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Toll-like Receptor Activation in Experimental Allergic Asthma

Bridge between skin and airways

ACADEMIC DISSERTATION
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1. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers:

I. Lehto M, Haapakoski R, Wolff H, Majuri ML, Mäkelä MJ, Leino M, Reunala T, Turjanmaa K, Palosuo T, Alenius H. Cutaneous, but not airway, latex exposure induces allergic lung inflammation and airway hyperreactivity in mice. *Journal of Investigative Dermatology* 2005, 125(5):962-8.

II. Haapakoski R, Karisola P, Fyhrquist N, Savinko T, Wolff H, Turjanmaa K, Palosuo T, Reunala T, Lauerma A, Alenius H. Intradermal CpG treatment reduces lung inflammation but induces IFN-γ mediated airway hyperreactivity in a murine model of natural rubber latex allergy. *American Journal of Respiratory Cell and Molecular Biology* 2011, 44(5):639-647.

III. Haapakoski R, Karisola P, Fyhrquist N, Savinko T, Lehtimäki S, Wolff H, Lauerma A, Alenius H. Toll-like receptor activation during cutaneous allergen sensitization blocks development of asthma through IFN-γ dependent mechanisms. *Journal of Investigative Dermatology* 2012, advance online publication, November 15, 2012.

2. ABBREVIATIONS

AHR	airway hyperresponsiveness
APC	antigen presenting cell
BAL	bronchoalveolar lavage
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CpG	cytosine-phosphate-guanosine
DC	dendritic cell
H&E	haematoxylin and eosin
ELISA	enzyme-linked immunosorbent assay
i.d.	intradermal
IFN	interferon
Ig	immunoglobulin
IL	interleukin
i.n.	intranasal
i.p.	intraperitoneal
ISS	immunostimulatory sequence
IT	immunotherapy
LN	lymph node
LPS	lipopolysaccharide
MCh	metacholine
NRL	natural rubber latex
ODN	oligodeoxynucleotide
OVA	ovalbumin
PAMP	pathogen associated molecular pattern
PAS	periodic acid-Schiff
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
TCR	T cell receptor
Th	T helper
TLR	Toll-like receptor

3. ABSTRACT

The increasing prevalence of asthma in western countries during the last decades continues to represent a significant public health problem, causing both economical and social burdens to society. Possible explanations behind this trend remain largely unknown, but both genetic and environmental factors are known to be involved. One of theories attempting to explain the rise in asthma prevalence is related to reduced exposure to microbial load and a change in Th1/Th2 cytokine balance towards the Th2-type immunity in early childhood ("hygiene hypothesis"). Micro-organisms present in the environment are recognized by different Toll-like receptors (TLRs), which bind viral and bacterial components and stimulate innate immune responses. TLR ligands have been used as immunotherapeutic agents to modify the response away from allergies and towards the Th1-type immune response. In spite of intensive investigation, the role of systemically or locally administered Toll-like receptor ligands in modulating asthmatic response is still controversial and more investigations are needed to clarify the detailed mechanisms.

The specific approaches used in this study included (i) characterization of mouse a model of natural rubber latex (NRL) –induced asthma by examining the effects of different exposure routes on airway inflammation and airway reactivity, (ii) investigation of the effects of CpG (ligand for TLR9), and (iii) the effects of LPS (ligand for TLR4), Pam_3Cys (ligand for TLR2) and Poly(I:C) (ligand for TLR3), on local and systemic inflammation and airway response using the mouse model.

In the first study, mice were sensitized by intracutaneous (i.c.), intraperitoneal (i.p.) and intranasal (i.n.) route with natural rubber latex (NRL), followed by three-day airway NRL challenge (**I**). Four weeks after NRL sensitization, mice sensitized by i.c. and i.p. route exhibited a drastic eosinophilic inflammation in BAL fluid, an enhanced amount of mucus around the bronchioles and increased airway reactivity to metacholine. Expression of Th2 type cytokines IL-4 and IL-13 and CC chemokines CCL1, CCL8, CCL11, and CCL24 in the lung tissue as well as IgE antibodies in the serum were also significantly elevated. On the other hand, i.n. NRL exposure induced the expression of regulatory cytokines IL-10, Foxp3 and TGF-β in

the lung tissue. These results highlight the role of skin as the primary site of antigen exposure and subsequent development of airway diseases.

In the second study, the effects of immunostimulatory CpG molecules were investigated in the murine asthma model characterized in the first study of the thesis (**II**). CpG significantly reduced airway inflammation, mucus production and Th2 type cytokines in the lung tissue, but unexpectedly enhanced airway hyperreacivity to inhaled metacholine. At the same time, the amount of the main Th1-type cytokine, IFN-γ, and CD8+ T cells were induced in the lungs after CpG administration. It was shown that CD8+ T cells were responsible for production of most of the intracellular IFN-γ. Finally, three strains of KO mice (RAG1-/-, STAT4-/- and STAT6-/-) and lung IFN-γ neutralization studies were used to verify the dependence of enhanced AHR on Th1-type immunity and increased production of IFN-γ in the lungs. These results indicate that treatment with Th1-inducing CpG nucleotides may also cause side-effects and should be taken into consideration when planning immunotherapeutic treatments for asthma.

In the third study, the effects of intradermally administered LPS, Pam_3Cys and Poly(I:C) were assessed in a murine model of asthma using ovalbumin as allergen (**III**). Administration of LPS and Pam_3Cys reduced total cell counts and eosinophils in BAL fluid, Th2-type cytokines in the lung and airway reactivity to metacholine, while Poly(I:C) had no clear effect on these parameters. During the early phase of the sensitization, both LPS and Pam_3Cys reduced the number of CD11c+ DCs in the lymph nodes, and especially LPS enhanced the expression of activation markers CD80 and CD86. Interestingly, the number of CD8+ T cells and their production of IFN-γ were significantly induced after LPS treatment. Using IFN-γ neutralization, it was revealed that the capacity of LPS to downmodulate inflammatory response in mice was critically dependent on enhanced production of IFN-γ in the airways. These studies are in line with the hygiene hypothesis suggesting that bacterial endotoxins, i.e. LPS, may induce Th1-type responses and protect from development of asthma later in life.

In conclusion, these results emphasize the importance of the skin as the primary route of allergen sensitization and subsequent development of airway responses to

inhaled allergen. The diverse immunomodulatory effects of intradermally administered TLR ligands on asthmatic response were critically dependent on the dose, amount and timing of ligand administration. The protective role of bacterial endotoxins on inflammatory response was in favour of the hygiene hypothesis, emphasizing the importance of host-microbe interactions in the development of airway diseases.

TIIVISTELMÄ

Astman lisääntyminen viime vuosikymmeninä on erityisesti länsimaissa esiintyvä ongelma. Syyt tähän kehitykseen ovat suurelta osin tuntemattomia, mutta sekä geneettisillä että ympäristötekijöillä tiedetään olevan merkitystä. Yksi allergioiden lisääntymistä selittävä teoria on nk. hygieniahypoteesi, jonka mukaan vähäinen mikrobialtistus varhaisessa lapsuudessa saa aikaan elimistön Th1/Th2 sytokiinitasapainon siirtymisen Th2- suuntaan johtaen allergioiden lisääntymiseen. Ympäristössä esiintyvät mikrobit tunnistetaan erilaisten Toll-like reseptorien (TLR) avulla, jotka tunnistavat virusten ja bakteerien rakenteita ja aktivoivat luonnollisen immuniteetin järjestelmän. Reseptoreihin sitoutuvia ligandeja on käytetty immunoterapiassa suuntaamaan immuunivastetta allergioilta suojaavaan Th1-suuntaan, mutta tulokset ovat olleet vaihtelevia erityisesti astman hoidossa.

Väitöskirjan ensimmäisessä osassa tutkittiin eri herkistymisreittien vaikutusta astman syntymiseen, sekä kehitettiin astman hiirimalli käyttäen luonnonkumia (NRL, natural rubber latex) allergeenina. Toisessa ja kolmannessa osakokeessa tutkittiin eri Toll-like reseptoriligandien (CpG; TLR9:n ligandi LPS; TLR4:n ligandi, Pam_3Cys; TLR2:n ligandi ja Poly(I:C); TLR3:n ligandi) vaikutuksia astman hiirimallia käyttäen.

Väitöskirjan ensimmäisessä osassa hiiret herkistettiin luonnonkumille ihon, vatsaontelon tai hengitysteiden kautta, jonka jälkeen hiiriä altistettiin luonnonkumille hengitysteitse kolmen päivän ajan. Neljän viikon herkistyksen jälkeen sekä ihon että vatsaontelon kautta herkistetyillä hiirillä havaittiin merkittävä hengitysteiden reaktiviteetin nousu metakoliinille sekä keuhkojen eosinofiilisten tulehdussolujen ja liman erityksen lisääntyminen. Keuhkojen Th2-sytokiinien IL-4 ja IL-13:n ja CC-kemokiinien CCL1, CCL8, CCL11 ja CCL24 lähetti-RNA -konsentraatiot sekä seerumin IgE vasta-ainepitoisuudet olivat myös selvästi koholla. Hengitysteiden kautta herkistettyjen hiirten keuhkoissa sen sijaan havaittiin immuunivastetta hillitsevien välittäjäaineiden, IL-10, Foxp3 ja TGF-β:n, lisääntyminen. Tulokset osoittivat että ihon kautta tapahtuva herkistys allergeenille ja sitä seuraava hengitystiealtistus sai aikaan merkittävän Th2-tyyppisen tulehduksen ja hengitysteiden reaktiviteetin nousun, osoittaen ihon kautta

tapahtuvalla herkistymisellä olevan merkitystä lisääntyneessä alttiudessa sairastua astmaan ja allergioihin.

Väitöskirjan toisessa osassa tutkittiin immunostimulatoristen CpG-molekyylien vaikutuksia astman kehittymiseen. Ihonsisäisesti annosteltu CpG vähensi selvästi keuhkojen tulehdusta, limantuotantoa sekä Th2-sytokiinien määrää, mutta tutkimuksessa havaittiin yllättävä hengitysreaktiviteetin lisääntyminen metakoliinille. Samanaikaisesti Th1-sytokiinin, IFN-γ:n, sekä IFN-γ tuottavien CD8+ T -solujen määrä lisääntyi keuhkoissa. Kolmea poistogeenistä hiirikantaa (RAG1-/-, STAT4-/- ja STAT6-/-) sekä keuhkojen IFN-γ neutralisaatiota käyttämällä voitiin osoittaa hengitysreaktiviteetin kasvun olevan suorassa yhteydessä Th1-vasteen nousuun ja lisääntyneeseen IFN-γ tuotantoon keuhkoissa. Tulokset antavat viitteitä siitä, että Th1-vastetta stimuloivien CpG nukleotidien käyttö voi aikaansaada myös ei-toivottuja sivuvaikutuksia jotka tulisi ottaa huomioon immunoterapeuttisia tutkimuksia suunniteltaessa.

Väitöskirjan kolmannessa osakokeessa tutkittiin kolmen TLR ligandin (LPS, Pam_3Cys ja Poly(I:C) immunomodulatorisia vaikutuksia hiiren astmamallissa. Ihonsisäisesti injektoidut LPS ja Pam_3Cys laskivat kokonaissolu- ja eosinofiilimääriä BAL-nesteessä, Th2-sytokiineja keuhkoissa ja keuhkoreaktiviteettia. Poly(I:C):lla sen sijaan ei ollut vaikutusta näihin parametreihin. Herkistyksen alkuvaiheessa imusolmukkeista mitattujen CD11c+ DC solujen lukumäärä laski sekä LPS että Pam_3Cys -ligandia saaneilla hiirillä. LPS stimuloi aktivaatiomarkkereiden CD80 ja CD86 ilmentymistä, sekä lisäsi CD8+ T solujen suhteellista lukumäärää ja IFN-γ:n tuotantoa. IFN-γ:n neutralisaatiolla pystyimme osoittamaan LPS:n aikaansaavan inflammaation vähenemisen olevan riippuvainen IFN-γ:n lisääntyneestä tuotannosta keuhkoissa. Saadut tutkimustulokset tukevat nk. hygieniahypoteesia, jonka mukaan bakteeriperäiset endotoksiinit, kuten LPS, aikaansaavat Th1-vasteen nousua ja ehkäisevät astman kehittymistä myöhemmällä iällä.

Saadut tulokset osoittavat ihon merkityksen allergeenille herkistymisessä sekä astmaattisten oireiden kehittymisessä annettaessa allergeenia hengitysteiden kautta. Ihonsisäisesti annosteltujen TLR ligandien moninaiset vaikutukset astmaattisiin

oireisiin olivat riippuvaisia sekä ligandien annoksesta että annostelureitistä ja -ajankohdasta. Bakteerien endotoksiinien inflammaatiolta suojaava vaikutus tukee hygieniahypoteesia, sekä painottaa isännän ja mikrobien välisen vuorovaikutuksen merkitystä hengitystiesairauksien kehittymisessä.

4. INTRODUCTION

Asthma is a chronic inflammatory disease of the airways characterized by episodic attacks of airflow obstruction, wheezing, coughing and shortness of breath. Many risk factors contribute to the severity and expression of asthma and these include both environmental and occupational exposure as well as genetic associations. It is estimated that over 300 million people around the world suffer from asthma globally and the number of people affected with asthma with current rising trends will grow by more than 100 million by 2025 (World Health Organization 2007, Holgate *et al.*, 2011). One reason for this trend has been claimed to be restricted exposure to microbial infections during childhood considered to favour the development of Th2-biased immunity and allergic diseases later in life. This so-called hygiene hypothesis first proposed by Strachan (1989) is supported by the observation that children growing up on a farm are exposed to more Th1-skewing cytokines in response to invading pathogens and thus have a reduced risk of developing asthma and atopic disorders.

The sequential progression of allergic diseases from atopic dermatitis (AD) to allergic rhinitis and asthma is known as the atopic march (Spergel, 2010). It is estimated that 60% of children with AD will develop asthma, allergies or both by 3 years of age (Boguniewicz and Leung, 2010). The evidence suggests that impairment or a defect in the epidermal barrier promotes the delivery of allergens across epithelia, highlighting the importance of the skin in sensitization and subsequent development of airway responses to inhaled allergens (Akei *et al.*, 2006). In asthmatic patients, the airways become infiltrated by CD4+ Th2 cells, degranulated mast cells and eosinophils, which evoke the key pathological features of the disease such as mucus hypersecretion, airway inflammation and bronchial hyperresponsiveness. Th2 cells are involved in the initation and maintenance of the inflammatory response by producing cytokines e.g. IL-4, IL-5 and IL-13. Th2 cells also stimulate the activation and proliferation of B cells into IgE-producing plasma cells. In addition to the conventional Th1/Th2 paradigm, an increased amount of Th1-type cytokine IFN-γ in the lungs of asthmatics has been related to airway inflammation and airway hyperreactivity, thus supporting the existence of diverse forms of asthma.

Immune responses against pathogens are initiated by TLRs, a family of at least 12 membrane proteins that have a critical role in both innate and adaptive immune responses. TLRs recognize invading microbes such as bacteria, viruses, parasites and fungi since these micro-organisms possess common structures called pathogen-associated microbial patterns (PAMPs) (Akira *et al.*, 2001; Janeway and Medzhitov, 2002). Recognition of microbial components by TLRs initiates signal transduction pathways, which trigger the expression of genes controlling innate immunity responses (Akira *et al.*, 2006). Different TLRs serve as receptors for a wide variety of ligands, including lipoproteins (TLR2), lipopolysaccharide or LPS (TLR4), viral single stranded (ss)RNA (TLR7,8) and CpG motifs of bacterial DNA (TLR9). TLRs and their ligands play an important role in antigen presentation and in immune modulation through activation of dendritic cells and regulating the immune response towards Th1, Th2 or Treg immunity (Feleszko *et al.*, 2006). For these reasons, the use of various TLR ligands in immunotherapeutic studies of allergic diseases such as asthma has attracted considerable interest in recent years.

In the first part of the thesis project a murine model of asthma was characterized with intradermal allergen sensitization being used to induce a local and systemic allergic response (**I**). This model was used to investigate the immunomodulatory effects of several Toll-like receptor ligands (CpG, ligand for TLR 9), LPS (TLR4 ligand), Pam_3Cys (TLR2 ligand) and Poly(I:C) (TLR3 ligand), on the outcome of pulmonary inflammation, systemic response and AHR in mice (**II-III**). NRL was used as the antigen when studying the effects of CpG, and ovalbumin was utilized in the studies elucidating the effects of LPS, Pam_3Cys and Poly(I:C) on asthma.

