normal p^{42/44}ERK phosphorylation was observed when recombinant IL-4 was pre-incubated for 1 hour with an excess of IL-4 neutralizing antibody (data not shown, n=2). However, culturing cells from children with atopic conditions with exogenous anti-

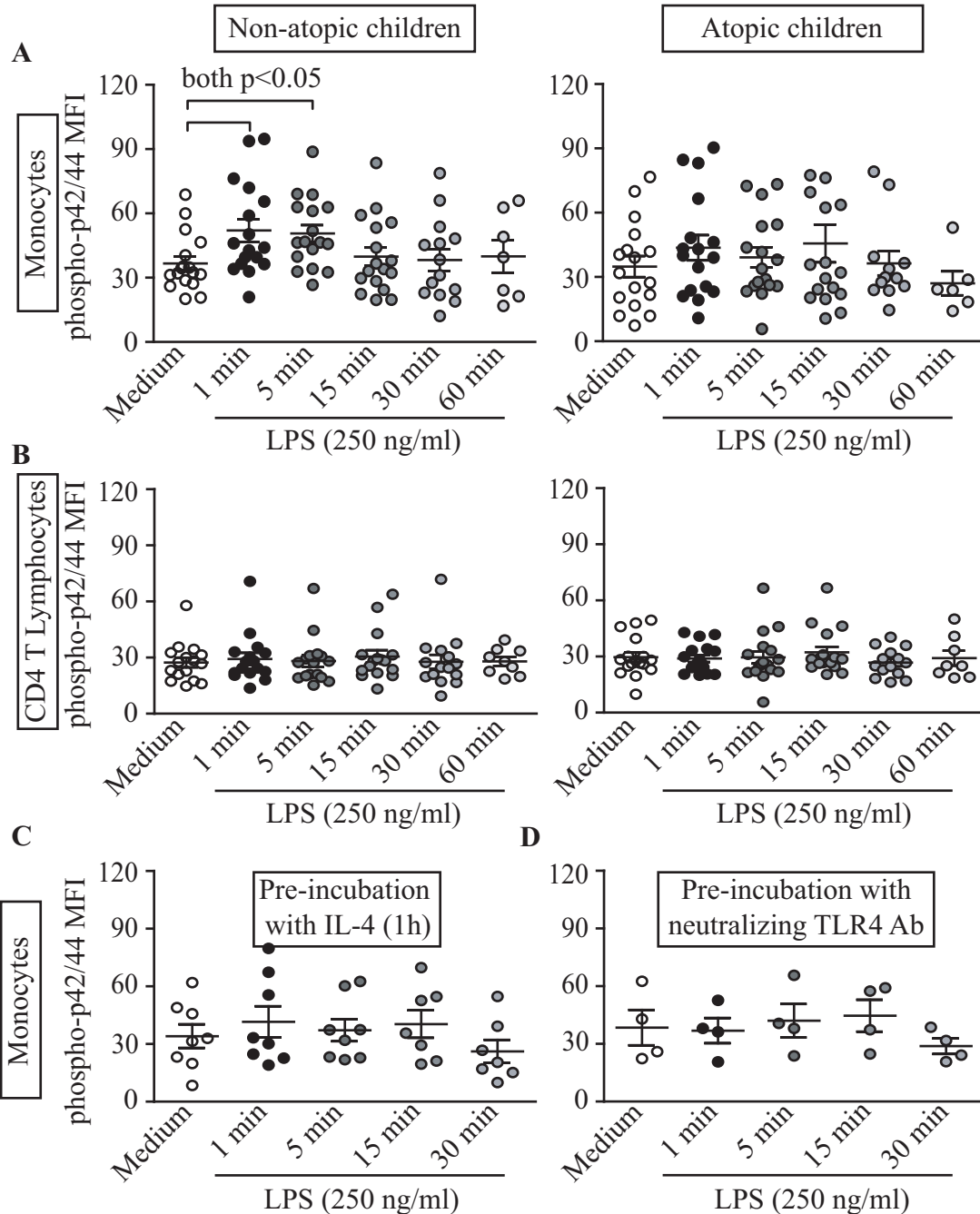


Figure 7. Impaired LPS-induced p42/44 ERK MAPK phosphorylation in CD4+ cells from children with atopic histories. PBMC pre-incubated with/without IL-4 were stimulated with 250 ng/ml LPS for 1 to 60 min, and then stained for CD4 and intracellular phospho-p42/44 ERK MAPK and analyzed by flow cytometry (section 2.2.9). Data are presented as MFI of phospho-p42/44 ERK MAPK detected in (A) CD4+Low monocytes and (B) CD4+High T ‘helper’ lymphocytes from non-atopic (left graphs; $n=16$; age: 12.1 ± 3.5) and age-matched children with atopic histories (right graphs; $n=17$, age: 12.4 ± 5.4). (C) Monocytes from non-atopics pre-incubated with recombinant IL-4 ($n=8$), or (D) TLR4 neutralizing antibody for 1h prior to LPS exposure ($n=4$).

IL-4 antibodies did not reverse the impaired LPS-induced phosphorylation (data not shown, n=2). The LPS-mediated effect on p^{42/44}ERK phosphorylation in non-atopic monocytes was TLR4-dependent since it was inhibited by pre-incubation with TLR4 neutralizing antibody (Figure 7d), not by an isotype antibody (data not shown, n=2). Taken together, these results demonstrate that LPS can activate at least two major signal transduction pathways downstream of TLR4 in CD4-expressing peripheral monocytes from non-atopic, but not in cells from children with a history of atopy.

3.1.2.6. A history of atopy in children associates with impaired LPS-induced decrease in TLR4 expression

It was previously reported that LPS stimulation induces rapid internalization of both LPS and TLR4 (759-761). We therefore assessed whether LPS could differentially affect TLR4 expression present at the surface of CD4⁺ leukocytes from age-matched children with and without history of atopy. Interestingly, LPS induced a stable and consistent decrease in surface expression of TLR4 in monocytes from non-atopic donors, but not consistently and either significantly in monocytes from children with atopic histories (Figure 8a vs. b). This phenomenon was independent on the LPS dose, between 50 and 5000 ng/ml. The LPS-mediated decrease in TLR4 surface expression was prolonged for 72h (Figure 8c), and was accompanied by a down-regulation of TLR4 mRNA in the whole PBMC fraction as reported elsewhere (981) (Figure

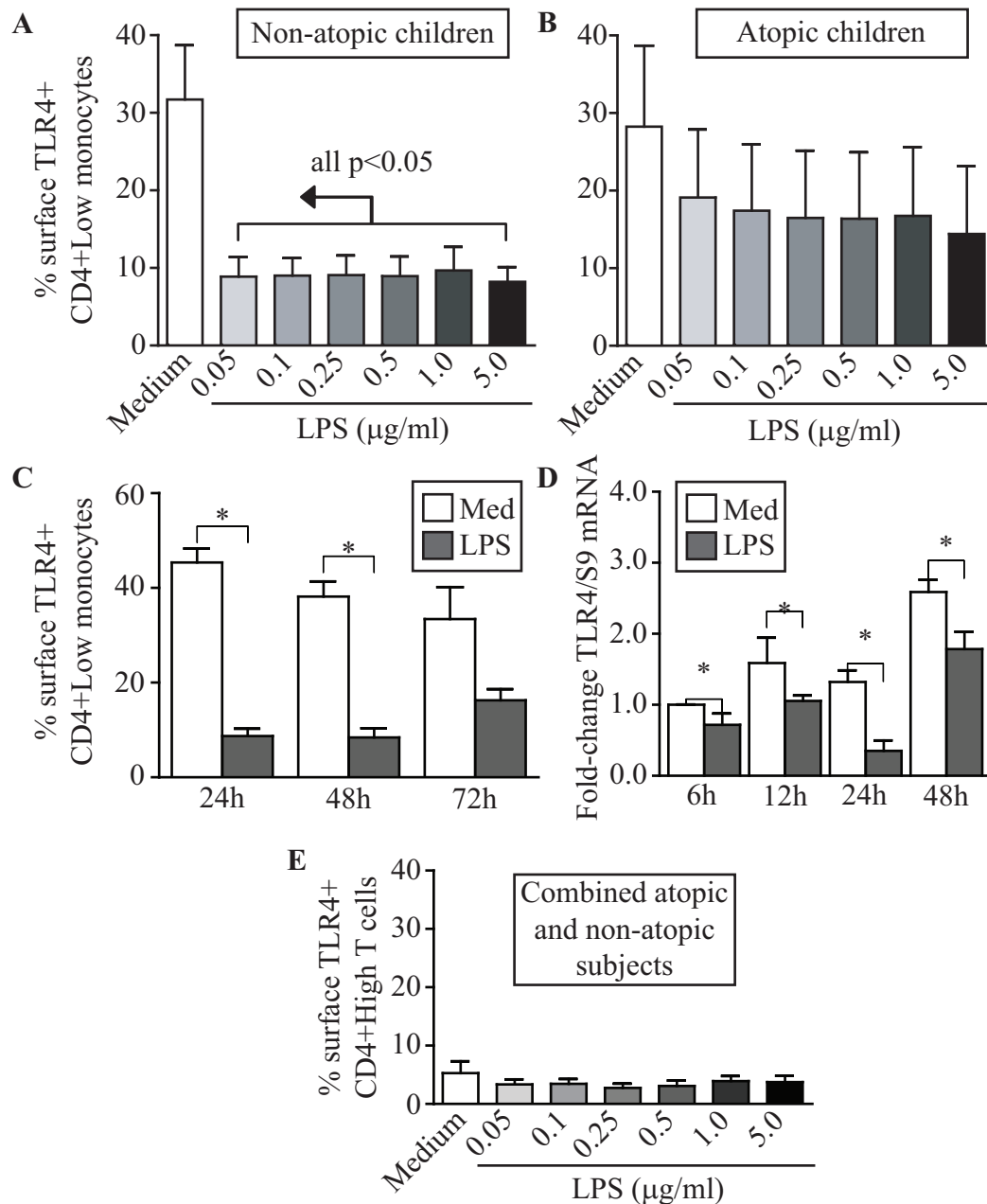


Figure 8. LPS decreases surface TLR4 expression only monocytes from non-atopic, not in those from atopic children. PBMC were exposed to LPS (0.05-5 μg/ml) for 24h followed by flow cytometric analysis of CD4/TLR4 expression. LPS-induced decrease in TLR4 expression was significantly greater in monocytes from non-atopic children (A) (n=4; age: 9.0±2.9) compared with cells from children with atopic histories (B) (n=5; age: 10.4±4). In C, LPS-induced decrease in TLR4 surface expression (C) and was TLR4 mRNA (D) for about 72h and 48h in non-atopic monocytes (n=4). LPS fails to reduce TLR4 surface expression in CD4+ T 'helper' lymphocytes, regardless of atopic status (n=7). Data are presented as mean±SEM and p-values < 0.05 were considered significant (*).

8d). LPS failed to significantly alter the lower TLR4 surface expression on T 'helper' lymphocytes, regardless of atopic status (Figure 8e). In summary, peripheral CD4⁺Low monocytes from both children with and without atopic histories display significant TLR4/CD14 expression (Figure 1f, 2 and Figure 4), compared with T 'helper' lymphocytes. However, only the monocytes from non-atopic children displayed significant LPS-induced signalling (Figure 6a, and 7a) and LPS-mediated reduction in TLR4 surface expression (Figure 8a vs. b) compared with atopic cells. These data support the notion that leukocytes, especially monocytes, from children with histories of atopic conditions display defective LPS responsiveness.

3.1.2.7. A history of atopy in children associates with impaired LPS-induced IL-10 gene expression from peripheral leukocytes

We finally sought to confirm if the impaired LPS-induced signal transduction (through MAPK-dependent pathways) in peripheral CD4-expressing leukocytes from children with histories of atopic diseases has an impact on the level of the induction of a well-described mediator released upon LPS exposure. Previous studies from our laboratory suggested that leukocyte-derived IL-10 would play a more prominent role at reducing Th2-type immune responses than IL-12, based on significantly greater numbers of IL-10-expressing cells compared with IL-12-expressing cells in LPS-stimulated nasal mucosa from children (736). We then decided to stimulate PBMC from children with/without atopic histories for

24h with LPS and to evaluate the IL-10 mRNA transcript levels by RT-qPCR using cDNA from both whole PBMC and CD4⁺ cell fractions isolated immediately after stimulations. As depicted in Figure 9a, PBMC stimulated with LPS (0.25 µg/ml) exhibit significantly up-regulated IL-10 mRNA expression levels, which contrasted with the concomitantly poor IL-10 induction in cells originating from age-matched children with histories of atopic diseases. We then compared the relative contribution of isolated CD4⁺ cell fractions to the LPS-induced IL-10 up-regulation in cell fractions prepared from atopic and non-atopic PBMC (Figure 9b). Interestingly, LPS slightly but not significantly up-regulated IL-10 mRNA levels among isolated CD4⁺ cells, independently of the subject's atopic status. These LPS-induced cytokine expression data in different cell fractions suggest that differential LPS-mediated IL-10 response of atopic and non-atopic subject's PBMC likely reflects the difference in LPS-induced signalling among monocytes from atopic and non-atopic children.

3.2. Transition

In the previous sub-chapter, we studied a cross-sectional paediatric cohort and demonstrated that circulating monocytes from children with a history of atopic diseases have impaired LPS responsiveness and decreased TLR4 expression with age. Furthermore, studies in our laboratories documented the colocalization of TLR4 on CD3⁺ T lymphocytes and CD4⁺ cells in the nasal mucosa of atopic and non-atopic children and adults (736, 888). Interestingly, the

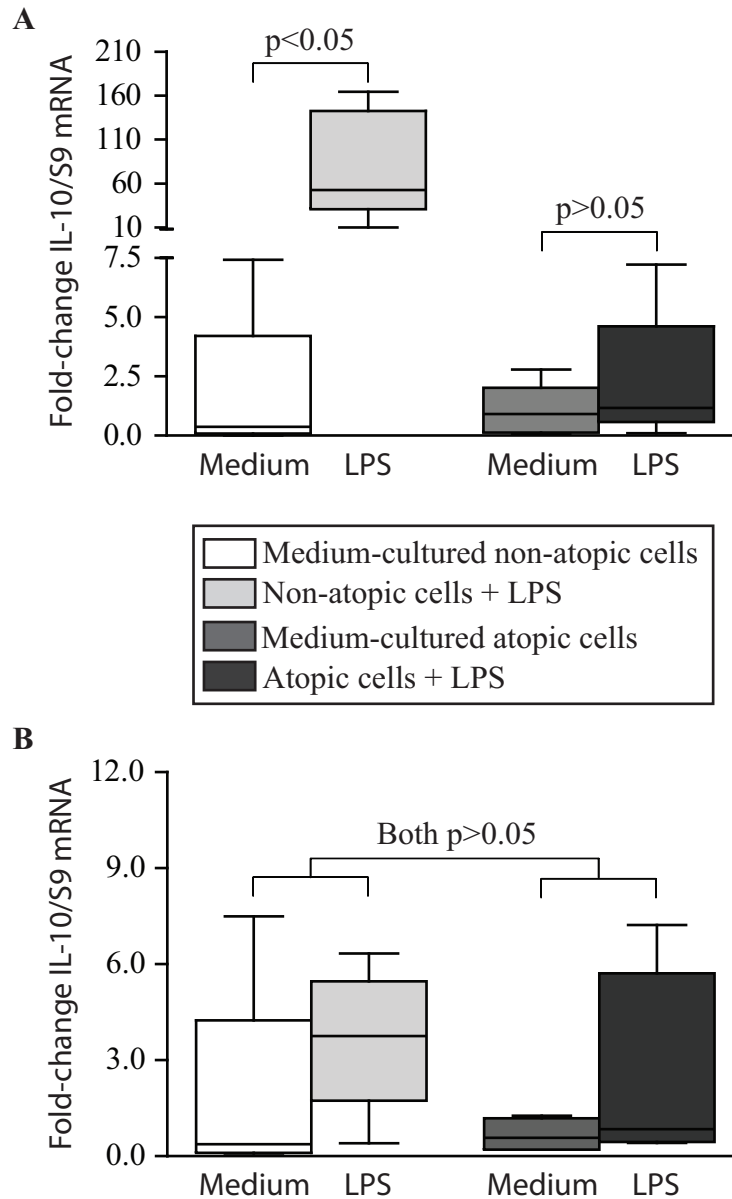


Figure 9. LPS differentially induces IL-10 in PBMC from non-atopic or atopic children: a possible reflection of impaired LPS-induced signalling of monocytes in atopy. PBMCs from age-matched non-atopic children and atopic children (n=5 per group) were stimulated with LPS for 24h (0.25 μ g/ml). In A, PBMC were lysed and processed for IL-10 mRNA quantification by RT-qPCR (n=5 per group). In B, CD4⁺ cells were isolated from PBMCs by immunomagnetic bead sorting prior to cell lysis and IL-10 gene expression profiling by RT-qPCR (n=4 per group). Data are presented as medians $\pm$ SEM, and were analyzed by ANOVAs (p-values < 0.05 considered significant).