5. REVIEW OF THE LITERATURE

5.1. The immune response

The immune response has classically been divided into two parts: innate and adaptive immunity protecting the host from micro-organisms present in our environment (Janeway *et al.*, 2005). The innate immune system serves as the initial immune defence against foreign material by targeting conserved structures of microbial components. Innate immune responses are initiated immediately after recognition of foreign antigens and they provide a fast and non-specific mechanism leading to the elimination of invading pathogens through several distinct effector mechanisms (Janeway and Medzhitov, 2002). Adaptive immunity, on the other hand, is orchestrated by two types of T lymphocytes, T and B cells, which recognize pathogens with high specificity. Adaptive immunity responds with relatively delayed kinetics and it involves cell proliferation, gene activation and protein synthesis leading to antigen-specific cell responses and finally to the induction of immunological memory (Cooper and Alder, 2006). Both innate and adaptive immunity depend on the ability of the immune system to distinguish between self and non-self molecules and to discriminate between host and pathogenic cells, which contributes to the induction of an appropriate adaptive immune response.

5.1.1. Innate immunity and Toll-like receptors

Innate immunity is an evolutionary ancient component of the host defence, since it is present in all multicellular organisms (animals and plants) (Hoffmann *et al.*, 1999). Innate immunity relies on two sets of cells, hematopoietic and nonhematopoietic cells. The hematopoietic cells include macrophages, dendritic cells, mast cells, neutrophils, natural killer (NK) cells and NK T cells, while nonhematopoietic comprise of intact skin and epithelial cells lining the respiratory, gastrointestinal and genitourinary tracts (Turvey and Broide, 2010). Macrophages, neutrophils and dendritic cells are able to phagocytose, i.e. ingest bacteria and other foreign cells, while NK cells release enzymes and other substances that damage the

outer membranes of foreign cells. Innate immune responses against pathogens are initiated by TLRs expressed on antigen presenting cells such as macrophages and dendritic cells.

TLRs are transmembrane proteins that are able to detect microbial components or pathogen-associated molecular patterns (PAMPs) of different pathogenic origins such as bacteria, viruses, fungi or parasites via their extracellular domains which are composed of leucine-rich regions (Akira *et al.*, 2006; Kawai and Akira, 2010). TLRs belong to the pattern recognition receptor (PRR) family which also has two other members, NOD-like receptors (NLR) and RIG-I-like receptors (RLR) (Fritz *et al.*, 2006; Yoneyama and Fujita, 2008).

So far, 13 TLRs (TLR1-TLR13) have been identified in humans and mice, although TLR11, 12 and 13 only seem to be expresssed in mice. These TLRs detect multiple PAMPs, including bacterial lipoproteins and lipoteichoic acids (TLR2), double-stranded RNA (dsRNA) (TLR3), lipopolysaccharide (LPS) (ligand for TLR4), flagellin (TLR5), single-stranded viral RNA (TLR7 and TLR8) and bacterial and viral CpG (TLR9) (Takeda and Akira, 2005) (Table 1).

The recognition of PAMPs by TLRs takes place in different cellular compartments such as plasma membrane, endosomes, lysosomes and endolysosomes. The TLRs that recognize microbial membrane components such as lipids, lipoproteins and proteins are expressed on the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6, TLR10 and TLR11). TLRs that recognize viral or microbial nucleic acids are localized inside intracellular vesicles such as the endoplasmic reticulum (ER) and endosomes and lysosomes (TLR3, TLR7, TLR8 and TLR9). TLR signal transduction originates from the action of TIR domain-containing adaptor MyD88 (*myeloid differentiation primary response protein 88*), TIRAP (*TIR-containing adaptor protein*)/Mal (*MyD88-adaptor-like*), TRIF (*TIR-containing adaptor inducing IFN-β*) or TRAM (*TRIF-related adaptor molecule*) leading to the activation of NF-κB (nuclear factor-κB) and subsequent expression of a wide array of proinflammatory cytokines like tumor necrosis factor (TNF)-α, IL-1 and IL-6 as well as regulatory cytokines IL-12 and IL-18 (O'Neill, 2008; Underhill and Ozinsky, 2002) (Fig. 1). Although the MyD88-dependent pathway is common to all TLRs,

there are also MyD88-independent signalling pathways. The TLR3-mediated MyD88-independent pathway is activated via the TRIF-adapter, leading to the activation of IRF (Interferon Regulatory Factor-3) and the induction of IFN-β and IFN-inducible genes. Although TLR4-mediated production of inflammatory cytokines depends entirely on the activation of MyD88-dependent pathway, TLR4 stimulation may also lead to the activation of IRF-3 and production of IFN-β (Kawai *et al.*, 2001).

Table 1. TLRs and their ligands (according to Elgert, 2009).

Receptor	Ligands	Ligand origin	TLR localization
TLR1	Triacyl lipopeptides	Mycobacteria	Cell membrane
TLR2	Lipoproteins, lipopeptides Peptidoglycan, lipoteichoic acid Zymosan, hemagglutinin Envelope protein	Mycobacteria Gram-positive bacteria Fungi Viruses	Cell membrane
TLR3	double-stranded RNA	Viruses	Intracellular
TLR4	Lipopolysaccharide (LPS) Mannan, glycoinositol phospholipids RSV fusion protein	Gram-negative bacteria Fungi Viruses	Cell membrane
TLR5	Flagellin	Bacteria	Cell membrane
TLR6/TLR2	Diacyl lipopeptides	Mycobacteria	Cell membrane
TLR7/TLR8	Single-stranded RNA	Viruses	Intracellular
TLR9	CpG DNA	Bacteria, protozoa, viruses	Intracellular
TLR10	Unknown	Unknown	Cell membrane
TLR11	Profilin	*Toxoplasma gondii*	Cell membrane
TLR12	Unknown	Unknown	Cell membrane
TLR13	Unknown	Unknown	Cell membrane

In the following section only the TLRs and their ligands studied in this thesis will be discussed in more detail.

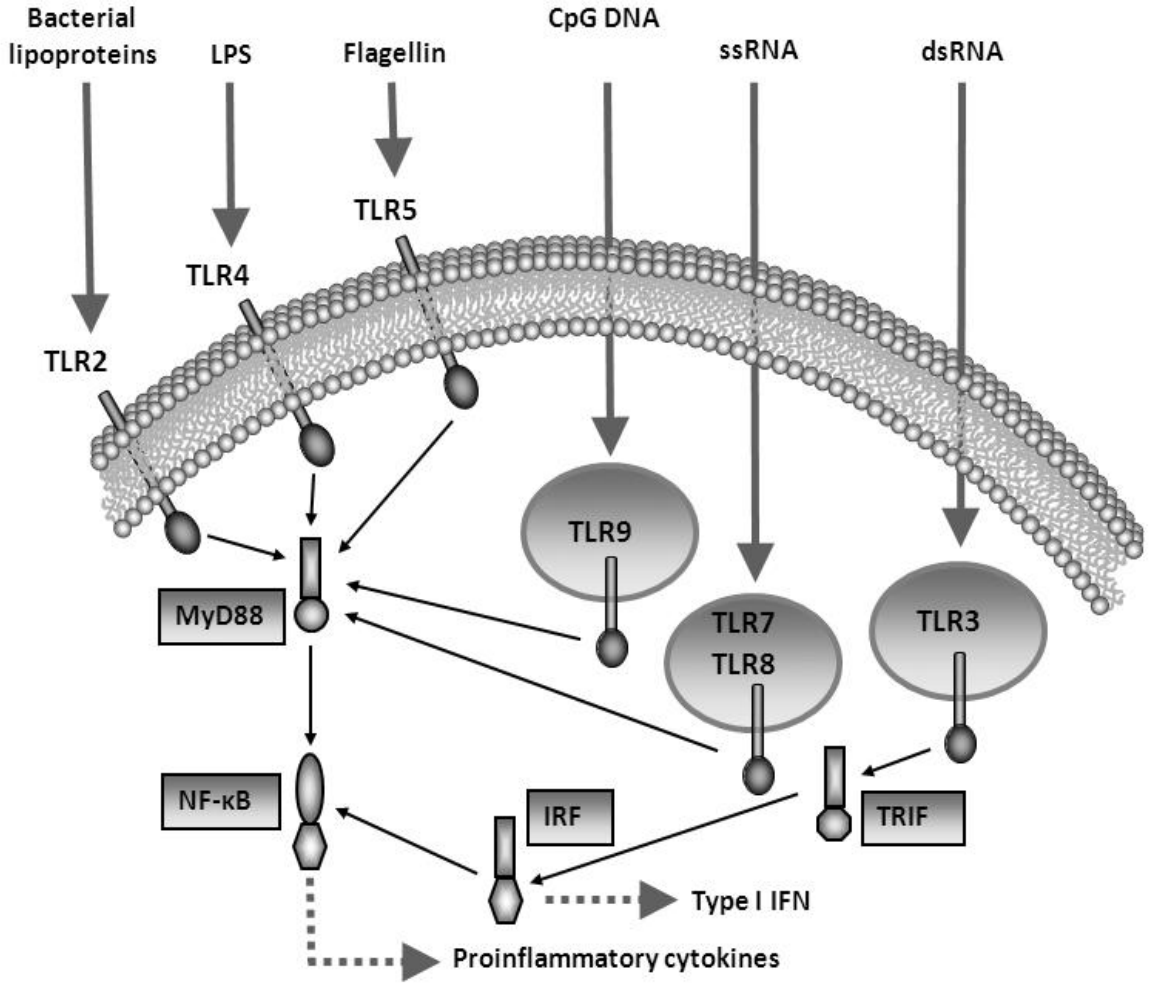

Figure 1. Signalling pathways of TLRs.

5.1.1.1. TLR2

TLR2, together with either TLR1 or TLR6, recognizes a broad range of bacterial tri- or diacetylated lipopeptides, lipoproteins, bacterial lipoteichoic acids (LTA), peptidoglycan (PG) and yeast molecules (Takeuchi *et al.*, 1999). In addition, some viruses are capable of stimulating TLR2, for example mouse cytomegalovirus (MCMV) (Szomolanyi-Tsuda *et al.*, 2006) hepatitis C virus (Chang *et al.*, 2007) and herpes simplex virus types 1 and 2 (Sato *et al.*, 2006). TLR2 forms heterodimers with TLR1 and TLR6, which functionally associate with TLR2 to allow it to discriminate the structural differences between triacyl and diacyl lipopeptides, respectively (Takeuchi *et al.*, 2001). The discrimination between lipoproteins takes place in different lipid-binding pockets of TLR1 and TLR6.

TLR2 is considered as a promiscuous member of the TLR family with a wide range of ligands capable of activating it, as reviewed by Zähringer *et al.* (2008). One of these ligands is a triacylated lipopeptide Pam_3CysSK_4 (also known as Pam_3Cys), which is a synthetic lipopeptide analogue of bacterial lipopeptide. When recognized on the cell surface, Pam_3CysSK_4 interacts with the TLR2-TLR1 ligand complex, where one of the chains of the peptide binds the hydrophobic channel of TLR1. TLR2 agonists primarily induce the production of inflammatory cytokines, but they can also induce type I interferon by inflammatory monocytes in response to infection with vaccinia virus (Barbalat *et al.*, 2009).

5.1.1.2. TLR3

TLR3 recognizes viral ds RNA and the synthetic analogue polyriboinosinic:polyribocytidylic acid (PolyI:C). Activation of TLR3 leads to the induction of type I IFNs, cytokine/chemokine production and DC maturation (Oshiumi *et al.*, 2003). In addition to Poly(I:C), TLR3 recognises other viruses such as respiratory syncytial virus, encephalomyocarditis virus and certain small interfering RNAs (Akira *et al.*, 2006). TLR3 is expressed both intracellularly and on the cell surface of fibroblasts and epithelial cells, and it is localized within the endosomal compartment of myeloid DCs. dsRNA activates TLR3 in endosomes or through cytosolic ribonucleic acid (RNA) helicases (RLR), such as retinoic acid-inducible gene-1 (RIG-1) and melanoma differentiation associated gene 5 (MDA5) (Kato *et al.*, 2006).

5.1.1.3. TLR4

TLR4 is the receptor for lipopolysaccharide (LPS), a compound present on the outer cell membrane of gram-negative bacteria. TLR4 is also involved in the recognition of endogenous ligands like heat shock proteins (HSP60 and HSP70), oligosaccharides of hyaluronic acid, heparin sulfate and fibrinogen, but requires high concentrations of these ligands in order to activate the receptor. The lipid portion of LPS, termed Lipid A or endotoxin, is responsible for the biological

activity of LPS and it is the only region of LPS to be recognized by the innate immune system (Alexander and Rietschel, 2001). TLR4 forms a complex with MD2 on the cell surface, which serves as the main LPS-binding component (Akashi-Takamura and Miyake, 2008). In addition, proteins like LBP (*LPS binding protein*) and CD14 (*Cluster of Differentiation-14*) facilitate the interaction with LPS and TLR4 (Gioannini and Weiss, 2007).

Activation of TLR4 induces the expression of pro-inflammatory cytokines (i.e. TNF-α, IFN-β) and potentiates diverse T helper lineages depending on the tissue microenvironment. LPS can also induce the production of type I interferons in macrophages and conventional DCs via the signaling adaptor TRIF (Yamamoto *et al.*, 2003). LPS is one of the most extensively investigated immunostimulatory components of bacteria known to have both detrimental (systemic inflammation and sepsis) and symptom-relieving effects in a host.

5.1.1.4. TLR9

TLR9 recognizes bacterial DNA containing unmethylated CpG motifs. In vertebrates, the stimulatory activity of CpG motifs is reduced due to the high level of methylation and reduced frequency of CpG dinucleotide motifs, while in viruses and bacteria, CpG dinucleotides are non-methylated and occur at a much higher frequency (Krieg *et al.*, 1995). CpG motifs can directly stimulate B cells and plasmacytoid dendritic cells (pDCs) and promote the production of type I interferons (IFN), Th1-inducing cytokines and chemokines and co-stimulatory molecules (Krieg, 2002). In contrast to humans, mice express TLR9 not only in pDCs and B cells, but also in monocytes and myleoid dendritic cells (Iwasaki and Medzhitov, 2004).

Synthetic CpG molecules have been studied as immunostimulatory adjuvant molecules for many years and are known to possess several beneficial properties. In mouse models, they have accelerated and boosted antigen-specific immune responses by 5- to 500-fold (Klinman, 2006). The strong adjuvant activity of CpG is attributable to the synergy between TLR9 and the B-cell receptor, inhibition of B-

cell apoptosis and dendritic cell maturation and differentiation, leading to enhanced activation of Th1- and cytotoxic T-lymphocyte (CTL) cells (Krieg, 2006). In studies investigating their potential to enhance CpGs immunostimulatory activity, CpG oligodeoxynucleotides (ODNs) have been conjugated directly to antigens, leading to an enhanced immunotherapeutic potential resulting in greater antigen uptake and reduction of antigen requirements (Tighe *et al.*, 2000a; Tighe *et al.*, 2000b). In addition, co-administration of CpG with adjuvants or in formulations has markedly improved its adjuvant activity (Ioannou *et al.*, 2002; McCluskie and Krieg, 2006). Due to its favourable immunomodulatory capabilities, CpG motifs have been used successfully in several immunotherapeutic studies in murine models of asthma to modulate the antigen-specific inflammatory response from Th2 toward Th1 (Hessel *et al.*, 2005; Kline *et al.*, 1998; Santeliz *et al.*, 2002). The therapeutic potential of CpG ODNs has also been studied in clinical settings with promising results, especially in the treatment of allergic rhinitis (Creticos *et al.*, 2006; Simons *et al.*, 2004).

5.1.2. Adaptive immunity

Adaptive immunity, also called acquired immunity, is comprised of B and T lymphocytes which provide an antigen-specific defence mechanism to protect the host from invading pathogens. B cells are responsible for humoral immunity while T cells orchestrate cell-mediated immunity. Activation of the adaptive immune response becomes effective only several days after the antigen encounter, this being the time required for antigen-specific T and B cells to proliferate and differentiate into effector cells (Janeway *et al.*, 2005).

The adaptive immune system consists of four defining characteristics: antigen specificity, diversity, immunological memory and self/non-self discrimination. One of the most remarkable features of the adaptive immune system is its ability to respond to an almost unlimited diversity of foreign antigens in a highly specific manner (Cooper and Alder, 2006). T and B cells possess receptors to distinguish self from non-self, T-cell receptor (TCR) and the antibody/ B cell receptor (BCR), respectively. Each T and B cell receptor is unique for a particular antigenic

determinant and there is a vast array of different antigen receptors on both T and B cells (Cooper and Alder, 2006). The enormous diversity of TCRs and BCRs are generated by the recombination of different germline gene segments, processes carried out by the products of the recombinase activating gene (RAG) as well as terminal deoxynucleotidyl transferase (TdT) (Hood *et al.*, 1985). Adaptive immunity establishes specific and long-term immunological memory responses that trigger clonal expansion of T lymphocytes, which in turn cross-talk with B-cells to produce antigen-specific antibodies. As part of the adaptive immune response, a subset of the activated specific lymphocytes proliferate and differentiate into memory cells, which remain ready to respond rapidly and efficiently to any subsequent encounter with the pathogen.

5.1.2.1. Antigen presentation and T cell differentiation

During the initiation of allergic immune response, allergens are captured and engulfed by antigen presenting cells (APCs), usually dendritic cells (DCs) residing on the cell surfaces like skin and mucosa. DCs process antigens into proteolytic peptides, and load them onto major histocompatibility complex (MHC) class I and II molecules. Processing of the antigen activates DC maturation where the expression of the co-stimulatory molecules and cytokines necessary to activate and differentiate naive T cells is upregulated. Activated maturing DCs migrate to secondary lymphoid organs such as lymph nodes, where they present antigens to naïve T cells and induce their differentiation into effector (CD4+ and CD8+) T cells or T regulatory cells (Guermonprez *et al.*, 2002; Janeway *et al.*, 2005; Lee and Iwasaki, 2007). DCs also present antigens to naïve B cells that have entered the lymph node via the high endothelial venule (HEV) (Qi *et al.*, 2006).

Antigen-loaded DCs provide naïve T cells with two signals required for their activation. The first is the antigen-specific signal received from T cell receptor (TCR) binding to peptide presented by the MHC molecules and the second is provided by the co-stimulatory molecules (e.g. CD40, CD80 and CD86) expressed by DCs. Depending on the density of the peptides presented, the types of co-stimulatory molecules expressed and cytokines released by the APC, naïve CD4+ T

helper (Th) cells start to proliferate and differentiate into different effector subpopulations such as type 1(Th1) or type 2 (Th2) cells, regulatory T-cells (Treg) or into recently reported Th17 cells (Harrington *et al.*, 2005; Murphy and Reiner, 2002; Romagnani, 2004a) (Fig 2). In turn, CD8+ T cells differentiate into cytotoxic T cells (Tcs), which can directly kill the targeted pathogen-infected cells (Podack and Kupfer, 1991). Th1 cells mediate immune responses against intracellular pathogens whilst Th2 cells mediate host defense against extracellular parasites including helminths (Mosmann and Coffman, 1989).

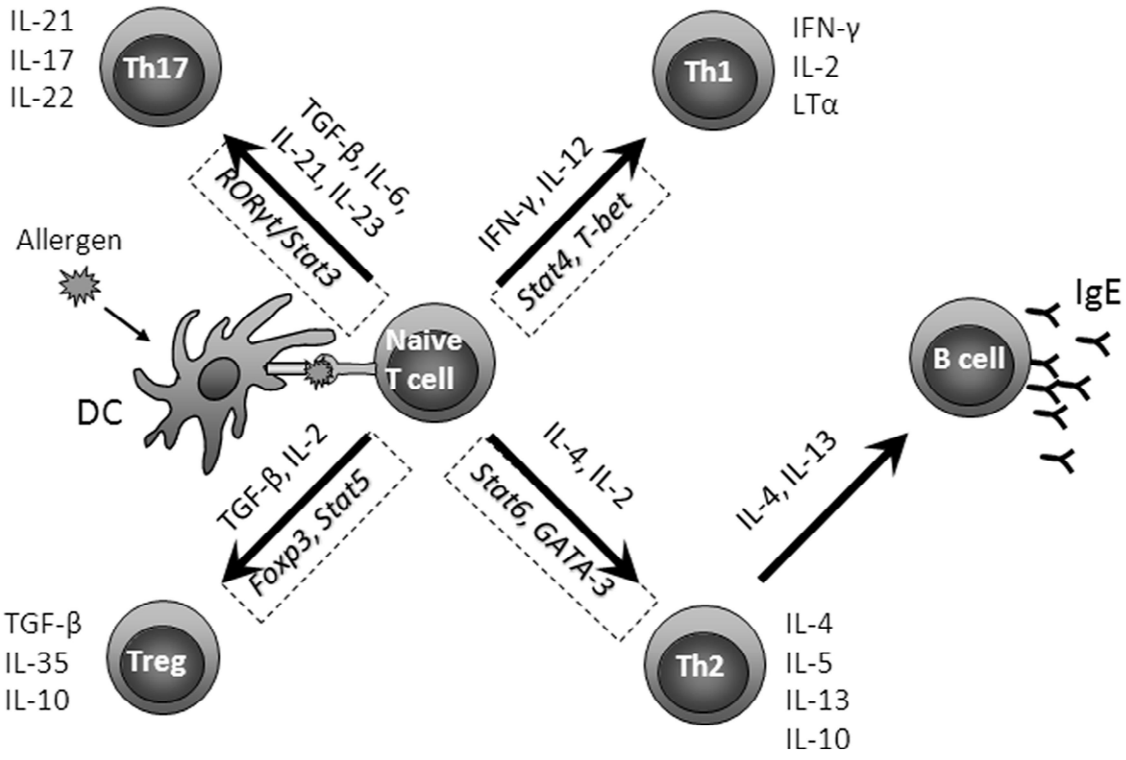

Figure 2. CD4+ T cell differentiation. Modified from Zhu and Paul (2008).

The signalling pathways that favour Th1 over Th2 differentiation are relatively well understood, with the best-characterised being those initiated by cytokines. IL-12 and IFN-γ are two important cytokines required for the efficient differentiation of Th1 cells whereas for Th2 differentiation, many cytokines including IL-4, IL-2, IL-7

and thymic stromal lymphopoietin (TSLP) may be involved (Zhu and Paul, 2010). A major means by which cytokines exert their effect on Th1/Th2 differentiation is through the activation of signal transducer and activator of transcription (STAT) family transcription factors. Th1 differentiation is regulated by transcription factors T-bet (T box expressed in T cells), Stat1 and Stat4, whereas Th2 differentiation is promoted by Stat6, GATA-3, c-Maf and NFATs (nuclear factor of activated T cells) (Agnello *et al.*, 2003; Farrar *et al.*, 2002). T-bet and GATA-3 have been claimed to be critical lineage-specific transcription factors sufficient to program Th1 and Th2 cytokine expression profiles, respectively (Szabo *et al.*, 2000; Zheng and Flavell, 1997). STAT4, in concert with STAT1, plays a significant role in Th1 cell differentiation by transmitting IL-12 signals to produce IFN-γ (Thieu *et al.*, 2008). Additionally, STAT-6 is the key player in Th2 cell differentiation and enhances the expression of IL-4, IL-13, and GATA 3 (Wei *et al.*, 2010).

T-cells can also differentiate into Tregs, which act to suppress immune responses and maintain the tolerance and homeostasis (Bopp *et al.*, 2007; Wan and Flavell, 2006). The key transcription factor and a master regulator of Treg cells is Foxp3 (forkhead box P3), that is required for the development, maintenance and function of regulatory T cells (Hori *et al.*, 2003). Treg cells can be divided into three main categories: the natural regulatory T (nTreg) cells, inducible regulatory T cells (iTreg) and Th3 cells. nTregs are self antigen specific CD4+ T cells that express CD25 in high levels and Foxp3 (Holm *et al.*, 2004), while iTregs are characterized by their elevated production of IL-10, TGF-β and IL-5 (Wu *et al.*, 2007). The Th3 cells are induced by oral administration of antigen and exert their suppressive activity by the production of TGF-β (Weiner, 2001). TGF-β is also a critical cytokine both in the differentiation of Th17 cells (Mangan *et al.*, 2006) and iTregs (Chen *et al.*, 2003) and is important for nTreg development (Liu *et al.*, 2008). Foxp3+ Tregs can suppress the activation, proliferation and effector of a wide range of immune cells, including CD4+ and CD8+ T cells, natural killer (NK) and NK T cells, B cells and antigen-presenting cells (APCs) both *in vitro* and *in vivo* (Sakaguchi *et al.*, 2008). In general, Tregs are known to inhibit allergen-specific effector cells and play a central role in the maintenance of peripheral homeostasis and in the establishment of controlled immune responses.

5.1.2.2. Activation of B lymphocytes

B lymphocytes are a major component of innate and adaptive immune system and responsible for the humoral immunity by producing antibodies (Manz *et al.*, 2005). B lymphocytes develop from haematopoietic stem cells in the liver of fetuses and later in the bone marrow of adults (Rajewsky, 1996). The humoral immune responses include pre-existing antibodies, memory B cells (MBCs) and long-living plasma cells (LLPCs), where the latter two cell types confer for the immunological memory. B-cell activation is initiated after engagement of the B-cell receptor (BCR) by a specific antigen with the help of CD4+ T helper cells. Following stimulation by the antigen, naïve B cells proliferate in the lymph nodes and spleen. Subsequently, activated B cells either differentiate into short-lived plasma cells or into high affinity MBCs and LLPCs or their precursors (Kalia *et al.*, 2006; O'Connor *et al.*, 2002). MBCs mediate rapid recall responses to infection by dividing and differentiating into antibody-secreting plasma cells, while LLPCs constitutively produce and secrete antibodies. The lifespan of plasma cells has been proposed to range from several days to several months (Manz *et al.*, 1997), and even in the absence of antigenic re-exposure, LLPCs and MBCs can still be maintained for decades (Maruyama *et al.*, 2000).

5.1.2.2.1. Immunoglobulins

There are five major types of immunoglobulins (Ig) or antibodies produced by B cells, which can be divided into five classes: IgG, IgM, IgA, IgD and IgE. They differ in their physicochemical properties (charge, size, and solubility) and behavior as antigens. (Janeway *et al.*, 2005) Each immunoglobulin consists of a basic unit of two heavy (H) and two light (L) polypeptide chains and variable domains (V_L and V_H). The large diversity of the immunoglobulin molecules lies in the variable domain, which is coded by a set of gene segments randomly selected and rearranged in a process called somatic recombination (V(D)J recombination). Through V(D)J recombination, which only functions in developing T and B cells, the enormous repertoire of antigen receptors essential to normal immune functioning is generated (Janeway *et al.*, 2005).

All naive B cells express cell-surface immunoglobulin isotypes IgM and IgD, with the class IgM being the earliest to appear after initial exposure to an antigen. IgM is normally present in the bloodstream, where it becomes attached to the antigen during the primary immune response and it then activates the complement system. IgG is the most abundant class of immunoglobulins constituting approximately 75% of total serum Igs. IgG is further subdivided into 4 subclasses: IgG1, IgG2, IgG3, and IgG4, which show more than 95% homology in their amino acid sequences of the constant domains in the γ-heavy chains. In humans, IgG1, IgG2, IgG3, and IgG4 are found in normal serum in the approximate proportions of 65%, 25%, 5% and 5%, respectively. IgG protects the body against bacteria, viruses, fungi, and toxic substances by binding to pathogens and protecting them by complement activation, opsonization for phagocytosis and neutralization of toxins. Immunoglobulin A (IgA) is the major class of antibody present in the mucosal secretions. IgA is also found at significant concentrations in the serum and milk of many species, where it functions as a second line of defence mediating the elimination of pathogens (Steward, 1984; Woof and Kerr, 2004).

IgE (immunoglobulin E) plays a significant role in a number of allergic conditions such as in the pathogenesis of asthma and other allergic diseases (Owen, 2007). IgE is the least abundant antibody class found in normal serum (concentration 0-0.002 mg/ml) (King *et al.*, 1991). Despite the low concentration in circulation, IgE possesses high biological activity. In type I hypersensitivity reactions occuring rapidly following allergen exposure, IgE binds to the high-affinity FcεRI receptors on the surface of mast cells and basophils. This triggers the production and release of inflammatory mediators, mainly histamine which, in turn, evokes a constriction of airway smooth muscle and an increased resistance to airflow. Th2 lymphocytes potentiate allergic responses by releasing Th2 type cytokines like IL-5 and IL-4/IL-13, which in turn regulate the recruitment and activation of eosinophils as well as enhancing the production of IgE antibodies, respectively (Platts-Mills, 2001).

5.1.3. Inflammatory mediators

5.1.3.1. Cytokines

Cytokines are soluble low-molecular weight glycoproteins or small polypeptides that act in an autocrine, paracrine or endocrine manner between leukocytes and other cells (Borish and Steinke, 2003; Steinke and Borish, 2006). Cytokines regulate the movement, differentiation, growth and death of many cell types being involved in virtually every facet of immunity and inflammation including innate immunity, antigen presentation and adhesion molecule expression. The nature of the immune response and the immune cells which are present determine which cytokines are produced and whether the response is cytotoxic, humoral, cell mediated, or allergic. Cytokines and their receptors exhibit very high affinity for each other, and thus very small concentrations of cytokines are needed to exert a biological effect. Cytokines are synthesized by lymphocytes and monocytes/macrophages and by non-immunologic cells such as keratinocytes and endothelial cells. In response to injury or infection, cytokines are secreted by inflammatory cells over several hours to days (Medzhitov, 2008).

Cytokines can be classified into different categories in numerous ways, mainly based on their structure, major functions or the cell type from which they derived originally. The nomenclature of cytokines is versatile due to the divergent functional behaviour of cytokines and the diversity of cytokine effects (Asadullah *et al.*, 2002). The major groups of cytokines are lymphokines, proinflammatory cytokines, inhibitory cytokines, growth factors, chemokines, cell- (Th1 and Th17) or allergic mediated (Th2) cytokines and regulatory cytokines (Tregs). These, in turn, can be categorized into different subgroups for example based on the type of response they mediate, i.e. those that activate mainly cytotoxic (IL-2, IL-6, IL-7, IL-10, IL-12, IL-15, TNF-α, TNF-β and the interferons), humoral (IL-7 and IL-11 in B cell maturation and IL-4 and IL-13 in isotype switching), cell-mediated (IL-2, IL-21, INF-γ, TNF-β, IL-15, IL-16 and IL-17) or allergic immunity (IL-4, IL-5, IL-13, IL-9, IFN-γ, IL-25, IL-3 and GM-CSF) (Borish and Steinke, 2003). In addition, several cytokines have predominantly anti-inflammatory effects, including TGF-β and IL-10 or they are proinflammatory, i.e. promoting systemic inflammation (IL-1, IL-6

and TNF-α). The most relevant cytokines participating in the allergic inflammation are listed in Table 2.

Table 2. Main cytokines involved in allergy and asthma (according to Akdis *et al.*, 2011).