TLR4 expression in nasal mucosa tissues was reduced in atopic patients compared with non-atopic adults suggesting that on-going disease may affect TLR4 expression *in situ*. Therefore, we sought to further investigate the *in situ* expression and regulation of TLR4 in nasal mucosa tissue specimens obtained from adult subjects with and without diagnosed allergic rhinitis.

Adult subjects undergoing endoscopic intra-nasal surgery were then enrolled with consent, and nasal mucosa tissue specimens were excised from the inferior turbinate, as described in the Materials and Methods section (sections 2.3.1 and 2.3.2). The subject's characteristics are presented in Table 8. The tissues were initially processed for histological evaluation of TLR4 and the presence of macrophages by immunocytochemistry. Infiltrating CD68-expressing macrophages were found in the nasal submucosa of both allergic rhinitis (Figure 10a) and control subjects (Figure 10b). There also seemed to be an increase in

Subject's characteristics	Normal subjects	Allergic rhinitis subjects
n (M:F)	7 (5:2)	10 (9:1)
Age (median and range)	35 (23-51)	36.5 (21-82)
Skin prick tested (yes:no)	6:1	9:1
Positively skin prick tested with allergen extracts (n)	None	Ragweed, tree or birch pollen (8), house dust mite (6), cat dander (2), dog dander (1)

Table 8: Subject's characteristics for the nasal mucosa study.

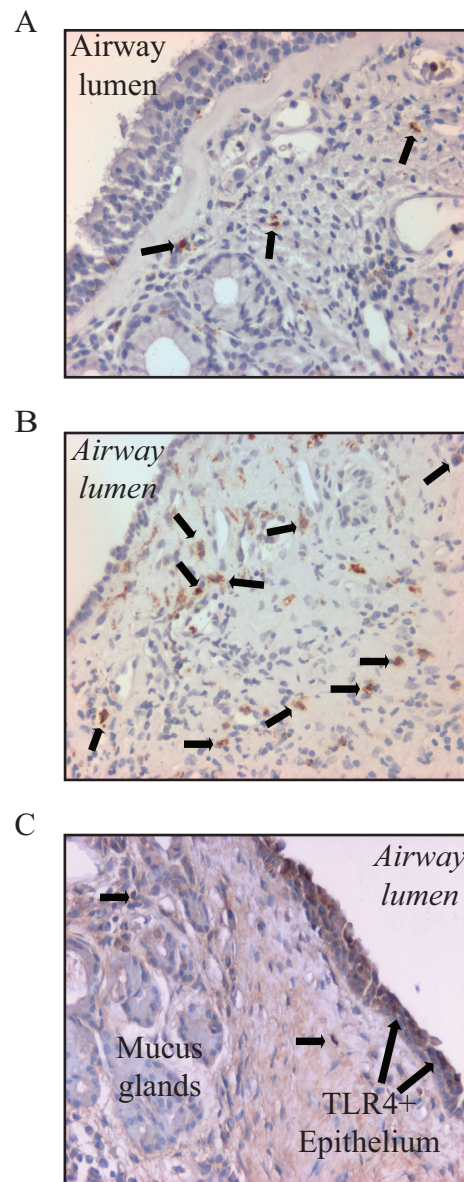


Figure 10. Identification of macrophages and TLR4-expressing cells in the nasal submucosa of non-atopic adults. Nasal mucosal tissue specimens were obtained from adults undergoing intranasal surgery and process for immunocytochemical analysis of CD68-expressing macrophages (A and B) and TLR4-expressing cells and using specific reagents; namely anti-human CD68 antibody and biotinylated LPS molecules, as described in the Materials and section 2.3.3. In A and B, representative micrographs depict CD68⁺ macrophages in nasal tissues of control and allergic rhinitis subjects, respectively. In B, TLR4-expressing cells in the nasal mucosa of non-atopic subject. The epithelial cell layer appears as the main TLR4-expressing structure.

the numbers of infiltrating macrophages in tissues from allergic rhinitis subjects compared to controls, as previously published by several groups (55, 128).

Tissue sections stained with isotype control antibodies displayed an absence of staining (data not shown). We then stained sections of nasal mucosa specimens from normal non-atopic adult subjects for the expression of the TLR4 protein (Figure 10c), using biotinylated LPS molecules. We realized that the majority of cells expressing the TLR4 protein were those forming the epithelial layer lining the airway lumen. However, these immunostaining results obtained with the biotinylated-LPS molecules needed to be confirmed using a TLR4 antibody, as described further. We therefore decided to focus on the epithelial expression of TLR4 as a more biologically relevant model representing the LPS-responsive cells in the airways of subjects with/without allergic diseases. In addition, the airway epithelium rather consists of the first structure to encounter inhaled LPS *in situ*, in contrast with submucosal cells (736).

3.3. Regulation of TLR4 in airway epithelial cells in asthma and rhinitis: the effect of Th2-type conditions and potential role for epithelial GATA-3 transcription factor

3.3.1. Introduction

Allergic asthma and rhinitis are both characterized by T 'helper' type 2-mediated i.e. Th2-type inflammation. Epithelial cells of the airways form the first line of defence against inhaled particles including pollutants, microbes and

allergens. However, the epithelium is no longer seen as a passive cell layer lining the airway lumen but rather as a major contributor to mucosal immunity. The airway epithelium contributes to the Th2-type inflammation seen in allergic diseases as they produce several pro-inflammatory mediators (1010), and display altered transcriptome (1011). Such phenotypic changes of airway epithelial cells in asthma are also mirrored by elevated expression of the signal transducer and activator of transcription 6 (STAT6), which drives the downstream effects of IL-4 / IL-13 signalling (343, 354). Epithelial cell-specific STAT6 expression was associated with chemokine production, mucus production and airway hyperresponsiveness in experimental murine asthma (343, 354, 356, 1012). In addition to STAT6, GATA binding protein-3 (GATA-3) is another important transcription factor regulating Th2-type immune responses (1013). Although it is considered mainly as a T cell-specific factor, GATA-3 expression was reported in other cells (like eosinophils and mast cells) (208, 1014) and organs during embryogenesis (1015). GATA-3 is expressed by skin keratinocytes (1016) and bronchial epithelial cells (345), but we currently have a poor understanding of GATA-3 regulation and function in epithelial cells.

Epithelial cells express a large number of receptors including Toll-like receptors (TLRs) which recognize pathogen-associated molecular patterns (745, 1017, 1018). TLR4 recognizes bacterial lipopolysaccharide (LPS, also called endotoxin) and is expressed by numerous resident and immune cells of the

airways, including epithelial cells. Epidemiological studies supportive of the hygiene hypothesis suggested that exposure to LPS early in life limits the development of allergic diseases (859, 1019). In experimental models, LPS was reported to either exacerbate or to limit allergic inflammation (736, 899, 900, 903-905, 919, 936) (Section 1.3.4.5). Interestingly, several TLR4-mediated immunoregulatory mechanisms were studied in epithelial cells, which appear to play roles in both immuno-suppressive (1020), or pro-inflammatory processes (1021). Interestingly, studies have associated allergic diseases and Th2-type microenvironment with impaired innate immune functions. Bronchial epithelial cells from asthmatic subjects display defective responses to virus infection (341, 1022), while immune cells from similar patients exhibit impaired cytokine responses to TLR ligands including LPS (734). Furthermore, intestinal epithelial cells exposed to Th2-type cytokines also display reduced TLR4 expression (971). The number of TLR4-immunoreactive cells in nasal mucosa tissue sections from non-atopic adults was reduced compared with tissues from atopic adults, both at baseline and after LPS exposure *in vitro* (736). Studies on monocytes reported that IL-4-induced STAT6 activation inhibits, but not fully abrogates, TLR4 expression (970, 972). Furthermore, our previous results showed that aging reduces the expression of TLR4 on peripheral monocytes only in subjects with atopic histories, not in non-atopic controls. These results suggest that on-going

atopy and allergic diseases may, especially in adults, be reflected by impaired TLR4 expression.

The objective of this study is to determine if Th2-type cytokines and/or Th2-type transcription factor GATA-3 (negatively) regulate TLR4 expression in airway epithelial cells of adult subjects with on-going airway allergic diseases (rhinitis and asthma), compared with controls.

3.3.2. Results

3.3.2.1. Decreased TLR4 expression by nasal epithelial cells in subjects with allergic rhinitis compared with controls

We first sought to compare the expression of TLR4 histologically, in nasal mucosa tissue specimens collected from atopic subjects with allergic rhinitis and non-atopic controls. As depicted in Figure 11a, both infiltrated leukocytes and the nasal epithelium of non-atopic controls exhibited significant immunoreactivity using rabbit anti-human TLR4 antibody. The TLR4 immunoreactivity mainly localized in the intracellular compartment, which corroborates previous observations (738). In contrast with tissues from non-atopic controls (Figure 11a), the epithelial cell layer of tissues from allergic rhinitis subjects displays markedly reduced TLR4 immunoreactivity (Figure 11b). When blindly scored, the intensity of epithelial TLR4 immunoreactivity in histological sections from allergic rhinitis subjects was indeed significantly decreased compared with those from non-atopic controls (Figure 11c, $p < 0.05$). These histological observations were

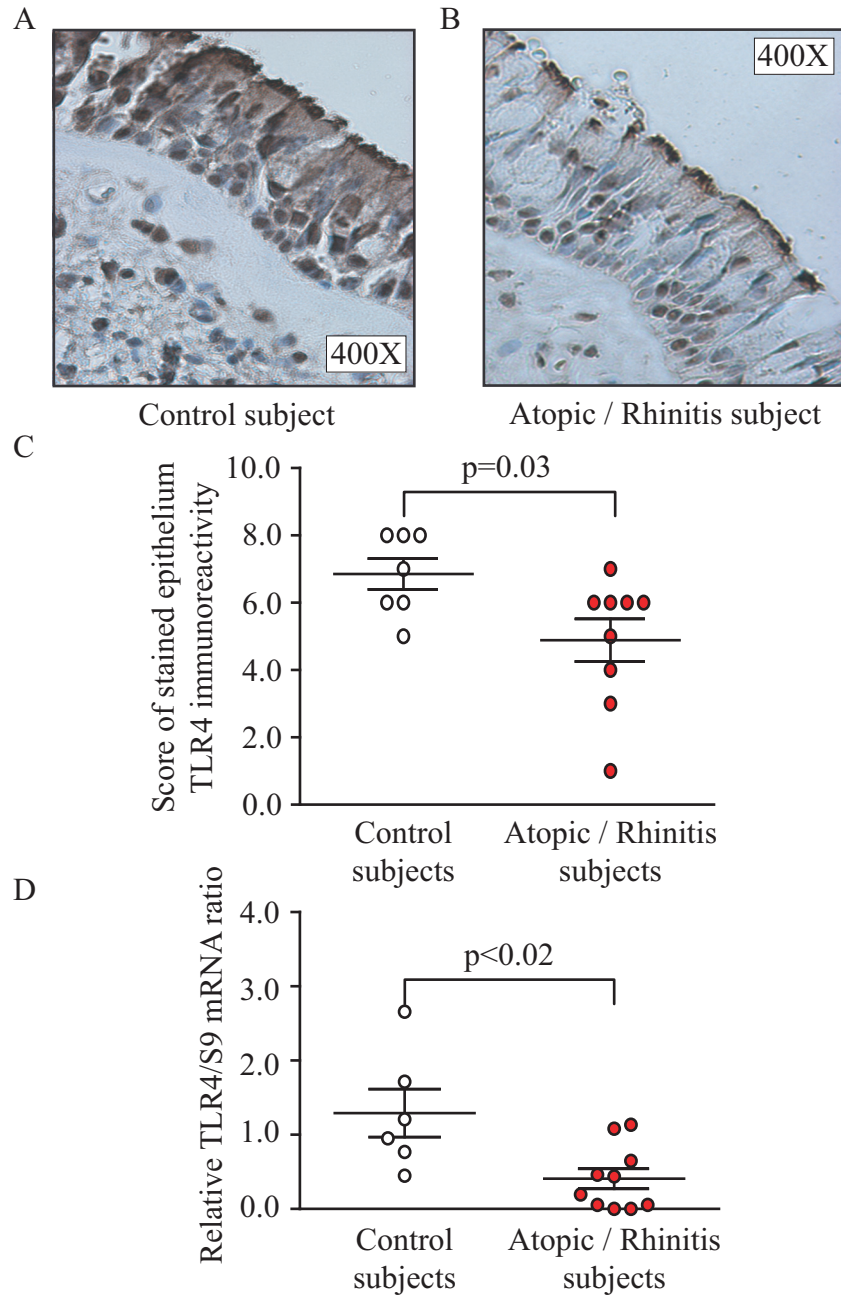


Figure 11. TLR4 expression by airway epithelial cells of the nasal mucosa is reduced in subjects with allergic rhinitis compared with non-atopic controls. Representative immunostaining for TLR4-expressing cells in nasal mucosa tissue using an anti-TLR4 antibody by immunocytochemistry: sections from healthy non-atopic (A) and from atopic / allergic rhinitis subjects (B). In C, percentages of TLR4-expressing epithelium were determined semi-quantitatively by blinded scorer using scores from 0 to 10, as detailed in the section 2.3.3. In D, TLR4 mRNA levels (normalized to S9 reference transcripts) were quantified by RT-qPCR using cDNA generated from homogenized nasal mucosa tissue specimens from non-atopic and atopic/allergic rhinitis subjects (n=6 and 10, respectively).

confirmed by RT-qPCR using cDNA samples generated from homogenized nasal tissue specimens: the TLR4 gene transcript levels (normalized to the S9 reference gene) is significantly lower in the nasal tissues from allergic rhinitis subjects compared to non-atopic controls (Figure 11d; $p < 0.02$). The normalized mRNA levels for other components of the LPS receptor complex, namely CD14, MD2 and MyD88, remained unchanged in both groups ($p = 0.23$, 0.93 and 0.82 , respectively; data not shown).

3.3.2.2. Assessment of Th2-type inflammatory profile in nasal mucosa of subjects with allergic rhinitis compared with controls

We then sought to characterize the inflammatory profile of the nasal mucosa from rhinitis subjects compared with controls (Figure 12 and Figure 13). In nasal tissues from subjects with allergic rhinitis, we observed massive infiltration of the subepithelium by mononuclear cells compared with the non-atopic control subjects, as depicted by H&E staining (Figure 12a). We also confirmed elevated numbers of CD4-immunoreactive cells (124) as well as the presence of CD4-expressing T 'helper' lymphocyte follicles in tissues from the rhinitis subjects, in contrast with those from controls (Figure 12b). Nasal mucosa sections from allergic rhinitis subjects also displayed increased numbers of infiltrating EG2-positive eosinophils compared to controls (Figure 12c and 12d; $p < 0.0001$). Using cDNAs generated from homogenized nasal mucosa tissues for gene expression profiling, we confirmed increased IL-5 and IL-4 mRNA levels in

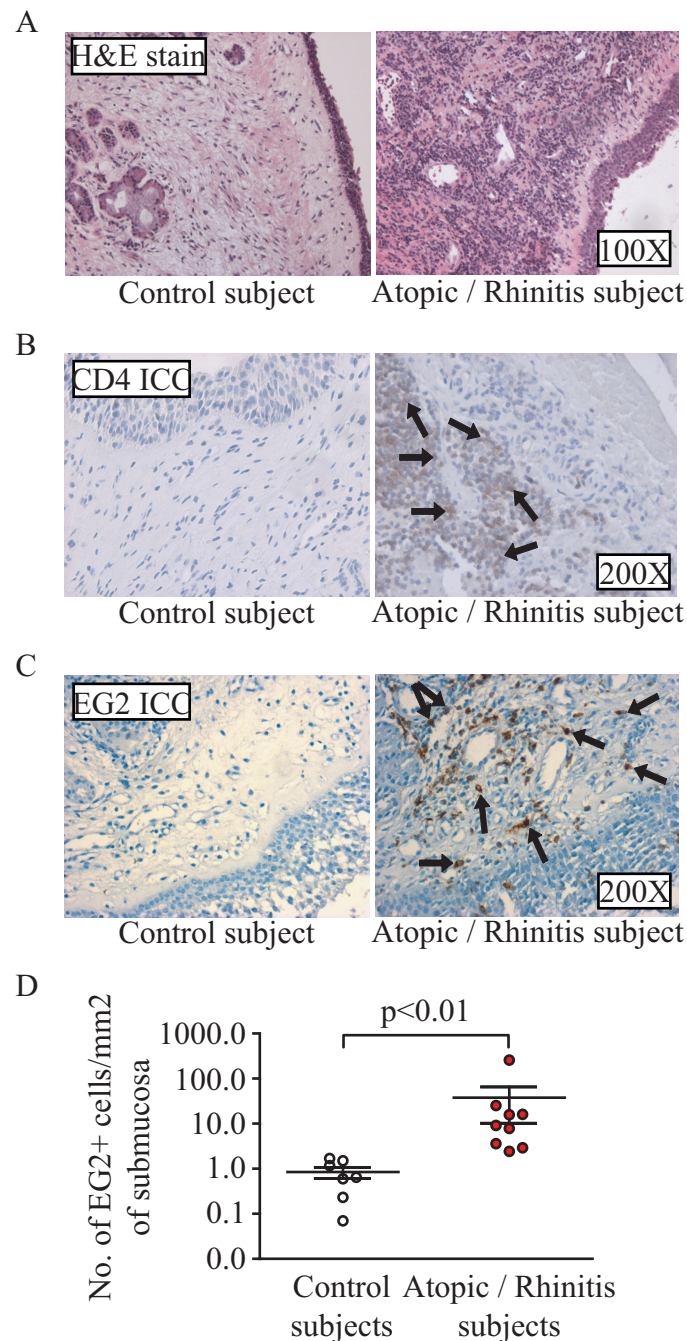


Figure 12. Histological characterization of inflammatory profiles in nasal mucosa biopsies from allergic rhinitis subjects compared with non-atopic controls. Biopsy sections obtained from the inferior turbinate of 7 non-atopic and 10 allergic rhinitis subjects sections were processed for H&E (A) and immunocytochemical staining detecting CD4 and eosinophil cationic protein (EG2) immunoreactive cells (B and C, respectively). D, the numbers of EG2+ eosinophils per squared mm of subepithelial surface were increased in rhinitis patients compared with non-atopic controls, as assessed by a blinded scorer ($p<0.05$).