Cytokine	Cell sources	Cell targets	Major functions
IL-1α, IL-1β	Macrophages, monocytes, lymphocytes, neutrophils	T cells, fibroblasts, epithelial cells, endothelial cells	Induction of proinflammatory proteins, hematopoiesis
IL-4	Th2 cells, basophils, eosinophils, mast cells	T cells, B cells	Induction of Th2 differentiation, IgE class switch, survival factor for B and T cells
IL-5	Th2 cells, activated eosinophils and mast cells	Eosinophils, basophils, mast cells	Chemotactic activity of eosinophils, remodeling and wound healing
IL-6	Endothelial cells, fibroblasts, monocytes/macrophages	Hepatocytes, leukocytes, T cells, B cells	T cell differentiation, activation and survival, B cell differentiation,
IL-9	Th2 cells, Th9 cells, mast cells, eosinophils	B cells T cells, mast cells	T and mast cells growth factor, proliferation of CD8+ T cells, IgE and mucus production
IL-10	T cells, B cells, monocytes, macrophages, DCs	Macrophages, monocytes, T cells, B cells, NK cells	Immune suppression
IL-13	T cells, mast cells, NKT cells, basophils, eosinophils	B cells, mast cells, epithelial cells, eosinophils	Switching to IgG4 and IgE, activation of eosinophils and mast cells, recruitment and survival of eosinophils
IFN-γ	Th1 cells, macrophages, NK and NKT cells, B cells	T cells, B cells, NK cells, macrophages, DCs, epithelial cells	Th1 differentiation, upregulation of MHC I and II, promotion of cytotoxic activity
IL-12 (p35/40)	Monocytes, macrophages, neutrophils, DCs, B cells	Th1 cells, NK cells	Induction of Th1-cell differentiation and cytotoxicity

Th1 cells promote cell-mediated immunity in order to combat against viruses and other intracellular pathogens but they also stimulate delayed-type hypersensitivity (DTH) skin reactions (Mosmann and Sad, 1996; Romagnani, 1996). The principal cytokine products of the Th1 cells are IFN-γ, IL-12, and IL-2. IFN-γ is important in activating macrophages assisting them to increase their microbicidal activity, while IL-2 production is crucial for CD4 T-cell memory. IL-12 is a strong activator of Th1-like immune responses since it induces IFN-γ production in NK, T, B, and APCs and regulating Th1 differentiation (Watford *et al.*, 2004). Th2 cells, on the other hand, play a triggering role in the activation/recruitment of IgE antibody producing B cells, mast cells and eosinophils and are critical for expelling extracellular parasites such as helminths. Th2-like responses are associated with

high production of cytokines IL-4, IL-5, IL-9 IL-13 and IL-25. IL-4 promotes B cell isotype switching to IgE, and recruits basophils, eosinophils and monocytes to the site of inflammation. IL-5 promotes eosinophil cell survival and activation (Gleich, 2000) and IL-9 and IL-13 are involved in the allergic response by promoting mucus secretion, airway inflammation, airway hyperresponsiveness and tissue fibrosis (Hauber *et al.*, 2004; Wills-Karp, 2004).

5.1.3.2. Chemokines

Chemokines are a group of small (8 to 12 kD) proteins and chemotactic cytokines which are able to induce chemotaxis in a variety of cells including neutrophils, monocytes, lymphocytes, eosinophils, fibroblasts and keratinocytes (Commins *et al.*, 2010; Esche *et al.*, 2005). Chemokines attract leukocytes into tissues and recruit and activate leukocytes to mount an immune response at the sites of infection or injury. Today, more than 40 chemokines have been identified and classified into four subclasses: C (γ-chemokines), CC (β-chemokines), CXC (α-chemokines), and CX3C (δ-chemokines) (X = variable amino acid), based on the positioning of the N-terminal cysteine (C) residues. The majority of the known chemokines belong to the CXC and CC families. Chemokines bind to receptors, which are divided into receptors for CC chemokines (CCRs), receptors for CXC chemokines (CXCRs), and the receptor for CX3C chemokine. In addition to their cardinal chemotactic features, chemokines also have additional homeostatic or housekeeping functions, i.e. trafficking of lymphocytes during hematopoiesis, antigen sampling in secondary lymphoid tissue, immune surveillance, and organ development (Moser and Loetscher, 2001).

Th1 and Th2 cells express a distinctive set of chemokine receptors that regulate the recruitment and localization of these cells to the site of inflammation. Th2 cells selectively express CCR3, CCR4, and CCR8 and induce the production of Th2 type cytokines IL-4 and IL-13. Increased expressions of CCR3 ligands CCL11, CCL24 (eotaxin-2), CCL26 (eotaxin-3), CCL13 (MCP-4), and CCL5 (RANTES), have been shown in the airways of individuals with asthma (Ying *et al.*, 1999) and CCR4 ligands CCL22 (MDC) and CCL17 (TARC) are known to be released from the

airway epithelial cells and DCs in the lungs of asthmatic patients (Panina-Bordignon *et al.*, 2001; Ying *et al.*, 2005). CCL5 and CCL11 are regarded as the most important eosinophil chemoattractants in allergic inflammation (Venge *et al.*, 1996), but also CCL3, CCL8 and CCL24 are known to attract eosinophils (Zimmermann *et al.*, 2003). CCL22 is postulated to have a critical role in asthma pathogenesis since it can induce the recruitment of Th2 cells and basophils expressing CCR4 (Lloyd *et al.*, 2000). Th1 cells, on the other hand, selectively express CXCR3 and produce IFN-γ, which induce the expression of XCXL10 (IP-10), CXCL9 (MIG), and CXCL11 (I-TAC). The role of CXCR3 chemokines in asthma is less well established, but CXCR3 ligands have been shown to antagonize CCR3, suggesting that they might suppress eosinophilic inflammation (Xanthou *et al.*, 2003).

5.2. Mechanisms of allergic reactions

Allergic (hypersensitivity) reactions are the result of exaggerated and inappropriate immune reactions that are triggered by substances which are normally innocuous to the host. They can be divided into type I, II, III and IV according to the classification proposed by Coombs and Gell (1963). Type I reactions are called immediate hypersensitivity or anaphylactic reactions that involve IgE-mediated release of inflammatory mediators from mast cells. Classic examples of type I hypersensitivity disorders are hay fever and allergic asthma. Type II hypersensitivity, also known as cytotoxic hypersensitivity, is primarily mediated by IgM or IgG antibodies and complement. Type III (immune complex) hypersensitivity reactions are characterized by antigen–antibody interactions and they provoke tissue injury by immune complexes, which are mostly of the IgG class, but also IgM may be involved. Type IV hypersensitivity consist of delayed reactions in which the immune system's response to specific antigens is slow to appear, typically occurring 1-2 days after antigenic exposure. These reactions are mediated by T-cells and monocytes and macrophages rather than by antibodies, and are often called cell-mediated immunity. Clinical examples of this type of hypersensitivity reaction are contact and atopic dermatitis (eczema).

5.2.1. Immediate and late phase hypersensitivity reactions

Type 1 (immediate hypersensitivity) reactions manifest themselves clinically as allergic asthma, allergic rhinitis, food allergy and atopic dermatitis. The early acute phase allergic reactions are mediated by the release of pre-performed and newly synthesized inflammatory mediators such as histamine, tryptase or proteoglycans from mast cells, which are responsible for clinical symptoms like vasodilatation, mucus secretion, increased vascular permeability and increased smooth muscle contraction (Broide, 2001; Turner and Kinet, 1999). This signalling cascade is triggered by the binding of IgE to high-affinity FcεRI receptors on mast cells, which occurs within minutes of allergen exposure. The late-phase reaction (type IV allergic reaction or delayed hypersensitivity) occurs 6 to 24 hours later after the encounter with the allergen. Late-phase reactions are caused by enhanced synthesis and release of inflammatory mediators such as prostaglandins, leukotrienes, chemokines and cytokines like IL-5 and IL-13 from the activated mast cells. In response to the early phase mediators, endothelial cells express several adhesion molecules (vascular cell adhesion molecules, VCAMs), which in turn alter the avidity and expression of the corresponding binding ligands on leukocytes (selectins and integrins). The interactions between these adhesion molecules result in the activation and recruitment of leukocytes, which move from the blood circulation to the site of inflammation (Bochner, 2004). This may eventually lead to a chronic allergic inflammation and in mucosal damage through effects of fibroblasts, vascular endothelial cells, and tissue remodeling (Williams and Galli, 2000).

5.2.2. Allergic asthma

Asthma is a chronic inflammatory disorder of the lungs that is characterized by recurrent episodes of airflow obstruction, bronchial inflammation, mucus hypersecretion and airway hyperresponsiveness (AHR) (Busse and Lemanske, 2001). Patients with asthma typically experience shortness of breath, wheezing, coughing and difficulty in breathing particularly after exposure to an allergen or non-specific irritants with symptoms often worsening at night. The most general form of asthma is allergic asthma which is attributable to airway inflammation

triggered by an environmental allergen. Allergic asthma can be defined by the positivity to skin prick test or via the presence of IgE antibodies to common environmental allergens. Other forms of asthma are non-allergic asthma where the cause of airway inflammation is unclear and mixed-type asthma where there is a combination of allergic and non-allergic factors. It is estimated that allergic asthma patients account for slightly more than half of all asthmatics (Handoyo and Rosenwasser, 2009).

The prevalence of asthma has increased during the last two decades in both the developed and developing countries (Eder *et al.*, 2006). According to the World Health Organisation (2007), over 300 million individuals are affected with asthma worldwide, and there are approximately 250,000 deaths attributed to the disease each year. ith the current rising trend it is estimated that the number of people with asthma will grow by a further 100 million by 2025 (Holgate *et al.*, 2011). The importance of both environmental exposure to the appropriate stimuli as well as genetic factors in the expression and progression of the disease has been recently highlighted (Yang and Holloway, 2007). If one considers the genetic factors, then susceptibility to develop IgE mediated reactivity to common aeroallergens is the most important factor in the development of asthma, especially in children. Other risk factors that may induce or exacerbate asthmatic attacks include allergen exposure in sensitized individuals, infections, aspirin, cold, exercise and tobacco smoke (Anderson and Daviskas, 2000; Jaakkola *et al.*, 2003; Jackson *et al.*, 2008; Jenkins *et al.*, 2004). The disease is often present concomitantly with other diseases such as allergic rhinitis, atopic dermatitis and allergic conjunctivitis. Atopic dermatitis is usually the first allergic disease which is followed by others like food allergy, rhinitis and asthma, with this progression being referred to the "atopic march" (Barnetson and Rogers, 2002).

The most popular paradigm regarding asthma pathogenesis involves allergen-specific CD4+ T helper (Th2) cells, which are thought to be present in the lungs of nearly all patients with asthma (Robinson *et al.*, 1992). In addition to CD4+T cells, CD8+ T cells, and γ/δ T cells have been detected in the airways of allergic asthmatics (Walker *et al.*, 1992). In genetically predisposed individuals, primary exposure to an allergen leads to the activation of Th2 lymphocytes, which produce

cytokines such as IL-4, IL-5 and IL-13 and stimulate IgE production from B cells (Fig 3). Subsequently, antigen exposure causes immediate release of biologically active mediators (histamine, leukotrienes) via mast cell degranulation, resulting in extensive vascular leakage, airway smooth muscle constriction, mucus hypersecretion, eosinophil inflammation and enhanced airway hyperreactivity. Mast cells are important contributors to both the initiation of asthma with release of acute-phase mediators and to the later stages where they produce a variety of inflammatory cytokines (Bradding *et al.*, 2006). Persistent inflammation lasting for years can ultimately lead to irreversible structural changes, such as airway smooth muscle hypertrophy and hyperplasia, thickening of airway mucosa, increased vascular hyperplasia of goblet cells and the development of fibrosis (Tagaya and Tamaoki, 2007).

Although the classical Th1/Th2 paradigm explains many features of asthma, recent evidence suggests that Th2 cells alone may not be sufficient to evoke the development of all types of asthma. For example, cytokines like IFN-γ and IL-17 are frequently found in the lungs of patients with severe asthma and on patients with corticosteroid-resistant asthma (Magnan *et al.*, 2000; McKinley *et al.*, 2008; Yang *et al.*, 2009). In addition, the degree of pulmonary inflammation does not always correlate with the severity of asthma and in some forms of asthma, neutrophils instead of eosinophils predominate in the airways (Brusasco *et al.*, 1998). Other non-Th2 factors connected to the pathogenesis of asthma are natural killer T (NKT) cells, which express features of both NK cells and conventional T cells (Meyer *et al.*, 2008; Umetsu and DeKruyff, 2006).

The current treatments for allergy and asthma are based on pharmacological interventions, such as treatment with antihistamines, glucocorticoids, beta-2-adrenoceptor agonists or leukotriene antagonists. These medications are used alone or in combination to control the asthma symptoms experienced by many asthmatics. Immunotherapy, on the other hand, is a causal treatment for allergic disorder since it is able to modify the immunological background of the disease and thus to provide long-lasting remission of allergic symptoms. However, there is a small but real risk of IgE-mediated reactions, including anaphylaxis when using immunotherapeutic strategies (mainly subcutaneous injections). At the moment, glucocorticoids are the

most effective and widely used therapy available for the control of asthma and are considered as the golden standard by which novel anti-inflammatory medications are measured.

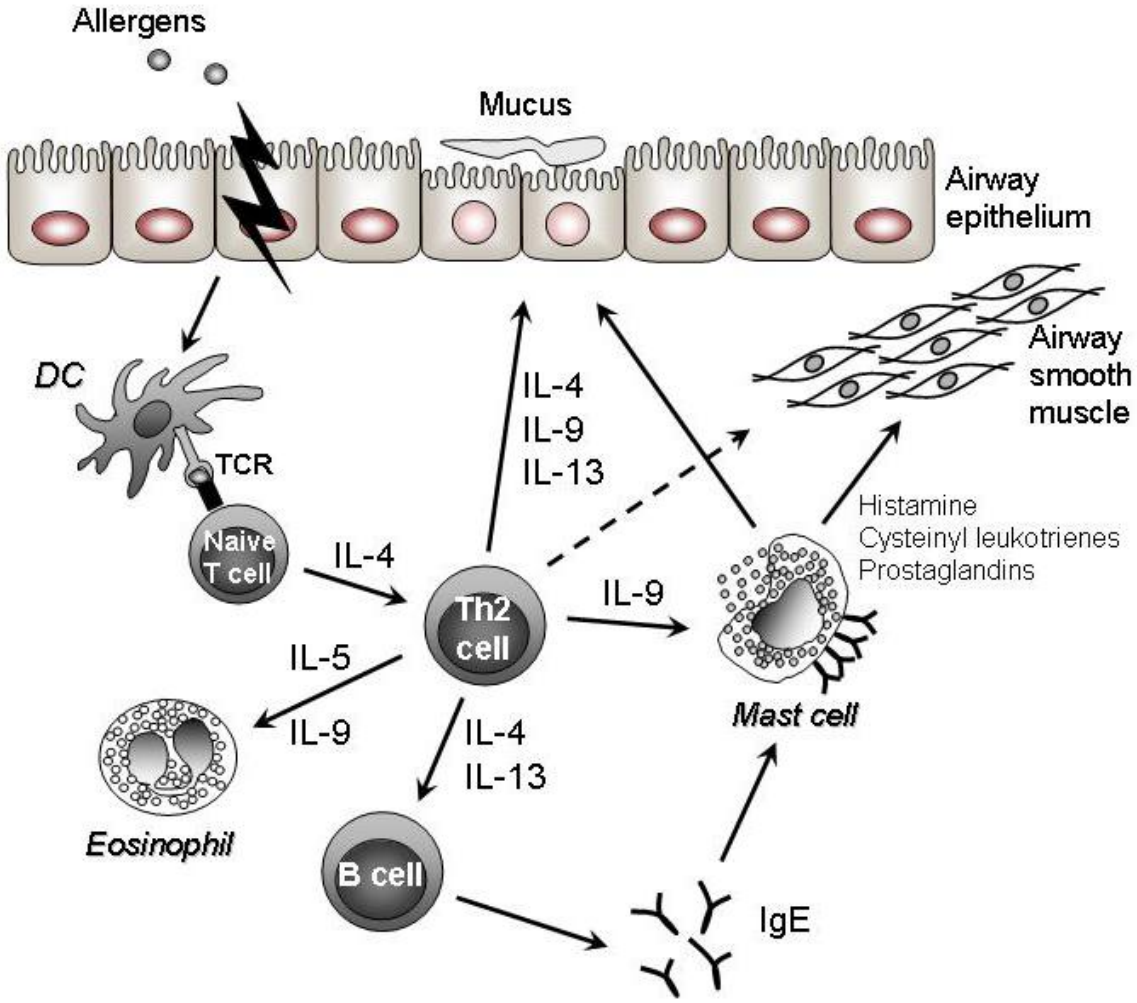

Figure 3. T cell -mediated interactions in allergic asthma (modified from Holgate and Polosa, 2008 and Robinson, 2010).

5.2.2.1. Immunomodulation of allergies and asthma

Allergen-specific immunotherapy (IT) constitutes a treatment method that modifies immunoreactive responses to specific allergens by administering allergens that provoke allergic diseases with the aim of inducing immunologic tolerance.

Allergen IT was first introduced by Freeman and Noon (Freeman and Noon, 1911) over a century ago and since then has been used as treatment option for allergic diseases. In current treatment practice, IT involves subcutaneous or sublingual administration of allergens leading to hyposensitization and improvement of clinical symptoms. In contrast to treatment with glucocorticosteroids which merely suppress the symptoms, immunotherapeutic strategies are the only curative choice targeting the underlying immune mechanisms and causing modifications in the disease course (Akdis and Akdis, 2007). Two basic forms of immunotherapy currently used are subcutaneous IT (SCIT) and sublingual IT (SLIT). SCIT can be used to treat inhalation allergies such as asthma and allergic rhinitis and SLIT is used for inhalation and food allergies. SCIT is normally based on the weekly injections of increasing doses of allergen extract until a standard maintenance dose is reached. Later, the injections are administered less often but on a regular basis, eventually leading to the reduction of both early and late phase responses and improved medication responses (Eng *et al.*, 2006). Termination of successful subcutaneous IT has been shown to lead to a long-term efficacy even lasting for years (Des Roches *et al.*, 1996; Durham *et al.*, 1999a), prevention of the onset of new sensitizations (Durham *et al.*, 1999b) and a reduction in the development of asthma in patients with allergic rhinitis (Moller *et al.*, 2002). In SLIT, on the other hand, allergens are administered to the oral mucosa as drops, sprays or tablets. In clinical trials, IT via the sublingual route has been shown to exert greater efficacy than placebo in grass pollen allergy (Lombardi *et al.*, 2009), asthma (Penagos *et al.*, 2008) and allergic rhinitis (Acquistapace *et al.*, 2009).

The immunoregulation induced by SCIT and SLIT achieves several mutual immunological changes, involving altered humoral and cellular responses. In successful immunotherapeutic treatment, the recruitment of effector T cells to the site of inflammation and the number of infiltrated eosinophils are reduced (Furin *et al.*, 1991; Rak *et al.*, 1991). Furthermore, an induced differentiation from Th2 to Th1 phenotype after immunotherapeutic treatments has been reported (Ciprandi *et al.*, 2008; Hamid *et al.*, 1997). This so-called immune deviation may be achieved especially with Th1-stimulating adjuvants, such as the TLR9 ligand CpG, which induces a robust Th1-like cytokine response (Chu *et al.*, 1997; Jahn-Schmid *et al.*, 1999). According to current knowledge, skewing of allergen-specific effector T

cells to a regulatory phenotype and development of peripheral tolerance appears to be the key event in the outcome of successful cases of SIT (Akdis and Akdis, 2009; Radulovic *et al.*, 2008). Treg cells produce regulatory cytokines IL-10 and TGF-β, which in turn induce switching of B cell responses in favour of IgG4 and IgA antibodies, respectively, and suppress survival and activation of IgE production (Jutel *et al.*, 2003; Meiler *et al.*, 2008; Wachholz and Durham, 2004).

One of the major problems with conventional IT is the possibility of triggering life-threatening systemic adverse reactions such as anaphylactic shock, and low allergenic and high immunogenic recombinant allergens have been developed. Recombinant allergen–based vaccines can be produced as variants with advantageous properties such as reduced allergenic activity or increased immunogenicity and in principle can be tailored to the patient's sensitization profile and sensitivity. Encouraging results have been obtained with recombinant allergen–based vaccines in the first proof of principle studies conducted for birch (van Hage-Hamsten *et al.*, 1999) and grass pollen allergy (Jutel *et al.*, 2005). In addition, site-directed mutagenesis techniques offer the exciting possibility of engineering allergens with low IgE binding capacity but containing most of the functional T cell epitopes, thus providing safer vaccines (Singh *et al.*, 1999). Both forms of current immunotherapeutic strategies continue to evolve, and on can predict that new forms of IT with alternative delivery routes will also be developed in the future. At the moment, the conventional injection-type IT is considered as the primary choice in the field of immunotherapeutic strategies with known effective doses and efficacy of the treatment indicated in various clinical trials. In contrast, sublingual IT is considered as safer and it also has the advantage of easier administration (Calamita *et al.*, 2006; Penagos *et al.*, 2008).

5.2.2.2. Hygiene hypothesis

The hygiene hypothesis was first proposed in 1989 by David Strachan (Strachan, 1989), who claimed that children with a higher number of siblings experienced a lower risk of hay fever and atopic sensitisation. Subsequently, hygiene hypothesis has gained considerable support with numerous epidemiological studies detecting an

inverse association between the number of siblings and atopy and a prevalence of allergic disorders and asthma among children raised in farm or rural areas versus those in urban areas (Ball *et al.*, 2000; Braun-Fahrlander *et al.*, 1999; Matricardi *et al.*, 2000; McKeever *et al.*, 2001). According to the hygiene hypothesis, reduced microbial exposure in early life lead to polarization of allergen-specific T-cell memory towards Th2 immune response and this predisposes the host to an increased risk of suffering allergic disorders. In turn, childhood exposure to microbiological stimuli induces the Th1-biased immunity characterized by the release of cytokines like IFN-γ, and confers a protective effect against the later development of atopy and asthma (Bach, 2002; Braun-Fahrlander *et al.*, 2002; von Hertzen and Haahtela, 2006).

TLRs are centrally involved in mediating immune responses between lymphocytes and microbial compounds. Several investigations have suggested that TLRs may have the potential both to inhibit and promote the development of allergic hypersensitivities and disease mechanisms by provoking Th1- or Th2-biased immune responses (Horner *et al.*, 2004; Schroder and Arditi, 2007). Exposure to house dust endotoxin or lipopolysaccharide (LPS; TLR4 ligand), a part of the outer membrane of Gram negative bacteria, is shown to be inversely associated with atopic sensitization against common aeroallergens, hay fever and atopic asthma. In these studies, the endotoxin level has been higher in house dust samples taken from large families (Wickens *et al.*, 2003), day care centers (Rullo *et al.*, 2002) and in the homes of farmers (von Mutius *et al.*, 2000). A correlation between higher levels of bacterial by-products and with lower levels found in urban homes is consistent with the hygiene hypothesis, indicating that endotoxin may be a good marker of bacterial load in certain environments. On the other hand, a number of studies has demonstrated inverse results showing that exposure to endotoxins increases the likelihood of an asthma-like syndrome and late-onset wheeze (Celedon *et al.*, 2007; Thorne *et al.*, 2005). When studying the immunomodulating effects of LPS on allergen-induced responses in animal models, variable and sometimes contradictory, results have been obtained depending on the route, dose and timing of LPS exposure. In studies using Th2-biased OVA/Alum protocol, systemic LPS has prevented allergen-induced responses (Aumeunier *et al.*, 2010; Rodriguez *et al.*, 2003), whereas airway LPS exposure has exacerbated the inflammatory responses

(Murakami *et al.*, 2007). The associations between endotoxin exposure and a lower prevalence of allergy and asthma are still unclear and further prospective studies in different locales and with agents acting through different molecular mechanisms will be necessary to determine if endotoxin is actually responsible for the protective effect against these diseases.

5.3. Natural rubber latex allergy

Occupational asthma (OA) is a major health problem around the globe, since the condition accounts for approximately 15% of asthma experienced by adults (Pawankar *et al.,* 2011). Exposure to natural rubber latex (NRL) has been recognized as one of the causes of occupational asthma, especially among health care workers (HCWs) with increased exposure to NRL products and patients with spina bifida due to the repeated exposure of their mucous membranes to latex during surgical interventions and procedures (Liss *et al.*, 1997; Turjanmaa, 1988). A history of atopy, previous hand dermatitis and allergies to foods known to share cross-reactive allergens with latex (i.e. chestnut, banana, kiwi fruit, avocado, and papaya) have also been reported as risk factors for latex allergy (Taylor and Erkek, 2004; Turjanmaa *et al.*, 1996). In the meta-analysis carried out by Bousquet *et al* in 2006, NRL allergy was found in 4.32% of HCWs and in 1.37% of the general population (Bousquet *et al.*, 2006).

NRL is a processed plant product manufactured almost exclusively from the rubber tree *Hevea brasiliensis*. Currently, 90 percent of rubber production takes place in the Southeast Asian countries of Malaysia, Thailand and Indonesia. NRL is a common component of a wide range of household and health care items, such as balloons, condoms, disposable gloves, intravenous tubing, syringes, catheters and other medical supplies. Latex gloves are the main source of allergic reactions among HCWs, and the proteins naturally present in NRL sensitize the wearer either through direct contact with the skin or by inhalation of the powder from latex gloves (Baur and Jager, 1990; Carrillo *et al.*, 1986). Exposure to powdered gloves may be associated with the development of asthmatic symptoms, such as allergic rhinitis and conjunctivitis (Taylor and Erkek, 2004). The prevalence of NRL-induced OA

has been significantly reduced during recent years, mainly due to increased awareness of the problem and by the substitution of powdered latex gloves with low protein, non-powdered latex gloves or latex-free (nitrile) gloves. Regular measurements of the allergen content of medical latex gloves and the availability of public information about these products have also been demonstrated to be effective ways to reduce the presence of high-allergenic glove brands on the market (Reunala T, 2004).

5.3.1. Natural rubber latex allergens

At present, the WHO/International Union of Immunological Societes (IUS) Allergen Nomenclature Committee (www.allergen.org) lists 14 NRL allergens (Hev b 1-14). The major NRL allergen is hevein (Hev b 6.02) which is present at the N terminus of prohevein (Hev b 6.01); this allergen is recognized by 70-80% of latex allergic patients (Alenius *et al.*, 1995; Alenius *et al.*, 1996; Chen *et al.*, 1997b). Both Hev b 6.02 and Hev b 5 are the most important NRL allergens for HCWs (Alenius *et al.*, 1995; Slater *et al.*, 1996) and Hev b 1 and Hev b 3 for children with spina bifida (Alenius *et al.*, 1993; Chen *et al.*, 1997a). The allergenicity of NRL products can be estimated by measuring the levels of the four major NRL allergens Hev b 1, Hev b 3, Hev b 5 and Hev b 6.02 (Palosuo *et al.*, 2007; Yip *et al.*, 2000). There are also other allergens detectable in NRL products e.g. Hev b 2 and Hev b 13 (Yeang *et al.*, 2004; Yeang *et al.*, 2002), but their role remains to be clarified. Altogether, there are estimated to be 250 proteins present in NRL and of these about 50 to 60 are able to trigger an IgE response (Kurup *et al.*, 1996).

Thirteen of the latex allergens (Hev b 1-13) have been cloned and are available in recombinant form. The major conformational IgE-binding B cell epitopes (Beezhold *et al.*, 1997; Karisola *et al.*, 2002), the dominant T-cell epitopes (de Silva *et al.*, 2004) and T-cell responses and IgE-binding capacity (Raulf-Heimsoth *et al.*, 2004) of Hev b 6 have been characterized in detail.In addition, linear B and T-cell epitope analysis data have been reported for Hev b 1, Hev b 3, and Hev b 5. The most recently identified latex allergens are Hev b 11-13 and Hev b 14; of these only Hev b 13 has been isolated and characterized in its native form. The allergenicity of

purified Hev b 10, Hev b11 and Hev b 12 has mostly demonstrated from recombinant froms (Yeang, 2004).

5.3.2. Symptoms, diagnosis and treatment

Three types of reactions to NRL have been described, namely type IV (delayed) hypersensitivity, type I (immediate) hypersensitivity and irritant contact dermatitis. The symptoms to NRL allergy range from mild clinical symptoms (contact urticaria, rhinoconjunctivitis, bronchial wheezing and mucosal swelling) to more severe systemic reactions such as bronchospasm and even anaphylactic shock. Most patients with latex allergy develop dermatologic symptoms as the first manifestation, with the most typical skin reaction being contact urticaria (Sussman *et al.*, 1991). Patients with delayed-type hypersensitivity reactions are at greater risk of developing immediate reactions due to skin breakdown and the resultant increased exposure to NRL (Charous *et al.*, 1994; Turjanmaa, 1994).

The diagnosis of latex allergy is based on the patient's clinical history as well as the results from skin prick testing and latex-specific serum IgE testing (Turjanmaa, 2001). The most sensitive method for diagnosis is the skin prick test, where the patient's arm is pricked with a lancet dipped into NRL extract causing a resultant wheal-and-flare reaction within 15 minutes of allergen application. Further *in vitro* diagnosis tests, i.e. RAST or ELISA, may be applied to confirm the presence of IgE to NRL in the patient's serum. In case of discrepancies or suspected false-negative or -positives between these tests it is recommended that a provocation or usage test with controlled exposure to NRL allergens should be applied to verify the diagnosis of latex allergy (Turjanmaa, 2001).

The best treatment for latex allergy is the total avoidance of exposure to materials containing latex allergens and avoidance of foods with cross-reactive proteins. The use of low allergenic NRL gloves or a change to latex-free gloves reduced the exposure to NRL allergens. The symptoms of contact dermatitis can be managed with the help of topical and oral corticosteroid medications. In the case of immediate hypersensitivity reactions and anaphylaxis, antihistamines, systemic

corticosteroids and injections of epinephrine can be used. An IgE blocking antibody (Omalizumab) has been investigated for the treatment of occupational latex allergy in HCW, although it has shown rather low clinical efficacy (Leynadier *et al.*, 2004). The IT of latex allergy has been attempted via the construction of a hevein (Hev b 6.02) with a highly reduced IgE-binding capacity and a low potential for adverse effects (Karisola *et al.*, 2004). There has also been identification of the hypoallergenic preparations of the major allergens Hev b 1, Hev b 3, Hev b 5 and Hev b 6.01 (Rolland *et al.*, 2005). In spite of these advances in the field of specific IT, further investigations will be required to broaden the knowledge of latex specific immunotherapeutic methods and to introduce new effective and safe specific hypoallergenic preparations to be investigated in clinical trials.

5.4. Mouse models of asthma

Experimental animal models provide important information on the outcome of a variety of diseases. Different animal models of asthma have been developed in recent years to study the pathophysiology of the disease, the activity and function of different genes and cellular pathways and to evaluate the safety of new drugs or chemicals before entering clinical studies. Allergic asthma models have been described in rodents (mice, rats and guinea-pigs) and dogs (Out *et al.*, 2002), sheep (Snibson *et al.*, 2005) and monkeys (Weiszer *et al.*, 1968). The mouse is being increasingly used as a model animal since it offers many advantages over larger species (Kips *et al.*, 2003). For example, there is worldwide availability of genetically well-characterized inbred mouse strains at relatively low cost as well as open access to the genetic map of the murine genome. The availability of genetically engineered transgenic and knock-out mice as well as a range immunological reagents available for analysis of cellular and mediator response further enhance the advantages of mice as valuable experimental animal, allowing the detailed investigation of the different mechanisms of allergic reactions (Shin *et al.*, 2009; Taube *et al.*, 2004).

Although experimental mouse models display many of the features of human asthma, including elevated levels of IgE, airway inflammation, goblet cell

hyperplasia and airway hyperreactivity to specific stimuli, a single animal model may not be able to reproduce all of the morphological and functional features of the disease. A large number of studies with asthma have been done by using models of acute allergic response, but chronic asthma models with clinical aspects related to human asthma have also been investigated by several research groups (Kumar *et al.*, 2008; Lloyd, 2007). One major drawback with these models has been the development of tolerance and downregulation of inflammation and AHR (Jungsuwadee *et al.*, 2004; Yiamouyiannis *et al.*, 1999). Moreover, the ability to induce airway hyperreactivity to inhaled metacholine varies greatly between the different mouse stains. This is exemplified by examining the situation with two of the most widely used strains; Balb/c mice exhibit robust AHR, while C57/BL6 mice have limitations in the development of airway responses to inhaled metacholine (Takeda *et al.*, 2001; Whitehead *et al.*, 2003).

Mice are usually sensitized by intraperitoneal (i.p.) injection of chicken egg ovalbumin (OVA), frequently given together with an immune-boosting adjuvant such as aluminium hydroxide (alumn). Other natural allergens or purified proteins derived from potent allergens including house dust mite (HDM), ragweed or fungi have also been increasingly used as sensitizing allergens in mice instead of ovalbumin (Chapoval *et al.*, 2002; Johnson *et al.*, 2007; Leino *et al.*, 2006). Sensitization may also be achieved by repeated administration of allergen via the skin (intradermally, subcutaneously or epicutaneously) or via the airways (intranasally, intratracheally). In order to mimic the symptoms related to atopic eczema i.e. skin irritation and thickening of the epidermis, the skin may be exposed to repeated tape-stripping before antigen administration, which evokes a dysfunction in the epidermal barrier making it more susceptible to allergens. After the sensitization period, mice are challenged with the allergen intratracheally, intranasally or by aerosol exposure. These conditions produce a robust eosinophilic inflammatory response and AHR, which is dependent on the presence of CD4+ T lymphocytes but independent of IgE, B cells or mast cells (Corry *et al.*, 1996).