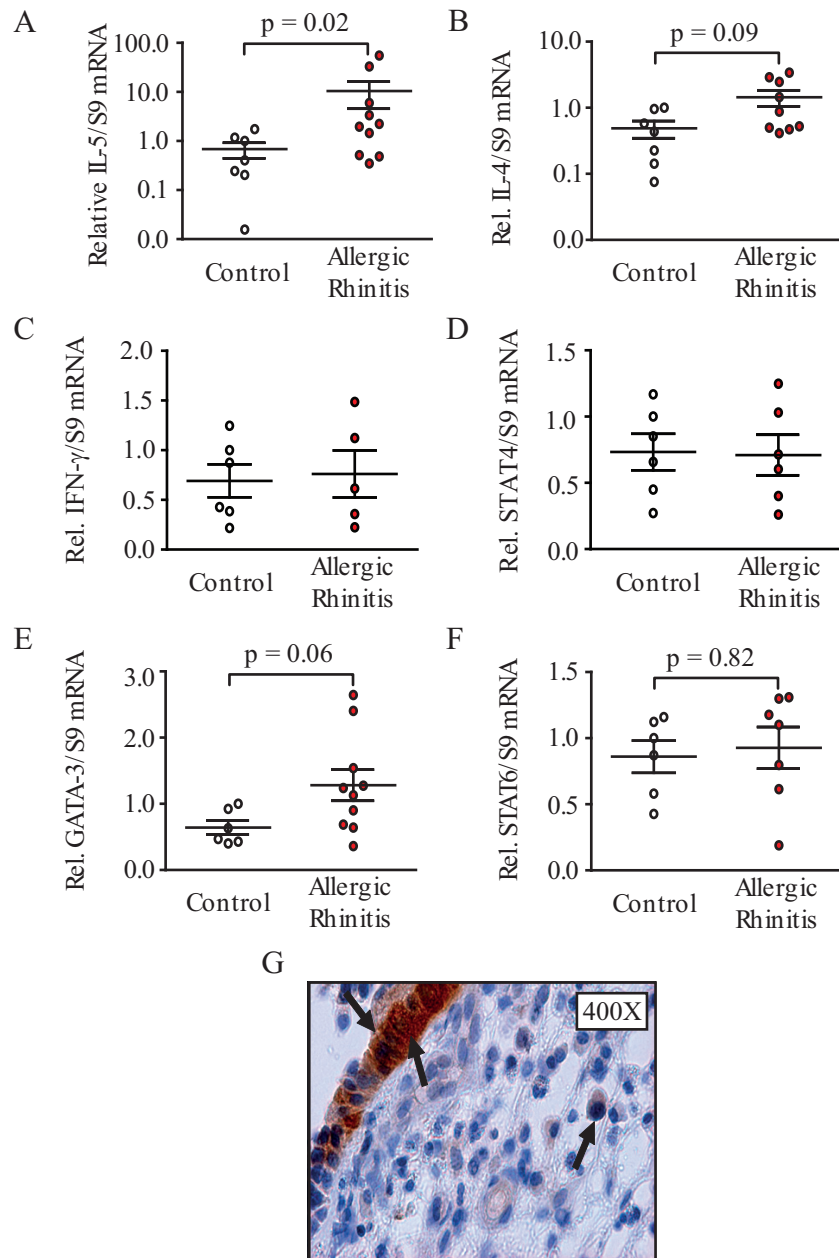


Figure 13. Characterization of inflammatory profiles in nasal mucosa biopsies from allergic rhinitis subjects compared with non-atopic controls, by RT-qPCR. RNase-free frozen nasal mucosa tissue specimens were homogenized and processed for gene expression profiling by RT-qPCR for IL-5 (A), IL-4 (B), IFN- γ (C), STAT4 (D), GATA-3 (E) and STAT6 (F) mRNA levels, normalized to S9 reference gene transcripts ($n \geq 6$ and up to 10 for non-atopic and rhinitis subjects, respectively). Data were analyzed using non-paired t tests, and p-values < 0.05 were considered statistically significant. In E, immunocytochemical evidence of GATA3 immunoreactivity by both epithelial cells and infiltrating mononuclear cells (arrows) in the nasal mucosa.

nasal tissue from atopic / rhinitis subjects, compared with non-atopic controls ($p=0.02$ and $p=0.09$; Figure 13a and b, respectively). In contrast with Th2-type cytokine transcript levels, the expression of the Th1-type cytokine IFN- γ mRNA were similar between nasal tissue from atopic / rhinitis subjects and with non-atopic controls (Figure 13c, $p=0.93$). When examining the expression of the mRNA levels of Th1- (STAT4) and Th2-type (GATA-3 and STAT6) transcription factors (Figure 13d to f, respectively), only a trend towards higher GATA-3 transcript levels was seen in rhinitis compared to control subjects ($p=0.06$) whereas no changes were seen in STAT4 ($p=0.94$) or STAT6 ($p=0.53$). Characterizing the GATA-3-expressing cells by performing immunocytochemistry of nasal tissue sections from rhinitis patients, we confirmed the presence of GATA-3 immunoreactivity not only in infiltrating immune cells (208), but also by epithelial cells lining the airways (Figure 13g) (345). Taken together, the previous results documented prominent Th2-type allergic inflammation but reduced TLR4 expression by nasal epithelial cells of subjects with allergic rhinitis compared with controls.

3.3.2.3. Opposite GATA-3 and TLR4 expression in bronchial epithelial cells from asthmatic and control subjects

We then assessed whether there was an inverse association between TLR4 and GATA-3 expression, using airway epithelial cells from patients with another Th2-type disease model, namely allergic asthma. Therefore, we obtained

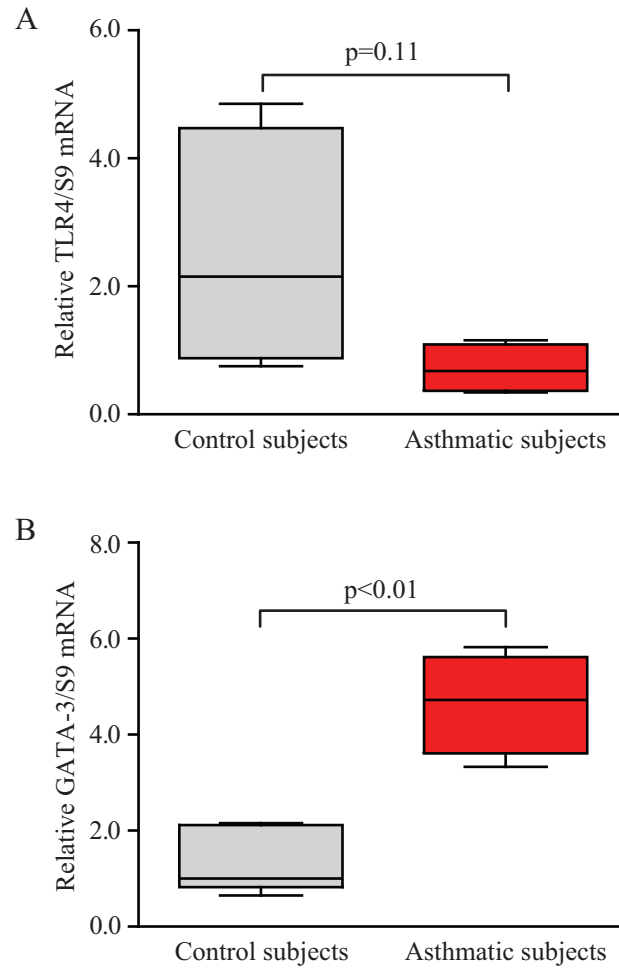


Figure 14. Opposite regulation of TLR4 and GATA-3 mRNA transcript levels in primary bronchial epithelial cells isolated from bronchial biopsy tissues obtained from subjects with or without asthma. The protocol followed for cell isolation is detailed in the Materials & Methods section 2.3.4, and transcript levels for TLR4 and GATA-3 were assessed by RT- and real-time quantitative PCR. Target gene transcript levels were normalized to those of S9 reference gene. Data from control and asthmatic subjects (n=5 and 4) were compared through non- paired t tests and p-values<0.05 were considered statistically significant.

primary bronchial epithelial cells isolated from bronchial tissues of subjects with allergic asthma and non-asthmatic controls (n=4 and 5 subjects per group, respectively) (999). These isolated cells were used to quantify concomitant expression of both TLR4 and GATA-3 genes by RT- and real-time quantitative PCR. The baseline TLR4 gene transcript levels in cultured bronchial epithelial cells from subjects with asthma were reduced compared to those from healthy controls, although the difference did not reach statistical significance (Figure 14a; $p=0.11$). In contrast, baseline GATA-3 mRNA levels of *in vitro* cultured bronchial epithelial cells from asthmatic subjects were significantly increased compared with cells of non-asthmatic subjects (Figure 14b; $p<0.01$). GATA-3 being a master transcription factor driving Th2-type immunity, these data suggest that elevated epithelial GATA-3 expression may reflect pro-allergic phenotypic alterations encountered in the asthmatic bronchi, but not in controls. These results also raised the question whether GATA-3 contributes to limiting TLR4 expression in the allergic patients.

3.3.2.4. IL-4 increases GATA-3 but decreases TLR4 expression in cultured epithelial cells

We then sought to establish the use of cultured airway epithelial cells as *in vitro* models to study the counter-regulation of TLR4 and GATA-3 under pro-allergic conditions. We first cultured commercially available immortalized bronchial epithelial (BEAS-2B) cells and normal human bronchial epithelial

(NHBE) cells and confirmed GATA-3 expression at the mRNA (Figure 15a) and protein levels (Figure 15b), therefore corroborating results reported by others (345). We further sought to test the ability of the Th2-type cytokine IL-4 to regulate GATA-3 expression in NHBE and demonstrated IL-4-induced dose-dependent increase in GATA-3 mRNA (Figure 15c), with IL-4 doses ranging from 1 to 100 ng/ml. Cultured NHBE were also incubated for 24h in the absence (Figure 15d) or presence of 10 ng/ml recombinant IL-4 (Figure 15e), followed by fluorescent immunostaining using AlexaFluor 555-labeled anti-GATA-3 antibody, or an isotype control antibody (Figure 15d insert). IL-4 exposure increased in GATA-3 protein immunoreactivity (depicted in red) in NHBE stimulated for 24h compared with medium-treated cells (Figure 15e and d, respectively). Part of these results were reproduced with permission from a previous publications (289) (see Research compliance certificates and permissions).

Cytokine-mediated regulation of GATA-3 and TLR4 was further characterized in NHBE and BEAS-2B culture experiments (Figure 16 and 17, respectively). In both cell types, IL-4 up-regulated GATA-3 mRNA in a time-dependent manner, but more strikingly in NHBE cells (Figure 16a and 17a, respectively). In contrast, exposing bronchial epithelial cells to IL-4 significantly down-regulated TLR4 transcript levels (Figure 16b and 17b). As a positive control in these experiments, stimulation of epithelial cells with the Th1-type cytokine IFN- γ had opposite effects as it respectively tends to decrease GATA-3 (Figure

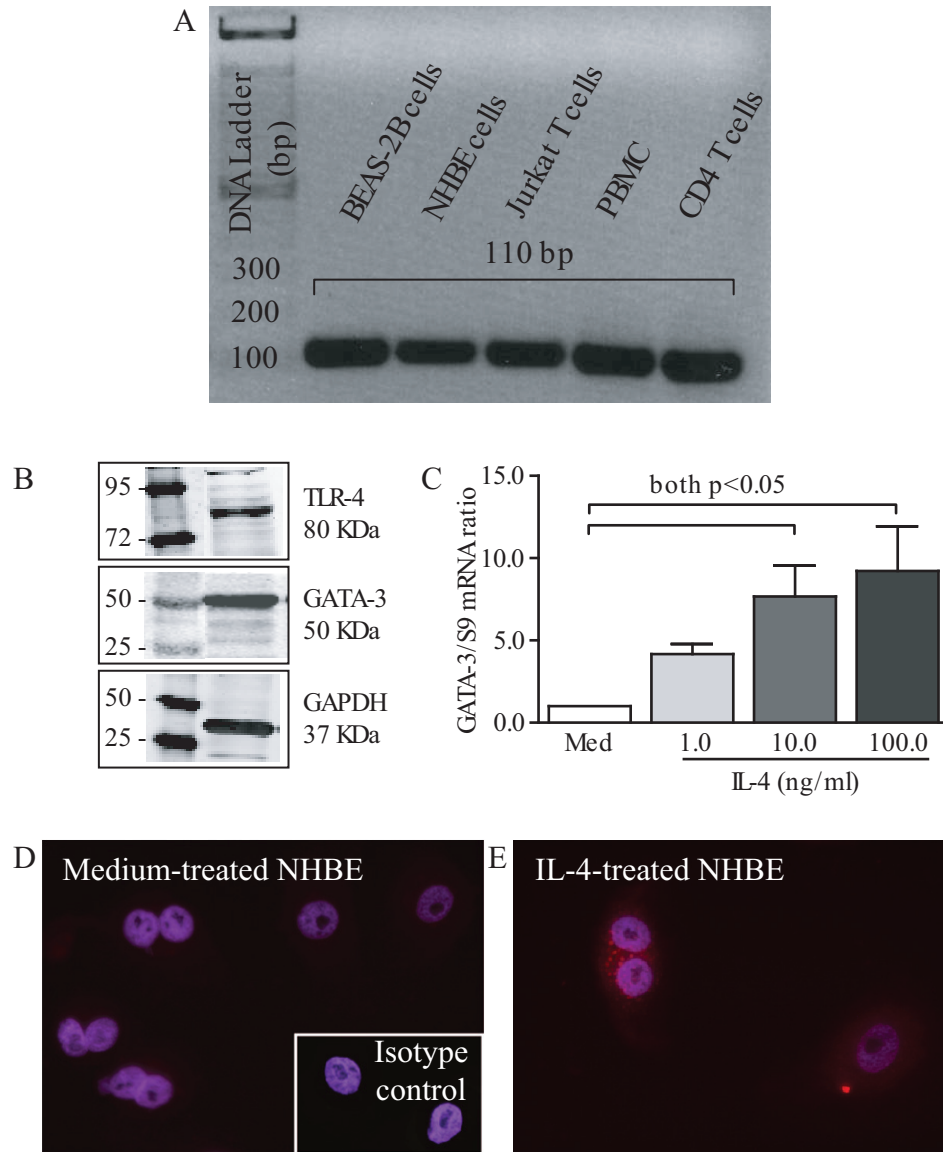


Figure 15. Cultured bronchial epithelial cells served as an *in vitro* model for studying the GATA-3 and TLR4 expression under Th2-type conditions. In A, the human bronchial epithelial cell line BEAS-2B and primary normal human bronchial epithelial cells (NHBE) expressed GATA-3 mRNA, along with the Jurkat T cells, PBMC and CD4⁺ cells from a control subject. In B, GATA-3 and TLR4 and GAPDH protein expression in NHBE lysates was tested by immunoblotting. Recombinant IL-4 dose-dependently increases normalized GATA-3 mRNA in NHBE cells stimulated for 24h (C). IL-4 increases GATA-3 fluorescent (red) immunoreactivity in NHBE cells grown on glass slides, and stimulated for 24h without (D) or with 10 ng/ml IL-4 (E) prior to fixation and immunostaining using isotype control (insert in D) or anti-GATA-3 antibody (D and E).