The measurement of lung function in mice poses a particular challenge, due to the small size of the animal (Irvin and Bates, 2003). Several techniques for measuring lung function in mice have been developed but, to date, none is

universally accepted. The techniques available can be divided into two main methods: the first is a direct or invasive measurement of lung resistance (Rl) and compliance (Cl), where mice are anaesthetised and tracheotomized, mechanically ventilated and placed into a whole-body plethysmography chamber (Lai and Chou, 2000). The alternative is to measure lung function via an indirect or non-invasive technique where an unrestained, spontaneously breathing mouse is placed in the main chamber of a whole-body plethysmograph, and the changes in the pressure fluctuations resulting from altered breathing patterns are monitored, and results expressed as Penh (enhanced pause) values (Hamelmann *et al.*, 1997). The application of Penh values as a lung function parameter is widely used in the study of AHR, as it is easy to accomplish and can be reproduced over time in the same animal. On the other hand, the validity of this method has been questioned as the Penh value is not based on actual physiological parameters (Lundblad *et al.*, 2007). For this reason, the direct invasive method to measure pulmonary mechanics is considered to be the golden standard in airway physiology since it provides more precise and clear-cut results.

6. AIMS OF THE STUDY

The specific aims of the study were:

I. To assess the differences in the development of skin inflammation and airway hyperreactivity in mice while using different exposure routes of natural rubber latex (NRL).

II. To investigate the immunotherapeutic effects of immunostimulatory CpG-ODN treatment in a murine model of NRL -induced asthma.

III. To elucidate the effects of three TLR ligands, namely LPS, Pam3Cys and Poly(I:C), on pulmonary inflammation and allergic responses in a murine model of ovalbumin-induced asthma.

7. MATERIALS AND METHODS

7.1. Establishment of asthma murine model

7.1.1. Animals (I-III)

Female Balb/c mice, 6-8 weeks of age, were obtained from NOVA-Scanbur ab (Sollentuna, Sweden) and used in experiments **I, II** and **III**. STAT6, STAT4 and RAG1 knock-out mice (Balb/c background) were obtained from the Jackson Laboratory (Bar Harbor, USA) and used in the experiment **II**. All animal experiments were approved by the Social and Health Care Department of the State Provincial Office of Southern Finland.

7.1.2. TLR ligands (II, III)

CpG oligodeoxynucleotides (ODNs) (5´-TCC ATG ACG TTC CTG ACG TT-3´) and mutated control ODNs (5´-TCC ATG AGC TTC CTG AGC TT-3´) were used in experiment **II**. CpG ODNs were synthesized, purified and phosphorothioate modified by Biognostic GmbH (Goettingen, Germany). The ODNs contained undetectable levels of endotoxin (<0.1 IU/ml) as determined by the *Limulus* amebocyte lysate assay (Biovian Ltd., Turku, Finland).

LPS, Poly(I:C) and Pam_3Cys (PamCys-SKKKK, L2000) were used in experiment **III**. Ultra Pure E. coli LPS (strain 0111:B4, version #06B24-MT), High Molecular Weight Poly(I:C) (endotoxin level <1.25 EU/ml, version #09L02-MT) were purchased from InvivoGen (San Diego, CA, USA). Pam_3Cys (PamCys-SKKKK, L2000) was purchased from EMC microcollections GmbH (Tübingen, Germany). All TLR ligands were prepared by aliquoting the lyophilized products in endotoxin-free PBS to provide a sterile stock solution.

7.1.3. Treatment protocols (I-III)

Mice were anesthetised with isoflurane (Abbott Scandinavia AB, Finland) and their backs were shaved and tape-stripped three to four times before the injections to evoke a skin injury. In order to produce a Th2-dominated skin inflammation, Balb/c mice were sensitized by intradermal (i.d.) injections of 40 µg of NRL (**I, II**) or 50 µg of ovalbumin (OVA, grade V; Sigma) (**III**) in 100 µl of PBS at weekly intervals for 4 weeks. In experiment **I**, part of the mice were sensitized by intranasal (i.n.) exposure to NRL once a week with 40 µg of NRL for four weeks or by the intraperitoneal (i.p.) route with 80 µg of NRL on days 1 and 14. One week after the sensitizations, on days 28, 29 and 30, all mice were challenged with 0.5% NRL via the respiratory route for 40 minutes using an ultrasonic nebulizator (Aerogen Ltd., Galway, Ireland) (**I, II**) or mice were challenged i.n. on days 28, 29 and 30 with 50 µg of OVA in 50 µl of PBS (**III**). On day 31, AHR was assessed, mice were sacrificed and the specimens collected for subsequent analysis (**I-III**) (Fig 1.)

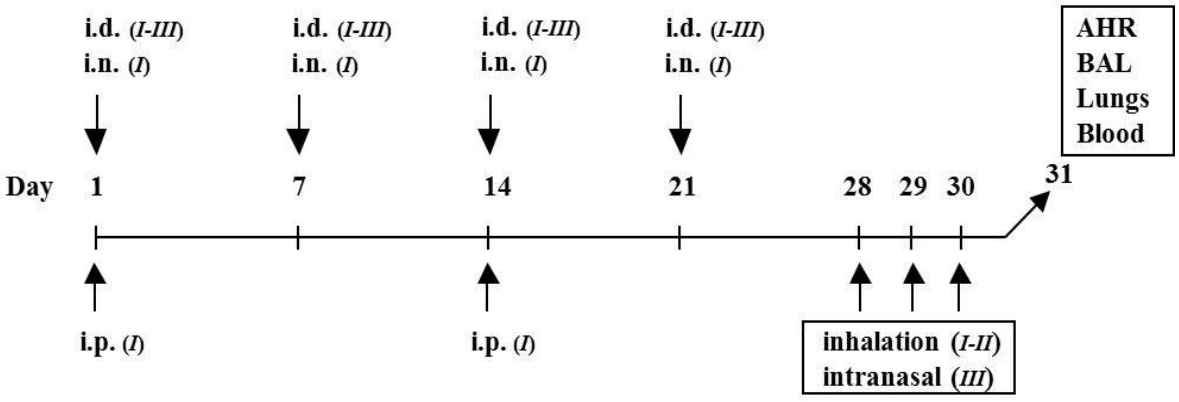

Figure 4. Sensitisation schedule used in experiments I-III. I.d; intradermal, i.n; intranasal and i.p; intraperitoneal route of sensitization.

7.1.4. Delivery of TLR ligands and antibodies (II, III)

Mice received either 10 µg of CpG (NRL + CpG group) or 10 µg of control, mutated CpG (NRL + muCpG group) i.d. together with NRL on days 1, 7, 14 and 21 (**II**). Negative controls (referred as muCpG) contain nucleotides with reversed base alignment (GpC), and are non-immunostimulatory. Part of mice were treated i.d. with 1 µg and 10 µg of LPS, Pam_3Cys or Poly(I:C) or with 100 µg of Poly(I:C) concurrent with 50 µg of OVA in 100 µl of PBS on days 1, 7, 14 and 21 (**III**). Control mice were given PBS only or PBS was given together with 10 µg of CpG or control CpG (**II**) or mice were given PBS and OVA only (**III**). Mice were challenged on days 28, 29 and 30 with 0.5% NRL by inhalation (**II**) or i.n. on days 28, 29 and 30 with 50 µg of OVA in 50 µl of PBS (**III**).

In experiment **III**, the immunomodulatory effects of Toll-like receptor ligands in the injection site and in the draining lymph nodes were analysed. In this experiment, mice were treated i.d. with 50 µg of OVA conjugated to Alexa fluor 647 (Invitrogen) or with 50 µg of OVA–Alexa fluor 647 concurrent with 10 µg or with 30 µg of LPS, Pam_3Cys or Poly(I:C). 2-48 h after the intradermal injections, the skin around the injection site and/or the brachial and axillary lymph nodes were removed, cells were stained and analysed by FACS Calibur.

7.1.5. Neutralisation of IFN-γ and depletion of CD8+ T cells (II, III)

For IFN-γ neutralization, mice treated with NRL + CpG were given i.n. 50 µg of anti-mouse IFN-γ mAb or 50 µg of IgG1a control mAb in 50 µl of PBS on three subsequent days just before the NRL inhalations (**II**) or 200 µg of anti-mouse IFN-γ or control IgG1 was given i.p. on day 27 (1 day before OVA airway challenge), and again 3 times i.n. (50 µg of anti IFN-γ ab or IgG1 in concert with 50 µg of OVA) on days 28, 29 and 30 (**III**).

In order to achieve the depletion of CD8+ T cells, mice were injected i.p. with 200 µg of anti-mouse CD8 mAb 4, 2 and 1 days before the OVA challenge. Control mice were treated with the same amount of rat IgG2b (**III**).

7.2. Sample preparation and measurements

7.2.1. Determination of airway reactivity to metacholine (I-III)

AHR was measured either using non-invasive (**I-III**) or invasive method (**II, III**) (Buxco Research Systems, Wilmington, North Carolina, USA).

In the non-invasive method, the responses to inhaled metacholine (Sigma-Aldrich, USA) were measured in conscious, unrestrained mice using whole-body plethysmography 24 hours after the last airway challenge. Briefly, mice were placed into the chamber and exposed for 5 min to PBS and increasing concentrations of metacholine (3, 10, 30 and 100 mg/ml) using an ultrasonic nebulizer (deVilbiss). Recordings were taken for 5 min after each nebulization. Airway hyperreactivity was expressed as the percentage of baseline Penh values (% baseline Penh).

For invasive measurement of lung resistance (R_L), mice were anesthetized with Hypnorm/Dormicum solution (10 ml/kg, VetaPharma, Leeds, UK) and tracheas cannulated. Mice were placed in a Resistance/Compliance (RC) collection station (FinePointe RC, Buxco Research Systems) and were mechanically ventilated at a rate of 150 breaths/min. PBS and increasing doses of aerosolized metacholine (3, 6, 12, 24 and 48 mg/ml) were administered via the trachea and changes in R_L were expressed as a mean value of 3 minutes of recording.

7.2.2. Sample collection (I-III)

Mice were sacrificed with isoflurane overdose and the blood was collected from vena cava using a 1ml syringe. The chest cavity was opened, the trachea cannulated and the lungs were lavaged with 800 µl of PBS. Part of the left lung was removed for RNA isolation and the other part was embedded in OCT (Sakura Finetek, The Netherlands) on dry ice. The right lung was perfused with 500 µl of 10% formalin.

7.2.3. BAL cytology, lung histology and immunohistology (I-III)

The bronchoalveolar (BAL) fluid cells were cytocentrifuged (Cytospin, Shandon Ltd., UK) and stained with May-Grünwald-Giemsa (MGG). Differential cells were counted in 15–20 high-power fields (HPFs) under light microscopy (Leica DM 4000B; Wetzlar, Germany). Lung samples embedded in OCT were cut into 5 µm cryostat sections, air dried, fixed and stained immunohistochemically with monoclonal antibodies against CD3, CD4, CD8, CD11c, Foxp3 (**II, III**) and F4/80 (**III**). Paraffin-embedded lung sections were cut into 4 µm sections and stained with haematoxylin and eosin (H&E) for analysis of lung inflammation (**I-III**) or with periodic acid-Schiff (PAS) solution to examine the amount of mucus producing cells in the lung bronchioles (**I-III**). Lung samples collected for RNA analysis were stored at -70°C until processed further **(I-III)**.

7.2.4. RT-PCR (I-III)

Total RNA from lung sections was isolated using Eurozol Reagent (EuroClone, Via Lombordia, Italy) and reverse transcribed to cDNA with MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA, USA). Real-time quantitative PCR was performed with AbiPrism 7700 Sequence Detector System (Applied Biosystems). PCR primers and probes were obtained as pre-developed assay reagents or they were self-designed using PrimerExpress (version 2.0) software and ordered from Applied Biosystems. The PCR products were detected by monitoring the fluorescence levels of the reporter dye labeled with FAM or VIC™. The FAM signals of the target gene were standardized to the VIC signals of the endogenous reference gene, 18S rRNA, to control sample loading and to allow normalization between samples. The final results were expressed as relative units (RU), i.e. ratio of PCR amplification efficiency in the sample cDNA compared to that of the calibrator. These were calculated by the comparative C_T method according to the manufacturer's instructions.

7.2.5. Flow cytometric analysis (II, III)

Analytical flow cytometry was carried out using a FACSCantoII instrument (Becton Dickinson, Franklin Lakes, NJ), and data was processed with the FlowJo Software (Tree Star, Ashland, OR).

Cells were washed once with ice cold PBS which contained 2% Fetal Bovine Serum (FBS) and Fc receptors were blocked with an excess of anti-mouse CD16/32 (eBioscience, San Diego, CA). In the evaluation of T cell subpopulations in the lung tissue (**III**) and in BAL fluid (**II, III**), cells were surface stained with APC (**III**) or FITC (**II**) -conjugated TCR-β, APC-Alexa750 (**III**) or PE-Cy5 (**II**) -conjugated CD4+ and with Alexa700-conjugated CD8+.

In the characterization of intracellular BAL cytokines (**II, III**), BAL cells were stimulated in the incubator at 37^{o}C for 4 hours with PMA (20 ng/ml) and ionomycin (1 µg/ml) (Sigma-Aldrich), and Brefeldin A (Sigma-Aldrich). After collection and washing with ice cold PBS including 2% FBS, BAL cells were Fc-blocked as described above and surface stained with PerCP (**III**) or PECy7 (**II**) -conjugated CD3+ and FITC (**III**) or PECy5 (**II**) -conjugated CD4+ (BD Biosciences, San Jose, CA). Cells were then permeabilized with intracellular Fix and Perm staining kit (Caltag, Burlingame, CA) and stained with PE (**III**) or APC (**II**) conjugated IFN-γ (BD Biosciences, San Jose, CA) or with PE (**III**) or APC (**II**) -conjugated IL-13.

In the investigation of OVA antigen uptake by dendritic cells (**III**), brachial and axillary lymph nodes from mice treated with OVA–Alexa fluor 647 (Invitrogen, Paisley UK) or with OVA–Alexa fluor 647 mixed with TLR ligands were removed, teased, counted and suspended in culture medium (PBS supplemented with 5% FBS). LNs were mechanically disrupted, single cell suspensions were obtained and total cell numbers were counted. Filtered single-cell suspensions were Fc-blocked and stained with mAbs for CD11c, CD11b, MHCII, CD40, CD80, CD86 and CD8α.

7.2.6. *In vitro* stimulation of spleen and lymph node cells (II, III)

Lymph node (LN) cells were collected from NRL and NRL + CpG -treated mice (**II**) or LNs and splenic mononuclear cells were collected from mice treated with OVA and OVA + different TLR-ligands (**III**). Pooled LN cells (n= 2-4) or spleen cells (n=1-2) were suspended in RPMI 1640 medium supplemented with 5% FBS, 1 mM sodium pyruvate, 0.05 mM 2-ME (2-Mercapto ethanol), 100 U/ml penicillin and 100 µg/ml streptomycin. A total of 3-5 x 10^6 cells were cultured for 24h in 12- or in 24-well plates in the presence of NRL (100 µg/ml) (**II**) or OVA (50 µg/ml) (**III**). Control wells contained RPMI only. The supernatants were used to measure protein concentrations by the ELISA method.

7.2.7. Measurement of cytokines (II, III) and serum antibodies (I-III)

Cytokine levels of IFN-γ, IL-4 and IL-13 were measured by ELISA from BAL fluids (**II, III**), from supernatants of cultured lymph node (**II, III**) and spleen cells (**III**) according to the manufacturer's instructions (eBioscience San Diego, CA for IFN-γ and IL-4 and R&D Systems, Minneapolis, MN for IL-13). The lower limits of detection were 8 pg /ml for IFN-γ and IL-13 and 4 pg /ml for IL-4.

Antigen-specific IgE (**I-III**), antigen-specific IgG2a (**II, III**) and total IgE (**I**) antibodies were measured from serum by ELISA. For antigen-specific IgE and IgG2a measurements, plates were coated with NRL (20 µg/ml) (**II**) or with OVA (2 µg/ml) (**III**) and bound antibodies were detected either with biotin-conjugated rat anti-mouse IgE (clone R35-118) or with biotin-conjugated rat anti-mouse IgG2a (clone R11-89) (BD Biosciences, San Jose, CA). Streptavidin horseradish peroxidase followed by the substrate was used to detect the bound antibody levels. Optical density was measured at 405 nm with an ELISA plate absorbance reader (Multiscan MS, Labsystems, Helsinki, Finland). For total IgE measurements, plates were coated with rat anti-mouse IgE (clone 35-72) mAbs (BD Biosciences, San Jose, CA) and diluted serum samples were added to the plates. Purified mouse IgE (clone C38-2) was used as the standard. Biotinylated rat anti-mouse IgE (clone 35-

118) and streptavidin horseradish peroxidase, together with substrate, were used to detect the bound antibody levels measured at 405nm.