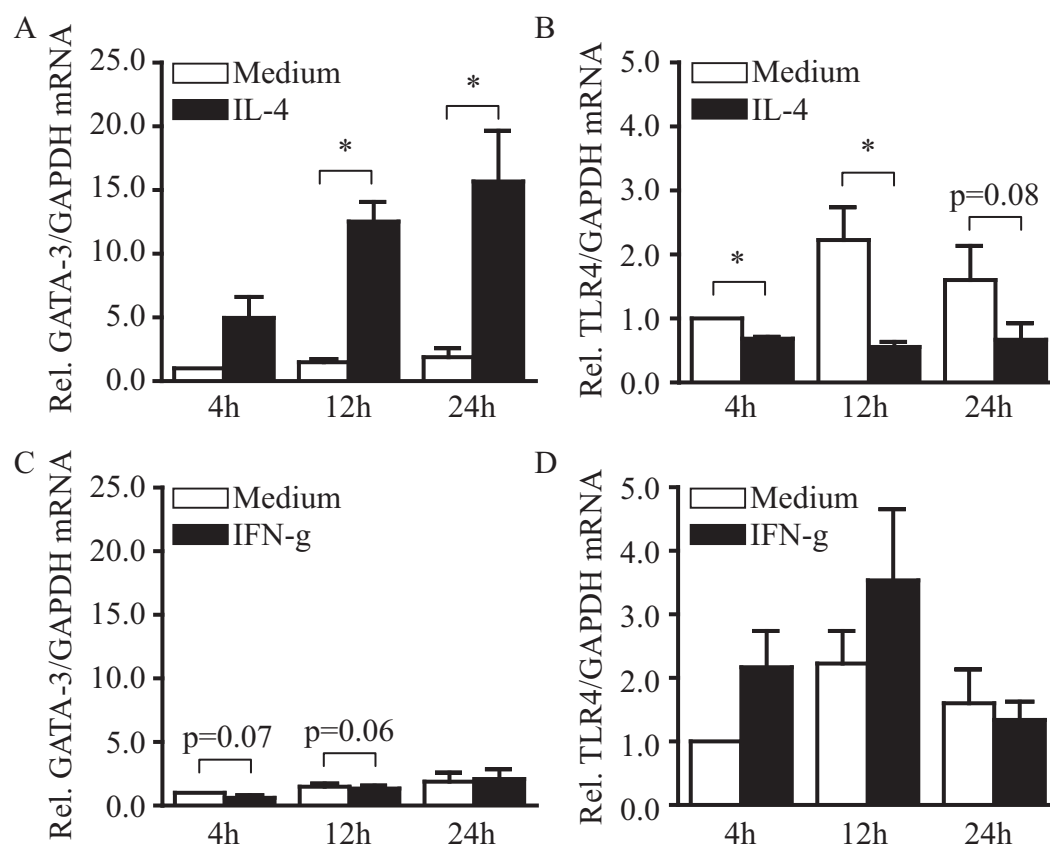


Figure 16. Time-dependent cytokine-mediated regulation of GATA-3 and TLR4 in cultured normal human bronchial epithelial (NHBE) cells. The cells were stimulated with recombinant IL-4 (A and B) or with IFN-g (C and D), both at 10 ng/ml for 4 to 24h. Cells lysates were processed for GATA-3 (A and C) and TLR4 (B and D) gene expression profiling by RT- and qPCR. IL-4 increased GATA-3 but decreased TLR4, whereas IFN-g tended to reduce GATA-3 and increase TLR4. Data are presented as means $\pm$ SEM of 4 independent experiments, and p-values <0.05 were considered statistically significant (*).

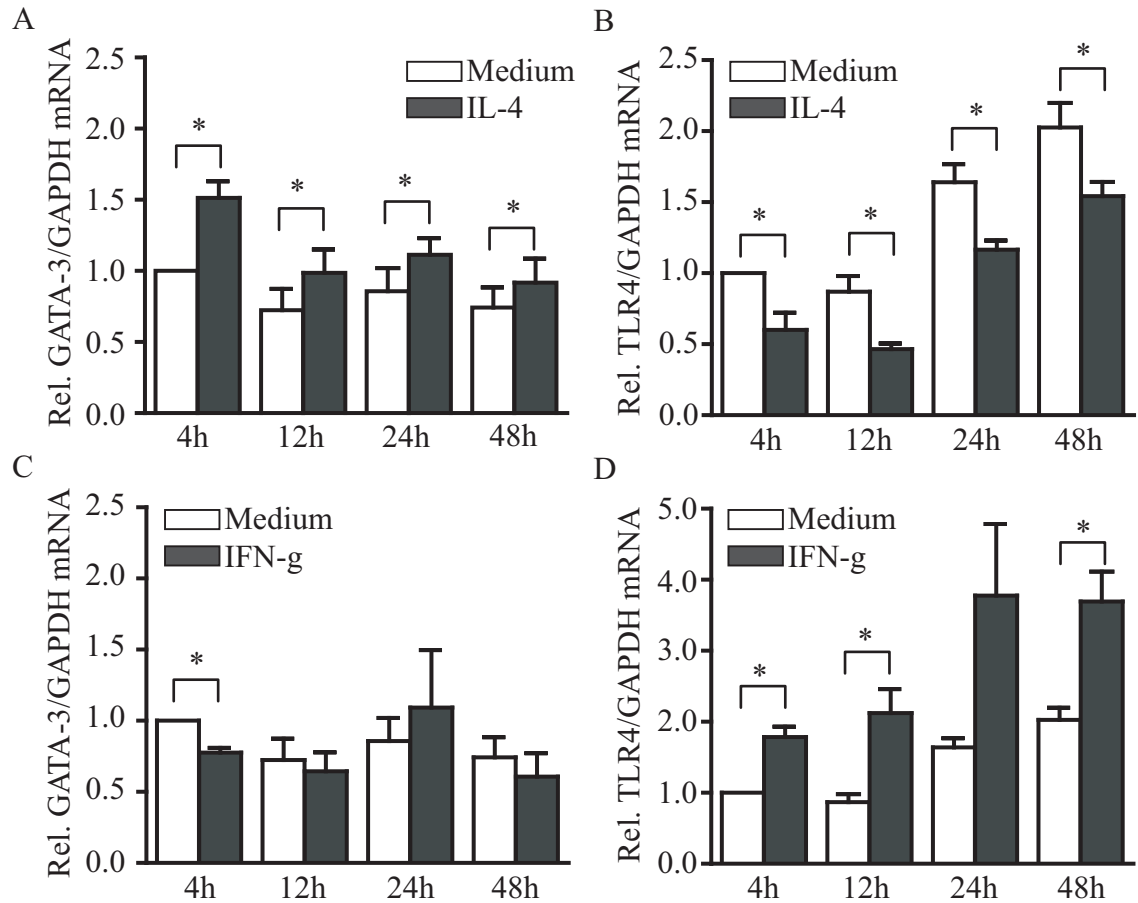


Figure 17. Time-dependent cytokine-mediated regulation of GATA-3 and TLR4 in cultured BEAS-2B bronchial epithelial cell line. Cells were stimulated with recombinant IL-4 (A and B) or with IFN-g (C and D), both at 10 ng/ml for 4 to 48h. Cells lysates were processed for GATA-3 (A and C) and TLR4 (B and D) gene expression profiling by RT- and qPCR. IL-4 increased GATA-3 but decreased TLR4, whereas IFN-g tended to reduce GATA-3 and increase TLR4. Data are presented as means $\pm$ SEM of 3 independent experiments, and p-values<0.05 were considered statistically significant (*).

16c and 17c), and increase TLR4 mRNA levels (Figure 16d and 17d) as reported by others (971). Most the effect of IL-4 and IFN- γ were sustained for the 4 to 24h of stimulation. Interestingly, IL-4 also down-regulated MD2 adaptor protein mRNA expression in BEAS-2B cells (data not shown). The Th2-type cytokine IL-13 has similar effects to those of IL-4 as it increased GATA-3, and reduced TLR4 mRNA transcript levels in both cell types (data not shown). IL-4-mediated regulation of GATA-3 and TLR4 in BEAS-2B cells was also confirmed at the protein level, as assessed by intracellular flow cytometry (Figure 18a) and Western blotting (Figure 18b). In summary, these data stress that the cytokine IL-4 (and IL-13) enhances epithelial GATA-3 expression but inhibits TLR4 expression, therefore confirming their simultaneous and opposite regulation in conditions that mimic Th2-type cytokine milieu seen in allergic diseases.

3.3.2.5. GATA-3 contributes to TLR4 down-regulation through GATA-3 silencing and over-expression *in vitro* experiments

In an attempt to determine whether epithelial GATA-3 expression has a direct implication in the down-regulation of TLR4, we sought to manipulate the expression of this transcription factor in cultured epithelial cells. First, we attempted to silence endogenous GATA-3 in BEAS-2B and NHBE cells using commercially available siRNAs targeting GATA-3 mRNA transcripts (from Qiagen), and a control non-silencing siRNAs. GATA-3 siRNA experiments successfully inhibited GATA-3 mRNA levels by more than 50% compared to

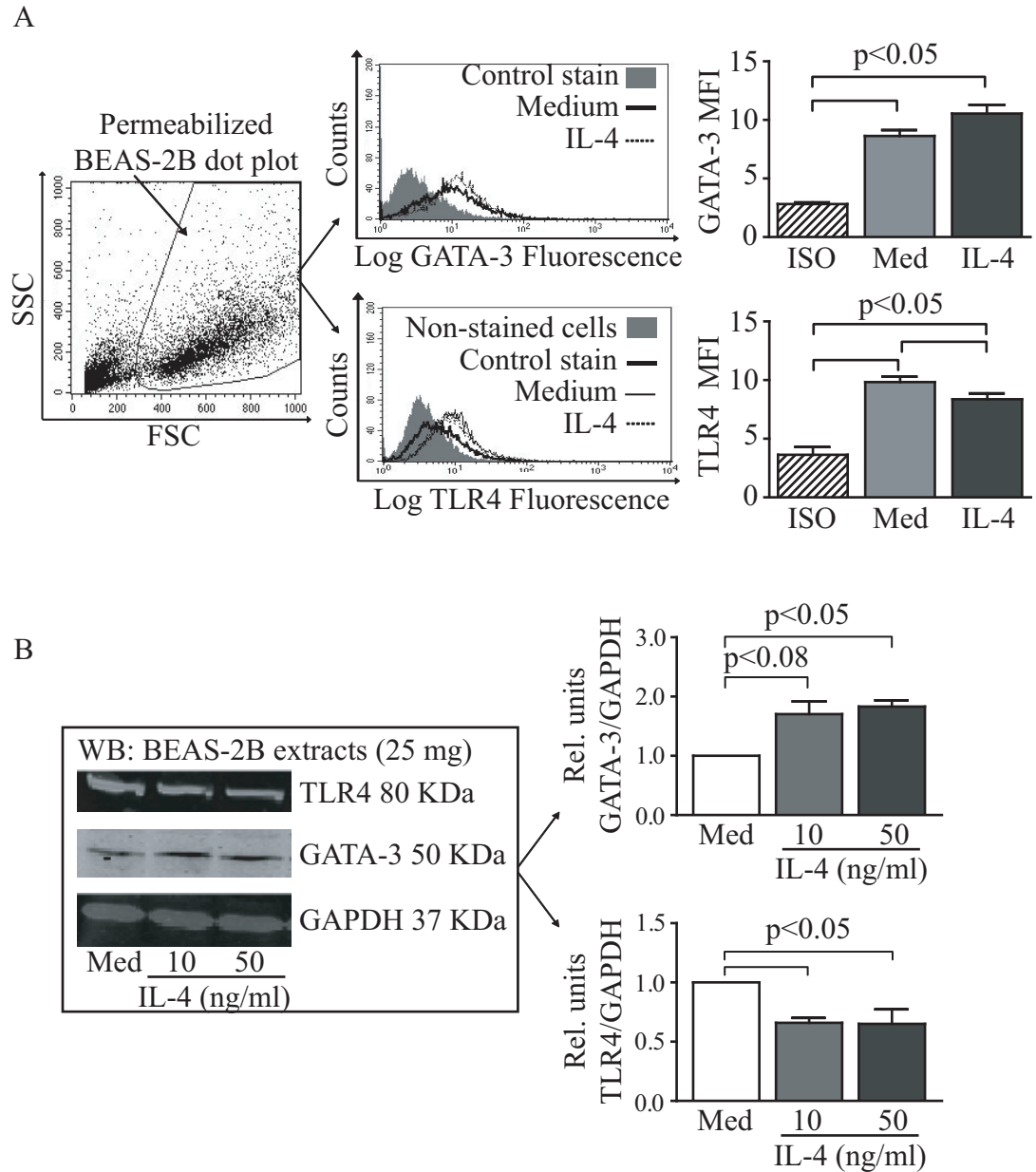


Figure 18. IL-4-mediated GATA-3 up-regulation and TLR4 down-regulation at protein levels in the BEAS-2B cell line. In A, cells were stimulated with/without IL-4 (10 to 100 ng/ml) and then analyzed by flow cytometry for intracellular GATA-3 and TLR4 immunoreactivity. Representative FSC/SSC dot plot shows permeabilized BEAS-2B cells stained for GATA-3 or TLR4 and analyzed based on their MFI for $n=3$ experiments. In B, representative immunoblotting experiment shows TLR4, GATA-3 and GAPDH protein in extracts from medium or IL-4-exposed BEAS-2B cells. Graphs display normalized densitometric means $\pm$ SEM, p -values < 0.05 were considered significant. Detailed procedures for flow cytometry and immunoblotting experiments are provided in the Materials and Methods (sections 2.3.9 and 2.3.10, respectively).

control siRNA-transfected cells after 72h of incubation, in both cell types (Figure 19a and b for BEAS-2B and NHBE cells, respectively). The effect of GATA-3 siRNA was less pronounced at earlier time-points (24 and 48h, data not shown), therefore justifying the use of 72h incubation periods to investigate its effects on TLR4 expression. GATA-3 silencing resulted in significant up-regulation of TLR4 gene transcription in the range of 30 to 80% for BEAS-2B and NHBE cells (Figure 19c and d, respectively). GATA-3 silencing also increased the mRNA levels of the MD2 adaptor protein (data not shown). Finally, we also aimed at exogenously over-expressing the human GATA-3 gene in cultured epithelial cells and determining its effect on TLR4 expression *in vitro*. To do so, we transfected cultured epithelial cells with either the transfection reagent alone (Superfect control), with pCMV5-empty or hGATA-3 gene containing vector-supplemented transfection reagent (pCMV5-empty or -GATA-3, respectively). In control experiments, BEAS-2B cells transfected with human GATA-3 gene open reading frame-containing pCMV5 showed significant GATA-3 over-expression at both mRNA (Figure 20a) and protein levels (Figure 20b) compared with pCMV5-empty plasmids transfected cells. Despite GATA-3 over-expression in BEAS-2B cells significantly up-regulated GATA-3 mRNA from 4 to 24h post-transfection (>5,000 fold-increase), it did not consistently alter TLR4 mRNA expression (data not shown). In contrast, normal human bronchial epithelial (NHBE) cells were then transfected using hGATA-3-pCMV5 plasmids for 4 and 24h, and displayed

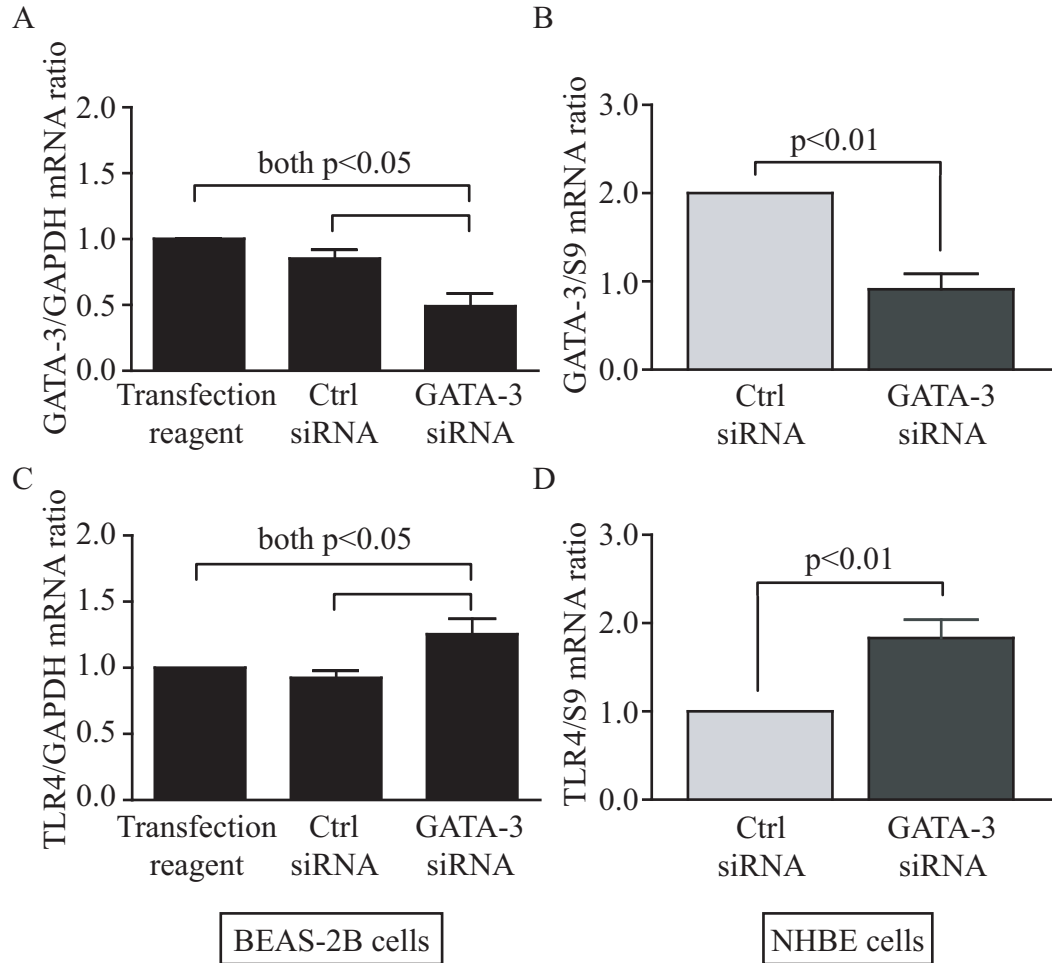


Figure 19. GATA-3 gene silencing through RNA interference augments TLR4 gene expression by human bronchial epithelial cells. In A and B, BEAS-2B (left graphs) and NHBE cells (right graphs) were transfected with either 5 nM of GATA-3-specific or negative control small interfering RNA (siRNA) vectors as described earlier (see sections 2.3.11 and 2.3.12). Total RNA was collected 72h post-transfection and gene expression profiling was performed by RT- and qPCR using GATA-3 (A and B) and TLR4 primers (C and D) along with reference genes (GAPDH and S9). The normalized data were presented as means of 6/4 independent experiments for BEAS-2B/ NHBE cells, and p -values < 0.05 were considered significant.

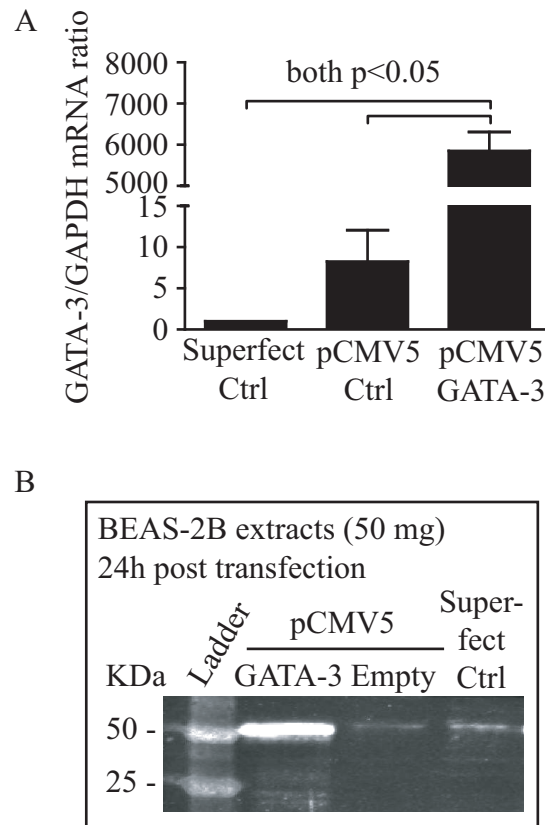


Figure 20. Confirmation of GATA-3 over-expression in transfected BEAS-2B cells. GATA-3 expression was assessed at both mRNA (A) and protein levels (B) in BEAS-2B cells transfected with either 0.35 mg of human GATA-3 gene open reading frame-containing pCMV5 or pCMV5-empty plasmids for 24h, as described in the Materials and Methods section 2.3.11. In A, RNA extraction was performed and GATA-3/GAPDH transcripts levels were assessed by RT- and qPCR. In B, extracts were tested for GATA-3 protein immunoreactivity by Western blotting. The presented data in A and B are representative of 4 and 2 independent experiments, respectively.

significant but lower GATA-3 mRNA over-expression by respectively 3 and 5.5-fold compared with cells transfected with pCMV5-empty (Figure 21a). GATA-3 over-expressing NHBE cells also showed significant reductions in TLR4 mRNA levels by 30 and 20% at 4 and 24h, respectively (Figure 21b), compared with pCMV5-empty-transfected cells. In summary, we report a decreased expression of TLR4 in airway epithelial cells in allergic rhinitis and asthma but increased expression of the Th2-type transcription factor GATA-3. Results obtained from our *in vitro* experiments suggest that GATA-3 contributes to TLR4 transcriptional down-regulation in airway epithelial cells and illustrates that phenotypic changes of epithelial cells in allergic diseases of the airways may affect innate immune functions.

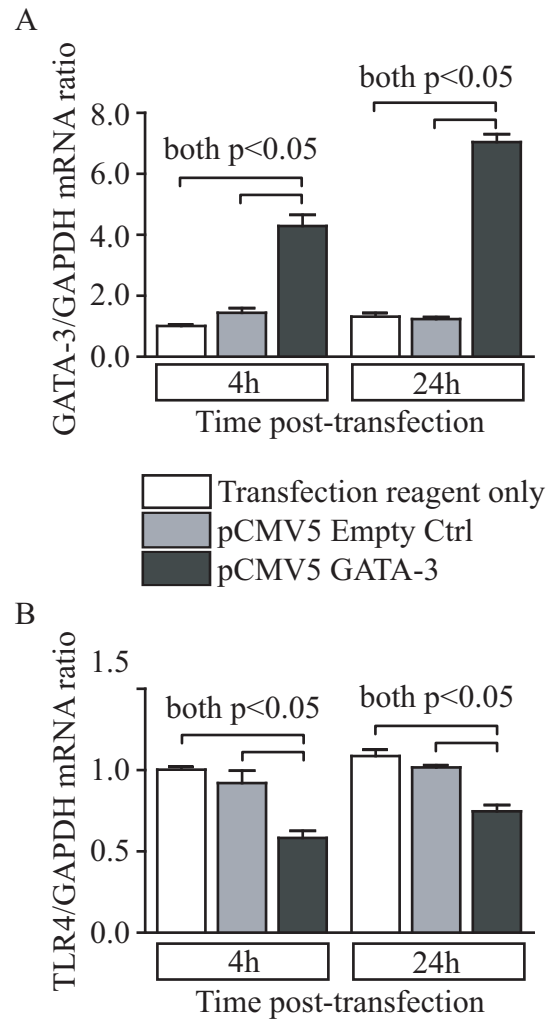


Figure 21. GATA-3 over-expression leads to concomitant increase in GATA-3 but decrease in TLR4 expression in NHBE cells. As described in the section 2.3.11, NHBE cells were transfected with either 0.35 mg of human GATA-3 gene open reading frame containing-pCMV5 or pCMV5-empty plasmids for 4 to 24h before RNA extraction was performed and the GATA-3/GAPDH (A) and TLR4/GAPDH (B) mRNA ratios were assessed by RT-qPCR. Data displayed are representative of 2 independent experiments ran in duplicate.

Chapter 4: Discussion

4.1. Introductory remarks

The incidence of allergic diseases has consistently and significantly been on the rise for the past few decades. Despite the strong association of several genetic traits with the development of atopy, it has been proposed that such rapid increases in the allergy rates are also linked to changes in our environment and our lifestyle. Numerous epidemiological studies conducted throughout the International Study of Asthma and Allergies in Childhood (ISAAC) documented marked increase of allergy incidence especially in paediatric populations. These studies also described important geographical variations in the allergy rates, the latter being strikingly elevated in westernized countries and urban environments, compared with developing countries and rural environments. The search for the causes of such geographical pattern in allergic diseases eventually led to the emergence of the hygiene hypothesis. This hypothesis arose from a series of environmental observations according to which atopy and allergy rates in children inversely correlate with family size, day care attendance, early exposure to and sharing of infections, exposure to the farming environment, livestock or pets (section 1.3.3.2.3). The hygiene hypothesis states that a reduced exposure to microbes would under-stimulate the developing immune system early in life and

lead to an increased propensity of the developing immune system to develop Type I hypersensitive reactions to innocuous allergens.

Upon the exposure to microbes, our innate immune system senses evolutionarily well-preserved microbe- or pathogen-associated molecular patterns (PAMPs), through a battery of conserved pattern recognition receptors (PRRs). One of the most ubiquitous and best characterized PAMP from our environments consists of the Gram-negative bacteria cell wall component endotoxin or lipopolysaccharide (LPS). Our immune system senses LPS via a receptor complex composed of the Toll-like receptor 4 (TLR4), together with CD14 and MD2 adaptor proteins, and which mediate signal transduction in the host cells. The LPS molecule typically triggers an inflammatory immune response, which further resolves through a negative-feedback loop preventing deleterious inflammatory burden and tissue damage. This initial inflammatory burst also influences the maturation and activation of antigen-presenting cells, therefore affecting adaptive immune response to foreign antigens. LPS/TLR4 signalling events also limit the inflammatory cascade induced by LPS exposures through the release of anti-inflammatory mediators, as well as through a process named endotoxin tolerance which will be discussed later. There is an extensive body of literature addressing the effects of LPS exposure against the development of allergic-type immune responses and disease phenotype. As reviewed earlier, a plethora of studies conducted both in human subjects and in animal models of

experimental allergies demonstrated that exposure of non-atopic individuals to environmental LPS doses, especially early in life, confers a protection against Th2-type immune responses to allergens and allergic disease manifestations. Most data obtained from humans were collected through epidemiological and observational studies taking into account ambient LPS levels. Nonetheless, several studies on human tissues but mostly in animal models demonstrated that LPS likely skews responses to allergens away from the pro-allergic Th2-type immunity and rather promotes Th1-type and/or T-reg-type adaptive responses. On another hand, numerous experimental models as well as studies in human subjects predisposed to develop atopy, that had on-going inflammatory diseases or histories of allergic diseases demonstrated that LPS exacerbates the diseases rather than preventing them. The mechanisms underlying this dual role of LPS in the context of allergic disorders remains the focus of an extensive research effort. However, few studies investigated whether a history of atopy and/or on-going Th2-type allergic disorders counter-regulated LPS-mediated innate immune functions. In this thesis, we decided to address whether a history of atopic diseases across childhood affect TLR4 expression and the ability of the immune cells to sense bacterial LPS. Furthermore, we sought to further characterize the mechanisms underlying the regulation of TLR4 in adult subjects with on-going atopy and allergic airways disease.

4.2. Discussion of results: Cross-sectional study on TLR4 expression and signalling in peripheral leukocytes of children with/without atopic histories

In our first study, we studied a cohort of children and demonstrated that atopic status is associated with reduced LPS responsiveness in circulating monocytes, as determined by impaired phosphorylation of MAPK pathways and impaired decrease in TLR4 surface expression upon LPS exposure. This report is also the first to use a large paediatric cohort to survey expression of TLR4 on CD4-expressing circulating leukocytes, including monocytes and T 'helper' lymphocytes. One limitation of this study was that atopic status and history was determined by questionnaire, as in the ISAAC studies (500). Within the limitation of our protocol, we screened several hundred children undergoing laboratory testing for other purposes. We accepted children as "atopic" primarily if they had history of allergic diseases and past skin prick testing. The consistency of the results appears to underline the accuracy of the screening process.

Previous work from our laboratory (736) detected significant TLR4 expression on CD4+ (also CD3+) T cells infiltrating the nasal submucosa in both atopic and non-atopic children. Similar to other studies, consistently low percentage of TLR4+ cells was detected among peripheral blood CD4+ T 'helper' lymphocytes, surface TLR4 expression being restricted to activated CD4+ T cells (735). Such discrepancies in the TLR4 expression by CD4+ T cells in the two tissue models likely reflects the naive to activated T cell ratio, which is greater in

PBMC than in mucosa-infiltrating T cells. In the PBMC, monocytes represented the most important TLR4+ circulating leukocyte population (728), and these monocytes also co-expressed the CD14 and CD4 marker (251). In our study, lymphocytes and monocytes were differentially gated using specific forward scatter/side scatter (FSC/SSC) characteristics, as previously published (990). Monocytes were enriched in TLR4+CD14+ cells, and did not express CD3 markers while T 'helper' cells expressed higher levels of CD4, uniformly expressed CD3 but mostly lacked TLR4 and CD14.

We addressed the possibility of an atopy-specific regulation of TLR4 expression using a large paediatric cohort, stratified by age and atopic status. In these subjects, we assessed both baseline TLR4 and expression following exposure to exogenous IL-4. No major variation in surface TLR4 expression could be seen across the whole cohort, and these results were not due to significant variations in cell numbers. However, we established that age differentially affected TLR4 expression on CD4+ leukocytes in children with/without atopic histories. Correlation analyses revealed that TLR4 expression by monocytes from children with atopic histories slightly but consistently diminished as a function of age compared with non-atopic children. In contrast, TLR4 expression by T 'helper' lymphocytes tended to increase with age in atopic, but not in non-atopic children. The trends towards decreasing TLR4 expression by monocytes from older children with history of atopy and increasing TLR4 on T

'helper' lymphocytes in older children with atopic histories compared with non-atopic children may consist of peripheral consequences of type-2 immune bias associated with atopy. In young children (2 to 12 years of age), TLR4 expression by T 'helper' lymphocytes tended to be lower in atopic than in non-atopic subjects. This was mirrored by the fact that IL-4-induced down-regulation of TLR4 complex-associated molecules at mRNA levels in children CD4⁺ T lymphocytes fractions. However, children with atopic histories likely display elevated proportion of circulating activated T 'helper' cells due to repeated inflammatory bursts, and as they get older. Indeed, the proportion of peripheral CD4 T cells (1023) and activated (i.e. CD25⁺) circulating T lymphocytes (1024) both increase in patients with asthma compared with controls (63, 100, 101). Activated CD4 T cells also display elevated surface TLR4 compared with naïve cells (735), which may reflect the increased proportion of TLR4⁺ T lymphocytes in older atopic subjects compared with controls. Nevertheless, the biological relevance of TLR4 regulation on T lymphocytes remains limited in light of the LPS signalling data that will be discussed later. On the other hand, the age-dependent decrease in the proportion of TLR4⁺ monocytes from atopic children, but not from controls, likely result from the chronic and/or prolonged type-2 conditions characterizing the natural history of atopic diseases in childhood. In these conditions, elevated IL-4 levels may affect TLR4 expression on circulating monocytes, only in the long term (or with increasing age). Similar results were obtained from the nasal

explant model (736), where the TLR4 expression was reduced in tissues from all adults compared with children (regardless from atopic status). In the same study, an atopy-associated decrease in TLR4 expression was only seen non-atopic vs. atopic adults, but not in non-atopic vs. atopic children. Our cohort data and correlation analyses of baseline TLR4 expression on monocytes also suggest that the age-specific reduction in TLR4 expression in atopic children only appear >13 year of age. In conclusion, data from our paediatric cohort support the concept that histories of atopic disorders and Th2-type environment affect TLR4 expression by monocytes in an age-dependent and progressive fashion which may alter LPS responsiveness.

With respect to the effect of type-2 cytokine environment on the LPS receptor complex, several studies previously documented IL-4-mediated decrease in TLR4 expression by monocytes, both using primary cells from PBMC and using a monocytic cell line (970, 972). Although IL-4 stimulation did not have significant effect on the baseline TLR4 expression by monocytes in our cohort, our results confirmed that IL-4 decreases CD14 expression by monocytes from non-atopic subjects (967). This was also the case for cells from atopic children. Finally, there was a trend toward decreasing baseline CD14 expression cells in children diagnosed with atopic conditions compared with non-atopic healthy children (both with and without IL-4), but the differences were not statistically significant. It would have also been interesting to determine the levels of sCD14

in atopic and non-atopic children, as this molecule may favour LPS responsiveness in cells lacking membrane-bound CD14. Furthermore, Zdzienicka and Jenmalm previously reported reduced levels of soluble CD14 in children with atopic children (986), which could therefore contribute to their defective LPS-triggered signalling.

We then used a subgroup of subjects to study LPS responsiveness in both children with/without histories of atopic diseases. We assessed the activation i.e. phosphorylation levels of two signal transduction proteins, namely the extracellular signal-regulated kinase (ERK 1 and 2, p42/44) and the p38 mitogen-activated protein kinase (MAPK). The choice of ERK and p38 MAPK signalling pathways was made on the basis of their central roles in both MyD88-dependent and TRIF-dependent signalling pathways (Diagram 10). Monocytes from non-atopic children significantly responded to LPS exposure by increasing p42/44ERK and p38-MAPK phosphorylation in a time-dependent manner, as confirmed by others (1008). We confirmed that the phosphorylation of p42/44ERK MAPK was dependent on TLR4 through the use of neutralizing antibodies, which excluded the possible contribution of LPS-induced TLR4-independent signalling pathways recently characterized (915). In contrast, LPS did not induce significant ERK or p38 MAPK activation among CD4⁺High T-helper lymphocytes regardless of atopic status, which reflects the low TLR4 expression in this population and their relative LPS unresponsiveness through the tested signalling pathways. The ability of

CD4⁺ monocytes to phosphorylate MAPKs upon LPS exposure was dependent on the atopic status of the patient. LPS indeed failed to trigger p42/44ERK and p38 phosphorylation in monocytes from children with atopic histories compared with cells from non-atopic children. These results are in line with Saghafian-Hedengren and collaborators which similarly observed impaired LPS-induced p38 MAPK phosphorylation in 24 months-old children from allergic mothers compared with those from non-allergic mothers (993). The difference in signalling between atopic and non-atopic children was not due to differences in ages, as suggested by Boehmer et al (1008), since the subjects studied in the signal transduction experiments were age-matched. Noteworthy, age-matched subjects selected for the signalling experiment had an average age of about 14 years, an age at which we began to observe an age-dependent dichotomy in the baseline TLR4 expression by monocytes. The previously-reported age-dependent decrease in TLR4 expression only in atopic children may therefore contribute to explain the reduced LPS responsiveness in cells from atopics. As discussed earlier, our cohort data on baseline TLR4 expression do not provide direct evidence that the negative regulation of LPS signalling in individuals with atopic histories is caused by consistent down-regulation of LPS receptor complex at least in children below 13 years of age. In support of our data, a recent study reported that circulating monocytes isolated from 5 years-old allergic children display TLR4 and CD14 expression levels that were equivalent to those of cells from age-matched non-

allergic children (1009). Our signalling data are also in line with the fact that children of less than 5 years of age from allergic mothers display impaired LPS-induced p38 MAPK pathway activation than children from non-allergic mothers (993).