7.2.8. Statistics (I-III)

Statistical analyses were performed by GrapPadPrism (version 4 GraphPad Software Inc.) (**I-III**). Single group comparisons were conducted by the nonparametric MannWhitney *U*-test. Results are expressed as mean ±SEM. A p-value of less than 0.05 was considered to be statistically significant.

8. RESULTS

8.1. Effects of different exposure routes (i.c., i.p. and i.n.) on airway, and on systemic responses in NRL-induced murine model of asthma (I)

After four weeks of NRL sensitization and subsequent three days' inhalation challenge, the amount of inflammation in the lung tissue was investigated by analyzing H&E -stained tissue sections and counting the differentiated cells in the BAL fluid. H&E staining revealed a significant perivascular and peribronchial inflammation in the lung tissue when mice were treated with latex by i.c. and i.p. route, while i.n. sensitization induced only a slight increase in perivascular inflammation (**I**, Fig. 1 and Suppl. Fig. 1). At the same time, i.c. sensitization was the only route triggering a dramatic enhancement of both perivascular and peribronchial eosinophils in the lung tissue, whereas both i.c. and i.p. exposures caused an increase in the numbers of lymphocyte and eosinophil in BAL fluid (**I**, Fig. 1B).

Airway reactivity to inhaled metacholine was measured in asthmatic mice by using non-invasive plethysmography (Buxco) 24h after the final NRL challenge. Both i.c. and i.p. NRL sensitization followed by NRL challenge were able to induce vigorous airway reactivity to inhaled metacholine. On the other hand, i.n. NRL sensitization failed to induce AHR (**I**, Fig. 2A). When analyzing mucus production around the bronchioles from PAS-stained lung tissue sections, it was noted that i.c. and i.p. exposures had stimulated a significant enhancement in the amount of mucus after NRL inhalation (**I**, Fig. 2B and Suppl. Fig. 2).

Chemokines are important mediators attracting inflammatory cells to the site of inflammation. Several CC chemokines are known to attract eosinophils (CCL3, CCL8, CCL11, CCL24) or Th2 cells (CCL1, CCL17). RT-PCR analysis of lung samples revealed that the expressions of most of the CCL chemokines studied were significantly higher after i.c. and i.p. NRL exposure, when compared to mice exposed intranasally to NRL (**I**, Fig. 3). In order to investigate the effects of different exposure routes on Th2- and regulatory cytokines in the lung tissue,

expression levels of IL-4, IL-5, IL-13, IL-10, TGF-β and Foxp3 mRNA were analysed. The expression levels of IL-4 and IL-13 mRNA were significantly induced after all exposure routes, while IL-5 mRNA showed a significant enhancement only after i.c. exposure (**I**, Fig. 4A). Interestingly, the induction of regulatory cytokine IL-10 mRNA was detectable in all NRL-treated groups, whereas TGF-β and Foxp3 mRNA were elevated after i.p. and i.n. exposure but not after i.c. exposure (**I**, Fig. 4B). Finally, a major elevation was detected in both total IgE and Hevb6.01 specific IgE levels after i.p. and i.c. exposure, while after i.n. NRL sensitization, the IgE antibody levels remained near to the baseline level as in control group (**I**, Fig. 5).

8.2. Effects of TLR9 ligand CpG on lung inflammation and AHR in NRL-induced murine model of asthma (II)

In order to investigate the effects of immunostimulatory CpG adjuvants on airway inflammation and asthma, a murine model of NRL- induced asthma (developed and characterized in study **I**) was used in the experiments. CpG and mutated (control) CpG were administered intradermally with PBS or NRL for 4 weeks, and the analysis of AHR and the collection of samples were conducted 24h after the final NRL inhalation challenge. Histological staining of the BAL fluid and lung samples revealed a significant reduction in eosinophilic airway inflammation and mucus production after CpG administration as compared to mice given NRL only (**II**, Fig. 1). At the same time, CpG elevated the number of lymphocytes and neutrophils in the BAL fluid, as well as inducing an influx of CD11+ dendritic cells into the lung tissue. Further analysis of the lung samples by RT-PCR showed a drastic downregulation in the expression of Th2 type cytokines IL-4, IL-5 and IL-13 and induction of Th1 type cytokines IL-12 and IFN-γ in the lungs (**II**, Fig. 2A, 2B).

Asthma is characterized by AHR, a manifestation of reversible airway obstruction resulting from smooth muscle constriction. Interestingly, a significant enhancement in the AHR to inhaled metacholine was detected after intradermal CpG administration using both non-invasive (**II**, Fig. 3A) and invasive (**II**, Fig. 3B) methods. FACS analysis of BAL fluid displayed an increased ratio of CD8+ T cells

over CD4 + T cells after CpG administration (**II**, Fig. 3D). In the subsequent experiments, it was shown that the levels of IFN-γ in BAL fluid were significantly induced (**II**, Fig. 3E), and that CD8+ T cells in BAL fluid were responsible for the production of most of the intracellular IFN-γ (**II**, Fig. 3F).

Three knock-out mice strains (RAG1-/-, STAT4-/- and STAT6-/-) were used in the following experiments to clarify the contribution of CpG-induced AHR on T- and B-cells, Th1/Th2 transcription factors and the levels of IFN-γ in the lung and in BAL fluid. AHR measurements revealed the unresponsiveness of RAG1-/- mice to inhaled metacholine in all studied mice groups (**II**, Fig. E2A). In contrast, STAT6-/- mice exhibited a markedly enhanced AHR after CpG treatment, an effect that was shown to be absent in STAT4-/- mice (**II**, Fig. 4A). When analysing Th1-type (IFN-γ) and Th2-type (IL-13) cytokines, it was shown that neither RAG1-/- nor STAT4-/- mice had any detectable amounts of IFN-γ mRNA or protein in the lung tissue and BAL fluid while in WT or STAT6-/- mice, this cytokine was clearly enhanced (**II**, Fig. 4B-C). On the contrary, the level of IL-13 protein in BAL fluid was clearly elevated in STAT4-/- mice but undetectable in STAT6-/- mice (**II**, Fig. 4D-E).

In an attempt to verify the contribution of increased IFN-γ on CpG-induced airway hyperreactivity, IFN-γ was neutralized by administering anti-IFN-γ into the lungs on three subsequent days prior to the NRL challenges. A significant decline in AHR was detectable in mice treated intranasally with anti-IFN-γ (**II**, Fig. 5A), and the levels of IFN-γ measured in BAL fluid were reduced after anti-IFN-γ treatment (**II**, Fig. 5B). Furthermore, after anti- IFN-γ treatment the ratio of CD4+/CD8+ T cells returned from the CD8+ T cell dominance observed in CpG-treated mice towards a CD4+ T cell dominance (**II**, Fig. 5D).

8.3. Effects of LPS (TLR4 ligand), Pam_3Cys (TLR2 ligand) and Poly (I:C) (TLR3 ligand) on lung inflammation and airway responses in a murine model of asthma (III)

The effects of three TLR ligands, i.e. LPS, Pam_3Cys and Poly(I:C), were studied by sensitizing mice once a week for four weeks i.d. with OVA or with OVA plus low (1 μg) and high (10 μg) dose of each ligand. The results revealed a significant attenuation in airway reactivity in mice given the higher dose of LPS and Pam_3Cys, while Poly(I:C) had no effect. Total cell numbers and the amount of eosinophils were also significantly reduced with both high and low dose of LPS and Pam_3Cys (**III**, Fig. 1C, 1D). Histological and immunohistological analyses revealed a reduction both in mucus production (**III**, Fig. 1E) and in the amount of macrophages in the lungs of LPS-treated mice (**III**, Fig. 1E, 1G). Analysis of serum antibodies revealed an enhancement of OVA-specific IgG2a (**III**, Fig. 1H) and on the other hand, a reduction in OVA-specific IgE levels compared to OVA group in all tested ligands (**III**, Fig. 1I).

Both LPS and Pam_3Cys decreased the expression of Th2 type cytokines IL-4, IL-5 and IL-13 in the lung tissue (**III**, Fig. 2A) and pro-Th2 IL-33 (**III**, Fig. 2B), while only LPS induced Th1 type cytokine, IFN-γ (**III**, Fig. 2C) after a 4 week sensitization protocol. To analyse the role of T regulatory cells in mediating the suppressive effect in the asthmatic response, the expressions of lung regulatory cytokines were investigated. The levels of Foxp3, IL-10 and CTLA-4 mRNA were all significantly reduced after LPS treatment, and the same trend was detected with the higher amount of Pam_3Cys for the expression of IL-10 and CTLA-4 (**III**, Fig. 3A-C).

The early events of sensitization phase were studied by analysing the total numbers and activation markers (CD80 and CD86) of dendritic cells in local lymph nodes 24h after i.d. injections with OVA and 10 or 30 μg of LPS or Pam_3Cys. The amount of OVA+ DCs was reduced after LPS and Pam_3Cys treatments, while the expression of both CD80 and CD86 were dose-dependently enhanced in mice treated with LPS (**III**, Fig. 4B, 4C). A kinetic study with LPS revealed the same

trend continuing from 2 to 48 hours, i.e. reduction of OVA+ DCs and an increase in the expression of activation markers (**III**, Fig. 4E-H).

To further elucidate the effects of TLR ligand activation on T cells, we investigated the T cell subpopulations in BAL fluid after 4 weeks of sensitization and challenge protocol. The results demonstrated that the number of CD8+ T cells and their IFN-γ production was clearly enhanced in BAL fluid of mice treated with OVA+LPS (**III**, Fig. 5D, 5E). In addition, a slight increase in the expression of CD69 was detected (**III**, Fig. 5F). Neutralization of IFN-γ before OVA airway challenge reduced the expression of IFN-γ and enhanced the production of IL-5 and IL-13 in the lung tissue as compared to mice treated with LPS and with IgG1 control ab (**III**, Fig. 6A-C). Finally, an increased amount of eosinophils as well as neutrophils and lymphocytes in the BAL fluid were detected after anti-IFN-γ treatment (**III**, Fig. 6D-F).

9. DISCUSSION

Asthma is a heterogeneous disease affecting over 300 million people worldwide. In addition to genetic factors, environmental factors influence the progression of asthma and other atopic diseases. TLRs and their ligands which are present ubiquitously in the environment play an important role both in stimulation of the immune system as well as immunomodulatory agents redirecting the immune response towards Th1, Th2 or Treg responses. In the present thesis, the effects of different exposure routes and different TLR ligands on the asthmatic response using experimental mice model have been studied in detail. The results show that allergen exposure through the skin was the most effective way to elicit a Th2-type inflammatory response and to enhance airway reactivity. In the following experiments, the intradermal sensitization model was used to investigate the immunomodulatory effects of different TLR ligands (CpG, ligand for TLR9, LPS, ligand for TLR4, Pam_3Cys, ligand for TLR2 and Poly(I:C) ligand for TLR3) on the outcome of asthma. In general, all of the tested ligands with the exception of Poly(I:C) evoked a clear reduction in eosinophilic airway inflammation, as well as decreasing Th2-type cytokines and mucus production in the lungs. Moreover, also adverse effects on AHR were detected with CpG.

9.1. Effects of different exposure routes on lung inflammation and AHR

NRL allergy has been a significant occupational health problem especially among HCWs with a peak in incidence occurring in the late 1980s and early 1990s. Since then, the prevalence of latex allergy has decreased substantially, mainly due to the switch to low-protein or latex-free gloves among the personnel working in health care industry. Today, the incidence of latex allergy in the general population is estimated to be less than 1-2%, and among HCWs, the sensitization rates is around 4-6% (Bousquet *et al.*, 2006; Taylor and Erkek, 2004). In general, the exposure to latex antigens may occur by the cutaneous, mucosal and parenteral routes. The aerosolized NRL particles from powdered latex gloves may act as a vehicle for latex allergens, causing asthmatic symptoms in a sensitized individual. In order to investigate the importance of different exposure route in the development of

asthmatic response after sensitization and challenge with NRL, three different routes, i.e. i.c., i.p. and i.n., were used in mouse models of asthma.

After four weeks of NRL sensitization and the subsequent airway challenge, major differences in the amount of lung inflammation and airway reactivity were seen depending on the route of exposure. After i.c. and i.p. sensitization, the overall inflammation in the lung tissue was clearly enhanced, and the lungs contained a large number of infiltrating eosinophils (**I**, Fig. 1A, 1B and Supplementary Fig S1). In addition, increased AHR to inhaled metacholine and mucus overproduction in the bronchus were seen after the i.c. and i.p. exposure routes (**I**, Fig. 2A, 2B and Supplementary Fig. S2). All these effects were either moderate or completely absent in i.n. exposed mice. Increased inflammation and the airway reactivity after sensitisation/challenge with allergen are well-established cardinal features of asthma. Both of these hallmarks were notably elevated after the i.c. sensitization route, demonstrating that sensitization via the skin without any adjuvant can contribute to the development of asthma using latex as the model allergen. On the other hand, sensitization via the airways using the same amount of allergen and sensitization period was not able to induce a clear inflammatory response in the lungs or airway reactivity to metacholine. It is generally accepted that repeated mucosal allergen challenge (i.n, inhalational or oral) leads to the formation of hyporesponsiveness, a situation known as mucosal tolerance. This immune state is characterized by specific antigen unresponsiveness, leading to a decline in airway reactivity and a protection against airway inflammation in asthma. For this reasons, immunotherapeutic methods via mucosal route have been used to protect against the development of asthma, and regulatory cytokines TGF-beta, Foxp3 and IL-10 play a major role in mediating the tolerogenic effects (Akbari *et al.*, 2001; Ostroukhova *et al.*, 2004).

Winkler et al. (2006) showed that i.n. administration of mice with Bet v 1 allergen was associated with the induction of TGF-β, IL-10 and Foxp3 in CD4+ T cells providing long-term prophylatic and therapeutic efficacy of immunosuppression (Winkler *et al.*, 2006). In addition, low-dose tolerance induction is mediated by both CD4+ and CD8+ Tr cells producing TGF- β (Alard *et al.*, 2004) and by CD4+ Tr1 cells producing IL-10 (Groux *et al.*, 1997). Similarly, it

was found that i.n. allergen sensitization with NRL induced the expression of TGF- and Foxp3 mRNA in the lung tissue, whereas i.c. NRL exposure had no effect on these parameters (**I**, Fig. 4B). Furthermore, the level of IL-10 mRNA was most enhanced in the lung of mice administered NRL intranasally (**I**, Fig. 4B), highlighting the involvement of regulatory cytokines in mediating the tolerogenic effect of mucosally delivered NRL.

Although intranasal allergen delivery usually leads to tolerance rather than induced airway inflammation, different results have also been obtained using other allergens. In the studies of Cates and coworkers (2003), house dust mite (HDM) and ragweed were used as allergens to develop a mouse model for asthma. Their results indicated that a short-term (10 days) daily i.n. administration of HDM extract elicited eosinophilic Th2-type airway inflammation and airway hyperreactivity in mice (Johnson *et al.*, 2004). Administration of purified ragweed, on the other hand, evoked only a mild airway inflammation and moderate production of Th2 type cytokines (Cates *et al.*, 2003). The magnitude of the response could be enhanced by enriching the airway microenvironment with GM-CSF. In another experiment, exposure of mice i.n. to *S. chartarum* spores three times a week during a 20 day sensitization protocol evoked a severe pulmonary inflammation (Leino *et al.*, 2006). All these discrepancies in the results of different studies clearly demonstrate that the inflammatory response by mucosal antigen administration may be affected by many factors such as the concentration of endotoxins in the allergen solutions, current airway microenvironment as well as the length of sensitization period. NRL-induced asthmatic response via i.n. route might thus require more i.n. instillations and/or adjusting the dose of antigen in a four week sensitization period to provoke a strong Th2-type immune reaction.