The inability of monocytes from children with histories of atopic diseases to properly activate MAPK pathways upon LPS exposure is likely reflected by dysregulated responses i.e. mediator production. Previous studies indeed reported that PBMC from asthmatic patients released significantly less IFN- γ , IL-10, IL-1 β and TNF- α compared with cells from controls when exposed to LPS (734, 990, 991). We confirmed the differential LPS-induced mediator expression results as we demonstrated the reduced LPS-induced IL-10 mRNA in PBMC from atopic subjects compared with PBMC from non-atopic children. We specifically selected IL-10 in light of our previous studies in the nasal explants model which demonstrated that this anti-inflammatory cytokine plays a critical role in the LPS-mediated prevention allergen-induced Th2-type inflammation *in situ* (736). Interestingly, studies previously documented the LPS-mediated anti-allergic effects were also studied in PBMC from both allergic asthmatics and controls patients (989). Min et al used PBMC from house dust (HDM) mite-sensitized asthmatic patients stimulated *in vitro* with HDM extracts (1025). HDM extracts induced IL-5 and CCL24 (eotaxin-2) up-regulation, a response which was reduced by adding LPS to the cultures; this immuno-suppression was dependent

on IL-10 production (1025). Interleukin-10 is quickly released from LPS-stimulated cells along with several pro-inflammatory cytokines. IL-10 is therefore associated with the negative-feedback loop contributing to attenuate and terminate an inflammatory response. The IL-10 cytokine exerts such anti-inflammatory effects by inhibiting APCs, and limiting their ability to activate T 'helper' lymphocytes and mount adaptive response to antigens. IL-10 reduces the production of pro-inflammatory cytokines/chemokines (1026) and the expression of MHC class II and co-stimulatory molecules by APCs (1027, 1028). IL-10 therefore promotes antigenic tolerance as it limits T cell's production of IL-2, and increases the expression of IL-1R antagonist (IL-1Ra) (832). In the context of allergies, IL-10 inhibits B cell's isotype swichting to IgE (1029), reduces eosinophils survival (1030), airway inflammation and hyperresponsiveness when instilled in the airways of animals with experimental allergic asthma (1031). The anti-allergic properties of LPS-induced IL-10 were also highlighted by the nasal explants experiments in our laboratory (736). Studies previously demonstrated that main sources of IL-10 are monocytes and macrophages (831, 990, 1032) and regulatory T (T-reg) cells (1033, 1034). The assessment of LPS-induced IL-10 production by CD4⁺ cell fractions ruled out the possibility that regulatory T cells significantly explains the differential cytokine production by PBMC from atopic and non-atopic children. However, this approach do not exclude the possibility that regulatory B cells play a role (1035, 1036): our preliminary results

indicate that a significant proportion of circulating B cells (i.e. 5 to 15% of PBMC) expresses TLR4 but further investigation is needed to determine whether B cells respond to LPS. Taken together, our results and those of others suggest that monocytes from children with atopic histories display impaired LPS responsiveness translating into defective induction of IL-10, a critical anti-inflammatory mediator in LPS-mediated protection against allergic inflammation.

Assessing the differential LPS-induced IL-10 expression by PBMC from atopic and non-atopic subjects is not only relevant due to its immuno-suppressive roles, but also considering the activation of MAPK pathways. LPS-induced IL-10 production by macrophages reportedly depends on the TRIF pathway, which activates TRAF3 and Type I interferon (779, 1037). Furthermore, LPS-induced IL-10 production is also reportedly dependent on p38 MAPK-driven pathways, as demonstrated through MAPK inhibitor-based studies (831, 1038). These observations should be put in perspective with the fact that TLR4-expressing CD4⁺ monocytes from atopic/non-atopic subjects were also discriminated on the basis of their loss of surface TLR4 expression upon LPS exposure. Monocytes from atopic children failed to significantly lose their surface TLR4 expression compared with cells from controls. Reduction in TLR4 surface expression (953) due to internalization takes place within minutes in a dynamin- and clathrin-dependent manner, and TLR4 is then trafficked to early/sorting endosomes (761). We described earlier the two phases of LPS/TLR4-mediated

signal transduction: the extracellular and mainly pro-inflammatory MyD88-dependent phase, and the endosomal/delayed TRIF-dependent anti-inflammatory phase (700, 1039) (section 1.3.4.3). The internalization of the TLR4 complex consists of a hallmark of endotoxin tolerized cells, which present with increased IL-10 expression during their delayed activation phase (831, 958). Therefore, our data not only support the concept that monocytes from atopic subjects failed to produce IL-10 upon LPS exposure, but also that pronounced and prolonged LPS-induced decrease in surface TLR4 may only takes place in non-atopic monocytes. Potentially, atopy may interfere with LPS/TLR4 complex internalization and, consequently, limit LPS-induced activation of delayed-phase MAPK-dependent signalling pathways responsible for IL-10 production. We currently have a limited understanding of the impact of a diminished internalization on the MAPK pathways. Nonetheless, endosomal trafficking of TLR4 was shown not to be necessary for triggering activation of the NF- κ B signalling pathway (757, 761). Interestingly, monocyte/macrophages that fail to internalize TLR4 display elevated activation of the NF- κ B pathway and increased secretion of the pro-inflammatory cytokine TNF- α , but reduced expression of anti-inflammatory IL-10 (1040). Whether a defect in LPS-induced TLR4 internalization is associated with the decreased expression of other mediators deserves more investigation. Similarly, the atopy-specific factors responsible for defective TLR4-

dependent MAPK-mediated signalling and endosomal trafficking of the receptor remain elusive.

Studies suggested that aging limits TLR4 expression (736) but also LPS responsiveness as assessed by LPS-induced cytokine production (1041).

Macrophage from aged mice (18-24 months) exhibited reduced TLR4 expression and LPS-induced IL-6 and TNF- α compared with cells from young animals (2-3 months) (1041). The previous results demonstrated an age-dependent decrease in TLR4 expression as well as an impaired LPS-mediated signalling and mediator production in monocytes from atopic but not from non-atopic children. Taken together, it is tempting to speculate that a history of atopic diseases during childhood is associated with an accelerated degeneration of LPS responsiveness of monocytes with aging.

After having studied TLR4 expression and LPS responsiveness in peripheral immune cells of subjects with/without histories of atopic disorders in a cross-sectional paediatric cohort, we attempted to address TLR4 expression and regulation in the context of on-going allergic disease, in adults. We initially sought to investigate TLR4 expression by monocytes/macrophages *in situ*. We therefore obtained nasal mucosa tissue specimens from adult subject with/without diagnosed allergic rhinitis undergoing intranasal surgery. Previous studies documented the infiltration of the nasal submucosa by increased numbers of macrophages in patients with allergic rhinitis (55, 126, 1042), compared with non-

atopic controls. Besides, numerous studies described macrophages as a critical immuno-regulatory cell type promoting antigen tolerance and limiting hypersensitive reactions. Lung macrophages-depleted animals indeed develop worsened allergic asthma inflammation and symptoms compared with control animals subjected to standardized protocols of allergen sensitization and challenge (262, 263). Despite we confirmed the presence of CD68-expressing macrophages in both groups of subjects, the assessment of TLR4 immunoreactivity revealed that nasal epithelial cells, rather than mucosa-infiltrating immune cells, consisted of the major TLR4-expressing cell population. In addition, cells lining the airway lumen are the first to encounter inhaled LPS, therefore representing a more biologically relevant cell type to study LPS receptor in airways tissue (749).

4.3. Discussion of results: Regulation of TLR4 in airway epithelial cells in asthma and rhinitis: the effect of Th2-type conditions and potential role for epithelial GATA-3 transcription factor

The epithelium lining the gastro-intestinal and the lung mucosa form the first immune cells to encounter the wide variety of microbes and PAMPs which are ingested and inhaled on a daily basis. Epithelial cells express the vast majority of PRRs, including TLR4. LPS-responsive peripheral immune cells like monocytes express TLR4 on their cell surface which enables them to quickly recognize bacterial invaders and trigger anti-microbial inflammatory response

preserving the blood microenvironment free of pathogens. In contrast, epithelial cells from the gut and the lungs are consistently exposed to large amounts and wide variety of PAMPs. Such exposure would logically activate pro-inflammatory pathways and create a persistent inflammatory milieu. In order to avoid deleterious inflammatory responses which would otherwise be sustained in these non-sterile environments, gut and lung epithelial cells adopted phenotypes rendering them more tolerant to TLR ligands, including LPS (1043). Expression of the LPS receptor by intestinal and bronchial epithelial cells is therefore normally confined to the endosomes (738, 744). This localization tolerizes without fully desensitizing the cells to LPS: epithelial cells would rather limit exaggerated or inadequate immune responses to this ubiquitous PAMP (1043). For example, bronchial epithelial cells respond to LPS (745) by preventing LPS-induced TNF- α production in a co-culture and co-stimulation model with surface TLR4 expressing-monocytes (1020). Bronchial epithelial cells also display a by-default immuno-suppressive phenotype which limits DC-mediated T lymphocyte proliferation, and promotes the development of regulatory T cells (1020).

The role of inhaled LPS in the development and manifestation of airway allergic diseases remains controversial. Endotoxin may either exacerbate experimental asthma or protect against its development (859). Studies on children and in early life animal models suggested that early life exposure to LPS prevents allergen sensitization and the development of airway allergic diseases

(637, 866, 871, 920, 921). When LPS is used as an oral adjuvant, it successfully prevents the development of food allergy to peanut extracts and of experimental asthma to ovalbumin (936, 937). In contrast, studies on atopic or allergic individuals reported LPS as a pro-inflammatory and an exacerbating factor of established diseases (631). Despite epithelial cells were earlier depicted as an LPS tolerant cell type, recent studies described that TLR4 expression by structural cells of the airways (1044), including epithelial cells (1021), are necessary for the development of Th2-type responses in murine models of experimental asthma. Furthermore, bronchial epithelial cells from asthmatic subjects an altered phenotype mirrored by the overexpression of Th2-type transcription factor STAT6 (343) and overabundant production of inflammatory mediators (Section 1.2.3.3.). In counterpart, studies reported that intestinal epithelial cells exposed to Th2-type cytokine milieu have impaired TLR4 expression (971). Bronchial epithelial cells from patients with asthma display defective innate immune functions (341, 1022). Our studies and others also demonstrated that circulating immune cells from subjects with allergic diseases have defective TLR4 expression (734), and display impaired responses to LPS or other TLR ligands (1045). In this study, we addressed the question whether Th2-type immune bias seen in subjects with allergic rhinitis and asthma affect the expression of TLR4 in airway epithelial cells. Using both *in situ* (mucosa) and *in vitro* culture models, we reported a reduced expression of TLR4 by nasal

epithelial cells from subjects with rhinitis, and by bronchial epithelial cells from subjects with asthma, compared with cells from non-allergic and non-asthmatic controls. Such reduced epithelial TLR4 expression in allergic diseases was mirrored by elevated GATA-3 expression as we confirmed expression of this Th2-type transcription factor by cells of the airway epithelium (345). Experiments led *in vitro* using in BEAS-2B and primary NHBE cells demonstrated that Th2-type conditions e.g. IL-4 which characterize the inflammatory milieu in rhinitis and asthma enhance GATA-3, but reduce TLR4 expression. Our experiments using either GATA-3 siRNA-transfected or GATA-3-over-expressing epithelial cells also supported a role for GATA-3 in the down-regulation of epithelial TLR4 expression.

Using nasal mucosa biopsies from adult subjects with allergic rhinitis and non-atopic controls, we observed a decreased proportion of TLR4-immunoreactive epithelial cells in the disease, as assessed by semi-quantitative blinded analyses of immunocytochemistry staining. Further homogenization of frozen nasal biopsies confirmed significantly lower TLR4 mRNA levels in nasal tissues from atopic subjects with allergic rhinitis compared with non-atopic controls, by RT-PCR. One limitation of TLR4 mRNA measurements in homogenized tissues consists of variations in cell composition between the different biopsy specimens. Fransson and colleagues reported unchanged TLR4 mRNA levels in homogenized inferior turbinate tissues from adult subjects with

allergic rhinitis using tissues also collected outside allergen season and compared with those from healthy controls (1046). However epithelium-specific TLR4 protein in was not assessed in this study. Interestingly, the authors noted increased mucosal TLR-2, -3 and -4 mRNA following allergen challenge of subjects with rhinitis, suggesting that allergen provocation can, at least temporarily, augment TLR4 mRNA in homogenized tissue possibly reflecting tissue infiltration by TLR4-expressing immune cells, including activated CD4 T cells (735). In an *ex vivo* study, Tulic and collaborators reported decreased numbers of TLR4 immunoreactive cells in explanted nasal tissues from atopic adults compared with those from non-atopic adults, both before and after LPS exposure (736). In our study, the reduced TLR4 expression in tissues from asymptomatic allergic rhinitis suggested that Th2-type microenvironment may limit TLR4 expression in the epithelium.

The use of nasal biopsy tissues collected from subjects with allergic rhinitis and healthy controls consists of a well-established upper airway model of allergic inflammation to study the development of early and late-phase reactions, the recruitment of submucosal immune cells exhibiting skewed Th2-type phenotype following allergen provocation and/or the effect of steroid therapy on the inflammatory processes (174, 1047-1049). We then sought to validate the state of allergic-type inflammation in rhinitis compared to controls to potentially explain differences seen at the levels of TLR4 expression. Although the nasal biopsies in

our study were collected outside the aeroallergen season, tissues from atopy patients with established history of allergic rhinitis exhibited increased infiltration by submucosal immune cells including CD4⁺ T lymphocytes and eosinophils. These histological data were in line with the mRNA levels for type-2 (or Th2) inflammatory markers detected in homogenized biopsies, including IL-4, IL-5 and GATA-3 transcripts. Nasal allergen provocation of rhinitis patients was previously shown to increase GATA-3 expression (131). Finally, we confirmed GATA-3 expression in nasal epithelial cells from subjects with rhinitis, although not all the cells seemed to express the transcription factor. These observations are in line with previous observations of GATA-3 expression in bronchial epithelial cells, also by immunohistochemistry (345).

On the basis of the reduced expression of TLR4 in the nasal epithelium of subjects with allergic rhinitis compared with controls, we sought to verify these observations in cells from the lower airways using bronchial epithelial cells isolated from healthy non-atopic adults and from subjects with asthma, another disease featuring Th2-type immune bias. Notwithstanding the observed trend toward decreased TLR4 mRNA levels in the cells from asthmatic subjects compared with controls, we detected significantly increased GATA-3 mRNA levels in epithelial cells from asthmatic subjects compared with those from controls. Caramori et al previously established GATA-3 protein expression by bronchial epithelial cells, but did not observe significant difference in the GATA-3

protein expression in homogenized bronchial biopsies from healthy vs. asthmatic subjects (345). To our knowledge, little is known about the regulation of GATA-3 expression in epithelial cells. Our *in vitro* experiments using both primary normal bronchial epithelial (NHBE) and the immortalized bronchial epithelial cell line BEAS-2B demonstrated dose- and time-dependent IL-4-induced GATA-3 expression at both mRNA and protein levels. These results are coherent with the increased expression of STAT6, the Th2-type transcription factor mediating the effect of IL-4, by asthmatic compared with non-asthmatic bronchial epithelial cells (343). In contrast with GATA-3, TLR4 expression was reduced upon IL-4 stimulation. IL-13, another pleiotropic Th2-type cytokine, had similar but less potent effects than IL-4 on both GATA-3 and TLR4. IFN- γ had a limited effect on GATA-3, whereas it augmented TLR4 expression. These results regarding cytokine-mediated regulation of TLR4 expression are in line with a previous report on intestinal epithelial cells (971), in which IL-4 and IFN- γ had opposite effects. Regulation of both markers at the protein level was confirmed by mean of Western blotting but also flow cytometry experiments. The use of flow cytometry confirmed intracellular localization of TLR4 presented by others (738). Our results provide with novel information on the regulation of GATA-3 in epithelial cells: they suggest that type-2 microenvironment reduces TLR4 but promotes GATA-3 expression. GATA-3 expression was indeed increased in asthmatic lung tissues (208), as well as in allergen-challenged nasal mucosa explants from rhinitis

(131). GATA-3 was also shown to correlate with IL-5 and IL-5R α in cells infiltrating asthmatic bronchial lumen investigated by induced sputum (1050). These observations are in line with our data on the increased eosinophil numbers, IL-5 and GATA-3 mRNA levels in nasal tissue from rhinitis subjects compared with controls. Nonetheless, the increased GATA-3 expression in homogenized tissue likely reflects both the recruitment of Th2-type inflammatory cells and phenotypic changes in mucosa resident cells including those forming the epithelium.

Aside from GATA-3, the signal transducer and activator of transcription 6 (STAT6), another Th2-type transcription factor, was widely associated with immunological features of allergic inflammation (1051). IL-4 and IL-13 share common signalling pathway which involves activation STAT6 transcription factor. For example, STAT6 promotes expression of chemokines (1052) and mucus-associated genes in epithelial cells. STAT6 axis was also shown to promote GATA-3 expression in differentiating T lymphocytes (173). Our data also suggested that IL-4 and IL-13 have a direct effect on GATA-3 expression in epithelial cells. Nonetheless, the contribution of epithelial GATA-3 to allergic disease-associated or IL-4-mediated TLR4 regulation is likely complementary to STAT6 (343). Studies using a monocytic cell model reported that STAT6 has a direct role in the IL-4-mediated down-regulation of TLR4, but failed to fully abolish its expression (970, 972). We then sought determine whether GATA-3 plays a

direct role in the regulation of TLR4 (independently of the IL-4/STAT6 axis) by mean of transient transfection experiments modulating GATA-3 expression. Silencing of endogenous GATA-3 expression (via siRNA) resulted in a significant enhancement of TLR4 gene transcription above the levels observed in non-specific siRNA-transfected cells BEAS-2B and primary NHBE cells. Furthermore, the transient over-expression of GATA-3 in both BEAS-2B and NHBE cells, and reduced, but did not abolish endogenous TLR4 expression compared with empty plasmid-transfected NHBE cells. Interestingly, 12 putative GATA-3 binding sites (including 3 bi-directional sites at -866, -3,675 and -6,244 bp upstream of the first start codon of the TLR4 gene) are located upstream of the human TLR4 gene using the MatInspector Release professional 6.0 (www.genomatix.de). As discussed later, it will be desirable to assess the activity of GATA-3 on its binding sites upstream of TLR4. Taken together, these data suggest that GATA-3 contributes to the regulation of epithelial TLR4 transcription. Further investigation is certainly warranted to confirm and describe additional functions of GATA-3 in the airway epithelium.

4.4. Summary

In our first study (Diagram 12a), we studied the ability of circulating CD4-expressing leukocytes from children with and without history of atopic diseases, especially monocytes, to recognize and initial signal transduction and mediator production following exposure to LPS. We clearly demonstrated that monocytes

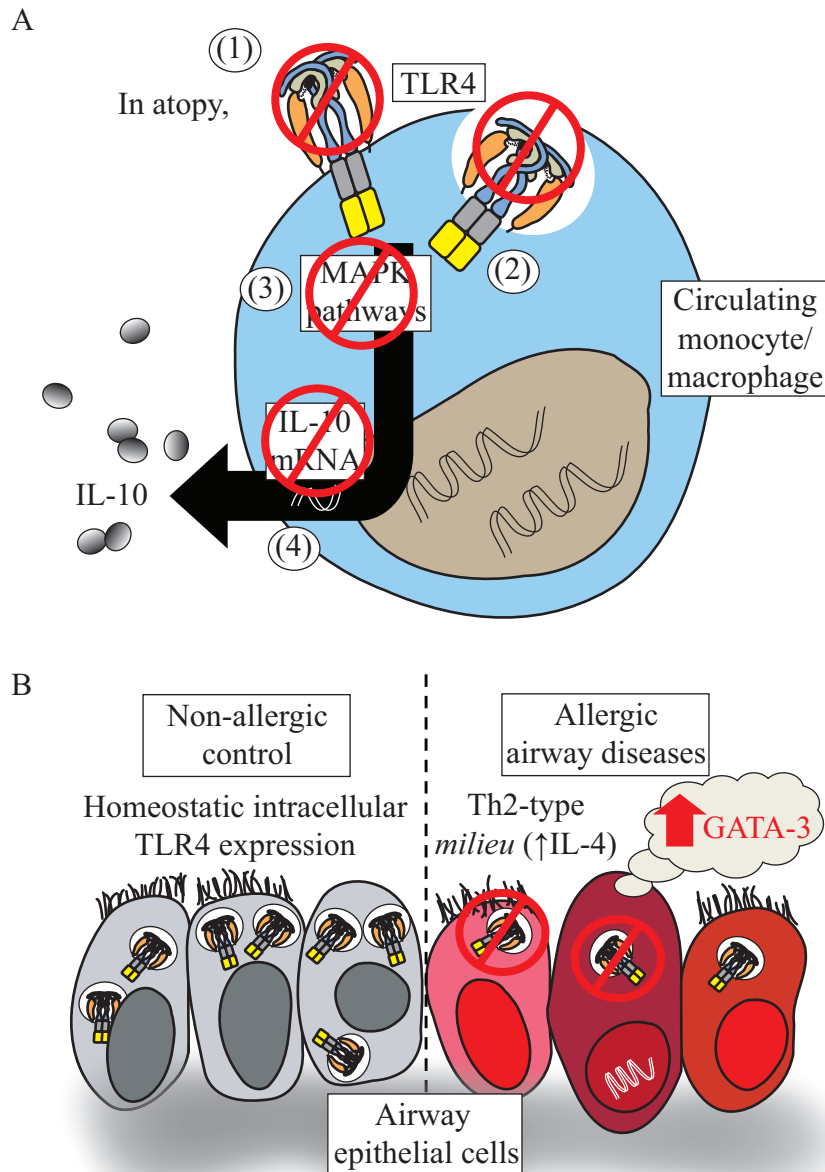


Diagram 12. Summary of results. A, Circulating monocytes from children with histories of atopic disorders display progressive age-dependent loss of TLR4 (1), defective LPS-induced reduction surface TLR4 expression (2) which suggest defective internalization, impaired LPS-induced signalling through the p42/44 ERK and p38 MAPKs (3), and impaired LPS-induced IL-10 mRNA expression when compared with cells from non-atopic children (4). B, Epithelial TLR4 expression in allergic rhinitis and asthma is reduced compared with non-allergic controls. The Th2-type conditions contribute to elevated epithelial GATA-3 expression. Our results suggest that elevated epithelial GATA-3 expression in allergies play a role in the TLR4 down-regulation.

from non-atopic children respond to LPS through $p42/44$ ERK and p38-MAPK protein phosphorylation, decrease in surface TLR4 expression, and initiate the expression of IL-10 anti-inflammatory cytokine expression. These processes were highly impaired in cells from children with atopic conditions. We also documented for the first time the TLR4 expression in a cross-sectional paediatric cohort and reported an inverse correlation between the age of subjects and TLR4 expression, only in monocytes from children with histories of atopic disorders. As this inverse correlation was not seen in controls, it may reflect a premature degeneration of TLR4-mediated responses to LPS in aging individuals with atopic histories compared with no atopic histories. This study provides a novel context for the hygiene hypothesis, which suggests bacterial LPS has immuno-regulatory effects during childhood and may limit the allergic phenotype later in life (592). Our results support other studies associating atopy with impaired TLR-mediated immune functions (1045) and LPS recognition by immune cells (734, 988, 991, 993), therefore suggesting that allergic histories may dampen innate immune functions associated to the LPS/TLR4 complex.

In our second set of experiments (Diagram 12b), we focused on the regulation of TLR4 expression by airway epithelial cells in the context of airway allergic diseases. The epithelial cells form the first physical and immune cell barrier interacting with inhaled bacterial invaders. We first demonstrated a reduced TLR4 expression by nasal and bronchial epithelial cells from patients

with allergic rhinitis and asthma, compared with non-allergic controls. Beside the predominant Th2-type inflammatory *milieu* observed in airway allergic diseases, elevated expression of the Th2-type transcription factor GATA-3 was detected in epithelial cells from asthmatic subjects compared with controls. Opposite regulation of TLR4 and GATA-3 could be reproduced *in vitro* using Th2-type cytokines, which respectively reduced TLR4 while increasing GATA-3 expression in epithelial cells grown *in vitro*. Finally, transient transfection experiments that aimed at silencing or over-expressing GATA-3 in cultured cells stressed that the transcription factor plays a role in TLR4 transcriptional regulation. These results also support the concept that epithelial innate immune functions may be impaired in airway allergic diseases and Th2-type inflammatory conditions (971, 1022). Despite GATA-3 expression by epithelial cells had been previously described (345), this study provides novel information about the regulation of its expression in allergic disorders and in Th2-type conditions. Finally, this study also provides the first evidence for a functional role for GATA-3 in epithelial cells.

4.5. Future directions

The current thesis raised several elements which would be of potential interest for further investigations. Some of these elements follow, along with brief background information and rationale.

- a) To investigate further the causes and consequences of a lack of TLR4 internalization in LPS-exposed monocytes from atopic/allergic subjects.

Beside the fact that LPS fails triggering the activation of MAPK pathways in monocytes from atopic children, LPS also fails to reduce TLR4 expression which likely reflect a defect in the TLR4 internalization process. It would be important to confirm this differential TLR4 trafficking through the use of confocal microscopy, and/or to study differential activation of dynamin proteins that regulate the budding of vesicles at the plasma membrane the formation of clathrin-coated pits (1053). Trafficking of LPS/TLR4 complexes through the endosomal pathway (761) is a critical step leading to late- or delayed-phase signal transduction and cell response to LPS via the TRIF pathway (section 1.3.4.3.). It would also be worth confirming whether the LPS-induced activation of the TRIF-dependent signalling pathways is also impaired in monocytes from atopic patients compared with those from controls. Early-phase signalling which is necessary for the maturation of antigen-presenting cells (1054), and for TLR4 complex internalization and signalling through TRIF (1055). This following delayed-phase signalling via the endosomal pathway critically regulates mediator production (765, 775, 776). TLR4 internalization also consists of a critical step in the phagocytosis of microbes and antigens by APCs. TLR4 trafficked to the late endosomes and lysosomes indeed colocalizes with functional MHC class II loading compartment therefore promoting antigen presentation and the development of adaptive responses through CD4+ T cells (761, 1056). The TLR4 internalization also correlates with endotoxin tolerance i.e. state of post-LPS

exposure unresponsiveness to further LPS stimulation as measured by altered mediator production (section 1.3.4.6.). Under normal conditions, TLR4 internalization reduces deleterious inflammatory burden potentially triggered by chronic LPS exposure. However, TLR4 internalization do not interfere with bacterial clearance through phagocytosis, neither with survival rates in sepsis models (1057). In contrast, pre-exposure of asthmatics to inhaled LPS limits APC-mediated phagocytosis of bacterial components (907). These observations suggest that atopy may not only limit TLR4 internalization but could also impair endotoxin tolerance mechanisms regulating phagocytosis, microbial clearance and the development of adaptive immune.

- b) To investigate further the molecular mechanisms or factors responsible for the atopy-specific dampening of LPS-induced TLR4-mediated signal transduction.

Allergic diseases including rhinitis and asthma typically feature Th2-type immune bias directed against innocuous allergens. In our study, the impaired ability of monocytes from children with history atopic conditions to recognize LPS and trigger signal transduction could be mimicked by pre-incubating cells from non-atopic donors with recombinant IL-4, but not with recombinant IL-4 that had been neutralized with an antibody. In line with this, data from previous studies demonstrated that monocytes exposed to IL-4 display reduced LPS-induced NF- κ B activation (972), TLR4 and CD14 expression (967, 970). Taken together,

these observations reinforce that Th2-type cytokine milieu, including IL-4, may negatively influence the expression and the function of the LPS receptor complex. However, our preliminary experiments suggested that neutralization of IL-4 in atopic cell cultures did not prevent the lack of LPS-induced signal transduction.

It would therefore be of considerable interest to study the differential expression of other factors modulating LPS receptor signalling in immune cells from both atopic/allergic subjects and non-atopic controls. Recent studies have described numerous modulators of innate immune functions which have not been extensively studied in the context of allergic disorders. For example, the suppressor of cytokine signalling-1 (SOCS-1) molecule, SOCS-1, has been associated with an inhibition of LPS signalling (1058, 1059). The expression of SOCS-1 is apparently inducible through IL-4 (1060). SOCS-1 would target the TIRAP adaptor protein for degradation, therefore reduce the activation of MyD88-dependent LPS-induced pro-inflammatory without altering the delayed-phase signalling pathways (1061). Atopy-specific regulation (possibly a down-regulation) of the IL-1 receptor-associated kinase M (IRAK-M) also remains an interesting hypothesis to explain changes in LPS responsiveness. The IRAK-M consists of a kinase-inactive protein repressing the activation of IRAK-4 and IRAK-1 part of the MyD88-dependent signalling pathway (1062) in monocytes/macrophages (1063).

Deficiencies in IRAK-M expression are associated with impaired endotoxin tolerance, the auto-inhibitory state of LPS responsiveness (1062).

With the emergence of microRNAs as critical regulator of immune response, it would be interesting to study the differential expression miR-147 in atopy and allergic diseases. miR-147 consists of a novel LPS/TLR4-induced microRNA which regulates excessive inflammatory responses through a negative-feedback loop (1064). Similarly, the nuclear factor-erythroid 2-related factor 2 (NRF2) is an LPS-induced transcription factor that regulates redox balance and stress responses. NRF2 would limit the activation of both MyD88- and TRIF-dependent pathways. NRF2 prevents excessive and deleterious LPS-induced inflammation, and sepsis-induced and mortality (1065). Furthermore, animals deficient in NRF2 display significantly worsened ovalbumin-induced experimental asthma symptoms compared with wild-type littermates (1066). It would therefore be interesting to also compare *in situ* NRF2 expression in atopic/allergic subjects and controls.

- c) To characterize the differential LPS-induced responses (i.e. early-, late-phase, and the endotoxin tolerance-associated responses) in cells from both atopic and allergic subjects by mean of genome-wide expression profiles

Although several studies and our results suggests differential LPS effects in atopy or allergic-type conditions (734, 988-991), there is currently a lack of

genome- and/or proteome-wide survey on the LPS-induced mediators comparing cells from controls to allergic patients. This could be achieved through the use of DNA microarrays (1067) and cytokine/chemokine arrays. It would be relevant to characterize the differential LPS response kinetics, especially discriminating the early- and late-phase LPS responses as well as the transcriptome of endotoxin tolerized cells from allergic and non-allergic individuals.

- d) To investigate additional functional roles of GATA-3 expression by epithelial cells

With respect to the project on epithelial cells and TLR4 expression in allergic disorders, our results support a role for GATA-3 in the transcriptional regulation of TLR4, possibly through its binding to putative consensus sequences detected upstream of the TLR4 gene. A first step would be to confirm such GATA-3 interactions with the TLR4 promoter, through the use of mutated TLR4 promoter sequences where the different GATA-3 sites would be deleted.

Transfecting these promoter variants within cultured epithelial cells using a luciferase reporter plasmid could serve to monitor the activity of the different GATA-3 binding sites, in the luciferase reporter assay. Chromatin immunoprecipitation and electrophoretic gel shift assays could also be used to confirm the binding of endogenous and recombinant GATA-3 to TLR4 promoter sequence either in live cells or *in vitro*. Furthermore, the identification of additional GATA-3 functions in epithelial cells is of definite interest considering

there is no information on any role for this lymphoid cell lineage-associated transcription factor in epithelial cells. Besides its role in the differentiation of Th2-type cells and the production of inflammatory cytokines, GATA-3 also likely play roles in the differentiation of skin stem cells into epidermal keratinocytes and the maturation of interfollicular epidermal cells surrounding the hair follicles (1016, 1068, 1069). It would be interesting to study GATA-3 in air-liquid interface cultured epithelial cells and determine its potential role in inflammatory mediator production (e.g. cytokines and chemokines), mucus production, epithelium integrity and repair (section 1.2.3.3.).

Beside its role as a promoter of Th2-type cytokine expression, GATA-3 in T lymphocytes also promotes the expression of ST2, a component of the IL-33 receptor (1070). It would be worth determining whether epithelial GATA-3 expression similarly promotes ST2 expression. IL-33 consists of a novel Th2-type pro-inflammatory cytokine whose role in allergies is not fully understood (188). The ST2 protein exists under two isoforms: one soluble form (sST2) which sequesters and limits the effect of IL-33, while the transmembrane form (ST2L) mediates IL-33-induced signalling. As an IL-1-type cytokine receptor, the ST2 signalling cascade shares many components with the TLR4 signalling and it was suggested that IL-33/ST2-mediated macrophage activation may regulate TLR4 expression, LPS/TLR4-mediated signalling and endotoxin tolerance in macrophages (1071, 1072). The IL-33/ST2 pathway induces Th2-type cell

differentiation (188), and our group recently demonstrated elevated expression of IL-33 in the lung of asthmatic subjects compared to controls (1073). We also demonstrated that IL-33 is predominantly expressed in the bronchial epithelium of asthmatics (1074). Furthermore, recent genome-wide association study conducted in subjects with asthma linked genetic variants near IL-33 to the disease (1075). Further studying of the interplay between GATA-3 and the ST2/IL-33 axis may shed light on the regulation of TLR4-mediated innate immune function in the asthmatic epithelium.

- e) To further characterize the regulation of GATA-3 expression by epithelial cells

Studies investigating the regulation of GATA-3 were mainly performed in CD4-expressing T cell models either *in vitro* or *in vivo*. As the master regulator of Th2-type cell differentiation (section 1.2.3.1.2.), GATA-3 expression increases upon T cell receptor (TCR) stimulation and proper co-stimulation of the T lymphocytes (152, 158). Additionally, IL-4-mediated activation of STAT6 also induces GATA-3 expression in T lymphocytes (173, 176). This mechanism is likely similar to what we demonstrated in cultured epithelial cells. In these experiments, Th2-type cytokines IL-4 and IL-13 both promoted GATA-3 expression. The specific contribution of STAT6 to these inductions is also worth being investigated in this cell type. Beside cytokines, it would be interesting to determine whether extrinsic stimuli contributing to phenotypic changes of the

asthmatic epithelium also regulate GATA-3 expression. House dust mite allergen-derived proteases (like Der p 1, 3 and 9) consist of interesting candidates since they activate epithelial cells through protease-activated receptors (PARs), induce the release of inflammatory mediators and drive Th2-type allergic immune responses *in vivo* (247, 313).

4.6 Implications of the results

Our first study described the defective LPS-induced cell signalling and peripheral immune cell responses in children with atopic histories compared with cells from non-atopic controls. These results should be considered in the context of TLR4 agonist-based immunotherapies or adjuvants destined to skew the developing immune system away from pro-allergic (Th2-type) responses to innocuous allergens. The efficacy of LPS to prevent the allergic sensitization and disease phenotype was extensively studied in animal models of experimental allergic asthma (section 1.3.4.5.). When used as a prophylactic adjuvant before allergen sensitization, inhaled LPS limited allergen sensitization, the allergen-induced Th2-type inflammation and prevented the development of airways response (905, 917-919). TLR4 was instrumental in mediating this protection, as demonstrated in knockout animal studies (899, 900). Similarly, lipid A extracts administered orally also prevented the development of OVA challenge-induced airway inflammation and hyperresponsiveness in sensitized animals without altering the IgE titers (937). In this model, lipid A increases the numbers of T-reg

cells in the airways. Interestingly, newborn animals from mothers that were exposed to Gram-negative *Acetivobacter* bacteria during pregnancy were protected against experimental asthma (both OVA sensitization and airways response) (924, 937). This protection was dependent on the expression of various TLRs, including TLR4, by the mother. Similarly, newborn animals receiving LPS intra-nasally during the first 2 weeks of life were also protected against the development of OVA-specific experimental allergic asthma (921, 924, 937). The LPS-induced allergen tolerance was reflected by the induction of activated T-reg-like cells producing IL-10. In our laboratory, LPS applied to explanted nasal mucosa explants from atopic children (i.e. 3-4 years of age) prevented allergen-induced Th2-type inflammation also in an IL-10 dependent manner, but this protective effect of LPS was not observed in tissues from 35 years-old adults (736). These results are in line with the controversial results obtained from protocols in which LPS was administered after sensitization: LPS applied shortly after sensitization remained protective while LPS administered 4 days after sensitization exacerbated allergic disease phenotypes (919). Taken together, these results suggest a yet to be defined window of opportunity for influencing i.e. tolerizing the developing immune system of children against allergens, possibly using TLR4 agonists. In this context, our results reinforce that the immune-modulatory and allergy-preventing effects of LPS would take place earlier than 13 years of age since we observed that LPS signalling and IL-10

induction was already impaired in 12 to 14 years-old children with atopic histories. Our study reinforces the concept that differences in TLR4 signalling due to atopy establish during childhood (<14 years-old). Furthermore, we similarly observed an inverse correlation between the age of atopic children, but not non-atopics, and the TLR4 expression by monocytes. Interestingly, it appears that TLR4 expression on monocytes began to decrease after 13 years age in atopic children, when compared with cells from non-atopic children. Our results therefore reinforce that preventive therapies during early life may be more efficient at limiting atopic disorders than therapeutic interventions in adolescent with documented histories of atopic disorders.

Common allergy and asthma pharmacotherapies include the use of anti-histamines, anti-inflammatory glucocorticoids (e.g. oral, topical or inhaled steroids) and β 2-adrenergic receptor agonists to relief symptoms allergic reactions. While the anti-histamines mainly block the histamine-induced swelling and vasodilation, they are also associated with drowsiness. Glucocorticoids consist of the gold-standard in term of anti-inflammatory, but they can be associated with side-effects including immune-suppression, resistance, growth delay in children, hormonal imbalance, etc. Finally, short and long-acting β 2 agonists limit bronchoconstriction during AHR episodes but some molecules were associated with increased risk of exacerbations. Besides the pharmacotherapies, specific immuno-therapy (SIT) (1076) aiming at progressively tolerizing the

immune system to allergens represents an attractive alternative to limit symptoms and progression of allergic diseases, as well as to reduce the use of other medications. However, SIT remains a long-lasting procedure with variable efficiency in the long-term. It is also inherently associated with a risk of systemic allergic reactions, significantly reduced when allergens are administered through the sublingual route. One of the new approaches for SIT consists of supplementing allergen extracts with TLR agonists (27) as adjuvants skewing the allergen-induced response away from type-2 immunity. Promising data were yielded using a prototypical TLR9 ligand, CpG oligodeoxynucleotides, to promote Th1-type responses and limit responses to allergens in human subjects (1077, 1078). Similarly, synthetic TLR7/8 agonist called resiquimod has also gone through successful animal testing using ovalbumin-specific experimental asthma immunization protocols (1079, 1080). One should however keep in mind the controversial LPS effects in experimental models of allergic diseases and asthma. Therefore, more studies are certainly needed to address the potential immuno-regulatory role of TLR4 ligand-supplemented SIT in animal models before it can be used on patients at the clinic.

Our second study described the up-regulation of epithelial cell-specific GATA-3 expression in the context of airway allergic diseases may be interpreted in the context of GATA-3 interference-based therapies against allergic diseases. Recent studies indeed validated the use of GATA-3-specific DNAzyme and of

RNA interference (1081, 1082) in murine models of experimental allergic asthma in. The DNAzymes consists of antisense molecules combining the specific of DNA base pairing with inherent RNA-cleaving enzymatic activity. The GATA-3-specific DNAzyme was delivered intranasally and successfully reduced OVA challenge-induced Th2-type airway inflammation and hyperresponsiveness in both acute and chronic models of allergic asthma (1082). The DNAzymes were also significantly taken up by surfactant-coated airway epithelial cells. Similarly, intra-tracheal instillation of GATA-3-specific short hairpin RNA (shRNA)-encoding lentiviral vectors also limited OVA-induced Th2-type inflammation and AHR compared with animals treated with mock vectors (1081). Our results showed that GATA-3 is induced by IL-4, the latter which mainly signals through STAT6 (section 1.2.3.1.2.). Inhibition of STAT6 expression and activation specifically in airway epithelial respectively limit IL-13- and OVA-induced experimental asthma features including inflammation and AHR (356, 1051). Interestingly, the mice that received intra-nasal administration of STAT6 inhibitory peptides (i.e. binds and sequesters phosphorylated STAT6) displayed predominant peptide localization to the airway epithelium (1051). Combined to our work on epithelial cells, these promising results in animal models suggest that targeting epithelial cell-specific GATA-3 expression may consist of another potential therapeutic target for treating allergic airway diseases. Future experiments using bronchial epithelial cell-specific GATA-3-deficient mouse subjected to classical OVA

sensitization/challenge protocol would certainly contribute determining the functional role(s) of epithelial GATA-3 in the context of these disorders.

To maintain homeostatic conditions, immune responses to pathogens (and LPS) have to be tightly regulated. On one hand, an uncontrolled inflammatory response can cause tissue damage, impair organ functions and septic shock. On the other hand, a failure to recognize and clear pathogens or an excessive microbial tolerance can promote persistent and deleterious infections similar to what is seen in TLR4-deficient animals (706) or immuno-compromised individuals (1083). Both uncontrolled and defective responses to microbes can lead to life-threatening conditions. One could therefore question whether our demonstration of an impaired TLR4-mediated innate immune function by monocytes, and TLR4 expression in epithelial cells of subjects with airway allergies imply a reduced ability of atopic individuals to clear bacteria and could reflect an increased susceptibility to bacterial infections. Some experimental and epidemiological evidence indeed suggest it may be the case. The airways and the blood are no longer seen as sterile tissues, since minute LPS amounts remain detectable in the serum of healthy subjects (section 1.3.4.4.) while recent studies have started characterizing the lung mucosa-associated microflora (1084). A recent phylogenetic screen of the microbes in human bronchial brushing samples revealed significantly different microbiota in the airways of patients with bronchial asthma compared with healthy controls. The asthmatic airways contained

significantly elevated proportion of Gram-negative *Proteobacteria* species (and Gram-positive species) compared with those of normal controls (1084).

Furthermore, a majority of subjects suffering from asthma additionally develop upper airway symptoms referred to as allergic rhinitis and/or chronic rhinosinusitis (CRS) (447, 1085). The latter is defined as a prolonged inflammatory condition of the paranasal sinuses and nasal passages, and often involved a persistent infectious component. The microbes associated with CRS infection commonly include *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Moraxella catarrhalis* and *Haemophilus influenzae* bacterial species, and/or fungal colonization of the mucus by *Aspergillus* or *Alternaria* species. There is also a growing debate regarding the incidence of sessile microbial biofilms in CRS; these *in situ* microbial reservoirs may contribute to the symptoms' persistence and resistance to pharmacological therapies (1086). It appears conceivable that dysregulated TLR4-mediated immune function may contribute to explain the incidence of recurrent bacterial infection in individuals with allergies and/or CRS.

Our work supported previous literature (734, 988, 990, 991, 993) and provided additional evidence that TLR4-mediated innate functions are impaired in atopy and airway allergic diseases. Experiments conducted in TLR4-deficient animals demonstrated that the lack of TLR4 signalling not only impairs the response to Gram-negative strain-derived LPS but also to the Gram-positive strain-derived *S. aureus* enterotoxin B (SEB; a bacterial superantigen) (1087).

Asthma is also associated with increased incidence of upper airway colonization by *S. aureus* producing *Staphylococcal* enterotoxin B (SEB) superantigen and driving the generation of the SEB-specific IgE (1088). Furthermore, studies characterizing the oropharyngeal, blood and cerebrospinal fluid microbiota of adults indeed described asthma as a risk factor for *S. pneumonia* colonization and infections (1089, 1090). Other groups have also demonstrated that cells from allergic asthmatic patients also display defective innate immune pathways, including impaired anti-viral responses (341, 1022) and dampened TLR7/8-mediated functions. Taken together, this thesis is in line with studies showing that atopy and inflammatory conditions associated with allergies may impair the ability of our immune system to respond and clear pathogens.

4.7 Conclusion and perspectives

This thesis should be put in the modern and revisited context of the hygiene hypothesis. Beside the strong genetic basis of atopy and observational studies reporting that infants from atopic families are at high risk of developing allergic diseases, the original skewing of the newborn's immune system toward a Th2-type phenotype is still considered as a relevant explanation for the increased allergy rates among paediatric populations. Patrick Holt's group provided immunologists with the first experimental evidence for a delayed postnatal maturation of adaptive immune functions in infants at high risk of allergies (1091). Soon after the discovery of the Th1/Th2 subtypes, they showed that their T cell

clones from high-risk children produced lower levels of Th1 cytokines, the latter which counter-balance the original Th2 skewing. Furthermore, allergic diseases are also associated with a failure to mount tolerant adaptive immune responses to allergens, due to impaired regulatory T cell-mediated functions (650). Taken together, this suggested a defect in the transition from the quiescent and Th2-polarized fetal immune system to the active and more balanced immune system of adults in high-risk and atopic individuals. Studies on the hygiene hypothesis greatly contributed to identify microbial infections and LPS exposure as factors driving this normal maturation of the immune system in early life.

Although T cells belonging to the Th2 phenotype orchestrate the allergic immune response, it is now well-described that T cells are only one component of a complex immune network of innate and adaptive immune cells and mechanisms likely also compromised in individuals genetically predisposed to atopy. Among the innate immunity mechanisms and cells critical to the development of allergies, the monocytes/macrophages were long described as immune-regulatory cells since lung macrophage-depleted animals displayed worsened asthma phenotypes to inhaled allergens (245, 260-263). Monocytes also consist of the most important LPS-sensitive cell population in the periphery. In the airways, epithelial cells are increasingly seen as critical immune players as they quickly respond to inhaled stimuli and environmental microbes through the expression of innate immune receptors, like TLRs and the LPS receptor TLR4.

The epithelial cell TLR4-mediated response produces various mediators influencing adaptive immune responses to inhaled allergens as well as the lung physiology (1021).

In this thesis, we first studied the LPS responsiveness of peripheral monocytes from children with histories of atopic diseases and demonstrated that their TLR4-expressing failed to properly activate some of the cell signalling pathways normally activated by LPS compared with cells from non-atopic controls. This impaired LPS response in cells from atopic children contributes explaining a failure to up-regulate their expression of the IL-10 immunosuppressive cytokine. Secondly, we confirmed that on-going allergic diseases in adults and Th2-type conditions reduced TLR4 expression by airway epithelial cells. Interestingly, this was reflected by an increased epithelial expression of the Th2-type transcription factor GATA-3, which potentially plays a role in the TLR4 down-regulation. Taken together, we demonstrated a negative-feedback phenomenon by which atopy and Th2-type inflammation limit the ability of our immune cells to sense LPS. This retroaction would take place through dysregulated LPS-induced signalling pathways in atopy and impaired TLR4 expression in epithelial cells in on-going allergic diseases.

Despite extensive research efforts, the role of LPS in allergies and the hygiene hypothesis remains controversial. Endotoxin is also not the only ligand for TLR4. One should therefore not exclude the plausible contribution of other

PAMPs (and PRRs) as immunological adjuvants in the development of allergic disorders. Our interpretation of the impaired TLR4 functions in atopy and allergies is two-fold. Based on the hypothesis that LPS can exacerbate on-going inflammation and allergic disease manifestations, a down-regulation of TLR4 functions and expression on immune cells of subjects with atopic histories and allergic diseases can be seen as a protective mechanism of our immune system to limit LPS-mediated worsening of hypersensitive reactions. On another hand, microbial and LPS exposures have been extensively associated with an immune skewing protective against the development of Type I hypersensitivity and allergic disorders. From this point of view, defective TLR4 expression and TLR4-mediated innate immune responses may consist of a negative-feedback loop reinforcing the allergic phenotype and the imbalance toward Th2-type immunity. In these conditions, TLR agonist-based therapies designed to prevent the development of allergic diseases would rather be more effective early during childhood and in the absence of atopy than in children (>13 years of age) with atopic histories or in adults with on-going allergic diseases. This thesis work provides insights into the counter-regulatory effects of atopy and the allergic inflammation on TLR4-mediated innate immune functions.

Contribution of authors

The following manuscripts, which have been published or submitted for publication in peer-reviewed journals, are part of the thesis work by David Préfontaine. The manuscripts are followed by contributions of each author:

1. Préfontaine D, Banville-Langelier AA, Fiset PO, Guay J, An J, Mazer M, Hamid Q, Mazer BD. Children with atopic histories exhibit impaired lipopolysaccharide-induced Toll-like receptor-4 signalling in peripheral monocytes. *Clin Exp Allergy*. 2010 Nov; 40(11): 1648-57.

David Préfontaine played the greatest role in the production of this manuscript. He designed and performed a major portion of the experiments, data collection and analysis, as well as writing the manuscript. Ms. Andree-Anne Banville-Langelier also contributed to collecting, processing blood samples and performing experiments. Dr. Fiset and Ms Guay contributed to elaborate the initial experimental design. Dr. An and Monty Mazer contributed to data analysis. Drs. Qutayba Hamid and Bruce D. Mazer were senior authors as they oversaw the work of this manuscript and were involved in the editorial process. Permissions of reusing parts of this published manuscript for this thesis can be found in the section on Research compliance certificates and permissions.

2. Préfontaine D, Mogas Andrea K, Al-Ramli W, Lajoie-Kadoch S, Semlali A, Chakir J, Lavigne F, Frenkiel S, Hamid Q. Down-regulation of epithelial Toll-like receptor 4 in asthma and rhinitis: a possible role for GATA-3. Submitted to *Clin Exp Allergy*.

David Préfontaine played the greatest role in the production of this manuscript. He designed and performed most of the experiments, data collection and analysis, as well as writing the manuscript. Ms. Andrea K. Mogas helped with some of the siRNA experiments while M. Wisam Al-Ramli performed some of the immunocytochemistry experiments. Dr. Lajoie-Kadoch contributed to produce and characterize the GATA-3 over-expression plasmid. Drs. Semlali and Chakir isolated the primary bronchial epithelial cells from asthmatic subjects and normal controls. Drs. Lavigne and Frenkiel conducted surgical resections of nasal mucosa tissues from patients. Dr Hamid was the senior author as he oversaw the work of this manuscript and was involved in editing it.

Publications during Ph.D. training

1. **Préfontaine D**, Banville-Langelier AA, Fiset PO, Guay J, An J, Mazer M, Hamid Q, Mazer BD. Children with atopic histories exhibit impaired lipopolysaccharide-induced Toll-like receptor-4 signalling in peripheral monocytes. *Clin Exp Allergy*. 2010 Nov;40(11):1648-57.
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