Induced amount of Th2-type cytokines and chemokines in the lungs and IgE production in the serum contribute to the outcome of allergic inflammatory response. The expression level of IL-5 mRNA, a key cytokine regulating eosinophil function and survival, was induced only after i.c. NRL exposure in the lung tissue (**I**, Fig. 4A). The mRNAs for IL-4 and IL-13, on the other hand, were enhanced after all exposure routes (**I**, Fig. 4A). These results support the finding that eosinophils were only present in the perivascular and peribronchial areas of the lung tissue in

mice injected i.c. with NRL, which may be due to the promotion of the survival rate of eosinophils by IL-5. Further studies have been conducted by investigating a variety of chemokines known to attract Th2 type cells (CCL1, CCL17) (Panina-Bordignon *et al.*, 2001) and inducing the recruitment of eosinophils to the site of inflammation (CCL3, CCL5, CCL8, CCL11 and CCL24) (Zimmermann *et al.*, 2003). The results show that CCL8, CCL1, CCL11 and CCL24 mRNA displayed significantly enhanced expressions after cutaneous sensitization in the lung tissue compared to i.n. exposed mice (**I**, Fig. 3A and 3B). However, increased amount of eosinophils in the lung of mice sensitized i.c. with NRL may not be explained only by the induction of certain chemokines, as all the tested chemokines were equally induced also after i.p. exposure. An analysis of systemic allergen-specific as well as total IgE antibody response after NRL sensitization demonstrated that the levels were significantly enhanced in the serum after i.c. and i.p. exposure, while the mice given NRL via i.n. route did not generate any IgE antibodies at all against the allergen (**I**, Fig. 5).

These results highlight the fact that both T- and B-cell responses were dimished after intranasal sensitization, providing an extensive suppression of inflammatory response. Taken together our data demonstrate that cutaneous NRL exposure without any adjuvant is a most effective and natural way of generating a strong Th2 type immune response against NRL in the current experimental settings. Intranasal NRL sensitization, on the other hand, produced more a tolerogenic state by reducing airway inflammation, mucus production, IgE levels and AHR and inducing regulatory mechanism through enhanced production of TGF-β, Foxp3 and IL-10.

9.2. Effects of intradermally administered CpG on lung inflammation and AHR

Allergen IT involves subcutaneous or sublingual administration of allergens in order to reduce the clinical signs and to modify the natural course of allergic diseases. Successful IT results in variety immunological changes to allergens and achieves an improvement of clinical symptoms and decreases drug requirements. Bacterial CpG -containing DNA sequences (CpG ODNs) are potent stimulators of

immune system and strong adjuvant molecules able to down-modulate Th2 and induce Th1 responses. For their well-known immunostimulatory capacity, CpG motifs are frequently used in immunotherapeutic studies for the treatment of variety of diseases (cancer, allergies, asthma) in murine models and more recently in clinical settings. In the second experiment of the thesis, the immunomodulatory effects of intradermally administered CpG motifs were investigated using the previously characterized latex-induced murine model of asthma (**I**).

After four weeks of NRL sensitization, intradermally administered CpG reduced the amount of eosinophils in the BAL fluid and in the lung tissue, mucus secretion and systemic IgE response (**II**, Fig. 1). In addition, the levels of Th2 type cytokines IL-4 and IL-13 were reduced and Th1 type cytokines IFN-γ and IL-12 induced in the lung tissue (**II**, Fig. 2A and 2B). Increased production of systemic IFN-γ was also demonstrated in stimulated lymph nodes after CpG treatment (**II**, Fig. 2C). These results are consistent with several other reports revealing the capability of CpG nucleotides to effectively promote Th1 responses and to reduce Th2-mediated inflammation in murine models of asthma (Hessel *et al.*, 2005; Kline *et al.*, 2002; Kline *et al.*, 1998; Santeliz *et al.*, 2002). In these models, CpG ODNs have reduced the manifestations of allergic disease and also to reverse an established allergic response by using different routes of CpG administration. The mechanisms that contribute to the ability of CpGs to induce prophylatic and therapeutic effects in allergies are believed to be connected to the selective down-regulation of IgE and Th2 cytokine production (Liu *et al.*, 2003). In addition, CpG preferentially induces a strong Th1 bias followed by TLR9 stimulation due to the enhanced production of IFN-γ by T cells and NK cells (Krieg, 2002).

In addition to modulating the cytokine milieu within the lung tissue, the ability of CpG´s to reduce the airway reactivity to inhaled metacholine has been demonstrated in murine models of both acute and established asthma (Hessel *et al.*, 2005; Jain *et al.*, 2002; Kline *et al.*, 2002; Kline *et al.*, 1998; Santeliz *et al.*, 2002). In contrast to these studies, a significant enhancement in airway reactivity to inhaled metacholine was observed in mice treated intradermally with CpG as measured by both invasive and non-invasive whole-body plethysmography (**II**, Fig. 3A and 3B). It should be noted that in the vast majority of the experiments studying the immunomodulatory

effects of CpG on asthma, the immunization has been conducted via the i.p. route with alum as the adjuvant which poorly reflects the sensitization route occurring in real life. In the present model of NRL-induced asthma, the inflammatory response was performed with natural allergen extract via the skin without any additional adjuvant molecule. Using this model, it was shown that intradermal sensitization was even more efficient in eliciting a severe inflammation in the lungs as compared to the i.p. route. This result suggested that i.d. CpG vaccination with allergen might not have merely beneficial properties on the development of the asthmatic response.

When analysing the possible cause for CpG-induced AHR, it was noticed that i.d. CpG treatment clearly enhanced the amount of IFN-γ in the lung tissue as well as in BAL fluid (**II**, Fig. 2B and 3E). In addition, the amount of CD8+ T cells were induced over CD4+ T cells and CD8+ T cells were confirmed to produce most of the intracellular IFN-γ in BAL (**II**, Fig. 3D and 3F). Experiments with knock-out mice with impaired Th1 (STAT4-/-) and Th2 (STAT6-/-) immunity revealed the dependence of airway hyperreactivity on Th1 cells and induced production of IFN-γ (**II**, Fig. 4). According to the existing data, cytokines that support Th1 milieu in the airways dampen down the inflammatory process and reduce the risk to develop asthma. For this reason, there has been considerable interest in using Th1-inducing agents like CpG in the prevention and treatment of allergic diseases characterized by excessive Th2-cell responses. On the contrary to clear beneficial effects, opposing results have also been shown regarding Th1 vs. Th2 cells in asthma and airway hyperreactivity. In clinical experiments, increasing numbers of IFN-γ producing T cells have been detected in the BAL fluid of human asthmatics (Krug *et al.*, 1996) and enhanced amount of Th1 cells in the lungs of asthmatic mice have been shown to induce AHR and airway inflammation (Hansen *et al.*, 1999). In addition, lung viral infections known to stimulate Th1 responses are one of the most commonly recognized triggers of asthmatic exacerbations (Busse *et al.*, 1997). These data clearly demonstrate that the conventional “Th2 theory” which postulates that mainly Th2-dominant allergen-induced inflammation accounts for airway responses in conjunction with other asthmatic markers may not be adequate to explain all the different forms of asthma. Therefore, the existence of diverse forms of asthma induced by different inflammatory subtypes or profiles needs to be recognized.

Overall, the data of the present thesis raise the concern that converting Th2-biased inflammation into a more Th1-type might not exert merely beneficial effects in asthma but may lead to even more severe problems. Extra concern should be addressed for adjusting the proper dosage and route of administration of Th1-inducing adjuvants when planning new immunotherapeutic trials for clinic.

9.3. Effects of intradermally administered LPS, Pam_3Cys and Poly(I:C) on lung inflammation and AHR

The prevalence rates of asthma have been increasing in the industrialized countries over recent decades, but the reasons underlying this trend remain poorly understood. The hygiene hypothesis postulates that the lack of exposure to endotoxin and other microbial derivatives in the increasingly sterile environment of western countries during infancy favours the development of Th2-type immunity and the development of asthma and other allergic diseases later in life (Garn and Renz, 2007; Okada *et al.*, 2010). TLRs and their ligands may affect the outcome of allergic responses by stimulating immune reactions and affecting the development of the host´s immune system by modulating Th1/Th2 cytokine balance. In the third experiment of the present thesis, the effects of i.d. admistered TLR ligands, LPS, Pam_3Cys and Poly(I:C), on the outcome of airway inflammation and asthmatic response were evaluated. The same murine model of asthma as described in previous experiments was modified by using OVA as the allergen instead of NRL.

In the present study, LPS injected concurrent with OVA effectively reduced airway inflammation, AHR and the levels of Th2-type cytokines IL-4, IL-5 and IL-13 in the lungs and IL-13 in *in vitro* stimulated lymph node and spleen cells after a 4 week sensitization period (**III**, Fig. 1B-1D, 2A and Supplementary Fig S4). In addition, a decreased number of mucus producing cells around the bronchioles and reduced levels of IgE in the serum were detected (**III**, Fig. 1E and 1I). The same trend showing downregulation of asthmatic markers was also detectable with Pam_3Cys, although mainly with higher ligand concentrations. Instead, Poly(I:C) had no clear effect on any asthmatic parameters in this model although it was able to reduce IgE and induce IgG2a concentrations in serum (**III**, Fig. 1H and 1I). These

results are in line with findings by (Gerhold *et al.*, 2002), who reported that systemic LPS administration before OVA sensitization can reduce airway eosinophilia, Th2 cytokines and IgE production and in mice. Instead, there have been more variable effects attained with inhalational LPS exposure with both exacerbation and inhibition of asthmatic responses (Hollingsworth *et al.*, 2006; Ng *et al.*, 2006). The TLR2 agonist, Pam_3Cys, also displays divergent effects on asthma depending on the route of exposure, i.e. s.c. administration promoting allergic responses (Redecke *et al.*, 2004) and airway delivery showing protection from the disease (Velasco *et al.*, 2005). These results show the variable effects of different TLR ligands on the outcome of asthma and highlight the importance of the route of TLR ligand administration. The dose of the TLR agonist is also crucial, since low doses of LPS induce allergen-specific Th2-type responses, whereas a high-dose of LPS seems to achieve a more Th1-type cytokine milieu which would be predicted to protect from asthma (Delayre-Orthez *et al.*, 2004; Eisenbarth *et al.*, 2002). With Poly(I:C) detecting viral DNA, it was found that administration of a 10-times higher amount (100 µg) of ligand induced an even more drastic eosinophilia in the lungs in the present mouse model (**III**, Supplementary Fig. S2). Therefore, in addition to the route and dose of ligand, the timing of exposure (i.e. before or after the sensitization period) critically affected on the outcome of asthmatic response.

Downmodulation of all Th2-type cytokines after a four week sensitization period was one clear finding in the present study, especially with LPS. While the Th2 type cytokine IL-5 acts as a major maturation and differentiation factor for eosinophils (Takatsu *et al.*, 2009), IL-13 is involved in mediating airway hyperreactivity and mucus production (Kuperman *et al.*, 2002). Therefore, the present results strongly suggest that inhibition of Th2 type cytokines by both LPS and Pam_3Cys serve as the major mechanism for downmodulation of asthmatic responses i.e. reduction in the numbers of lung eosinophils, PAS+ cells and decreased airway reactivity to metacholine. Along with reduction of Th2 type cytokines, an enhanced expression of Th1-type cytokine IFN-γ in the lung tissue and the increased amount of CD8+ T cells producing IFN-γ in BAL fluid were found in mice treated with LPS. By neutralizing IFN-γ in the lungs, it was possible to reveal that the attenuation of Th2-type responses was dependent on enhanced production of IFN-γ by LPS in the lungs (**III**, Fig. 6). Furthermore, LPS was the only ligand able to shift the Th1/Th2

cytokine balance towards a Th1-type. These data are in line with the hygiene hypothesis suggesting that continuous exposure to bacterial derivatives favours immune deviation from the Th2-type towards the Th1 phenotype which may protect from asthma later in life. The protective effect of infectious agents have an association with allergic diseases in children raised in farms and other rural areas with prolonged exposure to high levels of bacterial endotoxins or lipopolysaccharide in their environment (Ege *et al.*, 2011; Riedler *et al.*, 2001).

The exact mechanism of how reduced exposure to pathogens during childhood enhances Th2 responses and later susceptibility to allergies is still controversial. It has been postulated that bacterial endotoxins or bacterial DNA rich with immunostimulatory CpG dinucleotides could modulate innate immune responses towards Th1-type by production of IFN-γ and IL-12 and IL-18, thus providing protection from allergic airway diseases (Kline, 2007). One of the problems with the hygiene hypothesis and Th1/Th2 paradigm is that mostly Th1-cell mediated autoimmune diseases are protected by infections leading to a Th1 response, and parasite infections providing a strong Th2 response are associated with reduced rather than enhanced allergen sensitization (Okada *et al.*, 2010). Furthermore, reduced prevalence of infectious diseases is shown to be associated not only with increased prevalence of Th2-type allergic diseases but also with Th1-mediated diseases (Bach, 2002). These even contradictory epidemiologic data may partly be explained by the fact that several factors such as timing, route as well as dose of allergen exposure critically effect on the outcome of atopic diseases, in addition to the genetics and lifestyle factors.

Today, many findings support the multifactorial mechanisms providing the immunological basis for the 'hygiene hypothesis', suggesting the involvement of T regulatory cells and immune suppression in concert with Th1/Th2 paradigm (Cousins *et al.*, 2000; Romagnani, 2004b). According to this view, the exposure to variety of microbes in early life induces the activity of Treg cells and the production of immunosuppressive cytokines IL-10 and TGF-β. The present thesis shows that the expression levels of both Foxp3, a master regulator of Treg cells, and IL-10 were actually reduced in the lungs after four weeks of LPS treatment (**III**, Fig. 3A and 3B). Furthermore, the expression of CTLA-4, which is constitutively expressed

in Foxp3+ Treg cells and plays an important role for the suppressive activity of Treg cells, was significantly reduced in the lung tissue (**III**, Fig. 3C). The present results clearly show that Treg cells are not involved in mediating the suppression of the asthmatic response in the present mouse model.

With regard to LPS, the existence of endotoxin tolerance (ET) needs also to be considered. In ET, repeated exposure to low levels of LPS induces a state of hyporesponsiveness to subsequent endotoxin challenge. In ET, monocytes and macrophages decrease their expression of MHCII and costimulatory molecules such as CD80 and CD40, leading to the inhibition of antigen presentation to T cells (Wolk *et al.*, 2000). In the present experimental settings, expression of costimulatory molecules CD40 and MHCII in lung CD11c+ and CD11b+ cells and costimulatory markers CD80 and CD86 in draining LNs, were induced in LPS treated mice (**III**, Supplementary Fig. S5 and Fig. 4B and 4C). These results indicated that in the present mouse model the blockade of Th2 responses by LPS administration was not connected to ET. While inducing the activation of DC cells, i.d. administration of LPS also led to decreased numbers of OVA+ DC cells in skin draining LNs at each time point tested (2-48h) (**III**, Fig. 4E). This result seemed to be in contrast to published data, since LPS has been shown to activate DCs both *in vitro* and *in vivo* by inducing DC maturation and survival (Rescigno *et al.*, 1998). One explanation for this observation has been proposed earlier by De Smedt et al (1998), who showed that injection of LPS resulted in delayed apoptosis of DC cells, unless receiving a survival signal from T cells (De Smedt *et al.*, 1998). DCs that were rescued following T cell activation still underwent programmed cell death 24–30 h after administration of LPS and OVA peptide. Overall, the data of the thesis suggest that the mechanism behind the loss of DC number in draining LNs in the present experimental settings may be connected to either killing of DCs by activated T cells or decreased migratory ability of DCs to the draining LNs as introduced earlier by Granucci *et al.* (1999).

An attenuation of asthmatic responses in mice after pulmonary administration after both TLR4 ligand LipidA and TLR2 ligand Pam_3Cys has been shown earlier by Velasco *et al.* (2005). Cytokine analysis from BAL fluid revealed a very modest, i.e. close to the level of detection, enhancement of IFN-γ after treatment with both

tested ligands, suggesting that some mechanism other than Th1 skewing might be involved in their model. The present study clearly showed the dependence of LPS induced IFN-γ production on the outcome of airway inflammation and Th2-type cytokine milieu. However, it is possible that Pam_3Cys with even higher ligand concentrations might induce the effect similar to LPS in the present model, but this needs further investigation. It also cannot be ruled out that effect some other than Th1/Th2 cytokine balance may be involved in mediating the protective effects of Pam_3Cys on asthma.

As conclusion, the present data demonstrate that both TLR2 and TLR4 agonist are able to suppress the asthmatic response in mice, and that the effect at least with TLR4 ligand LPS results from shifting the cytokine balance from Th2-type into Th1-type. Therefore, our results are in favour of the hygiene hypothesis, suggesting that bacterial derivatives present in the environment are able to induce Th1-type responses and to confer protection from asthma later in life. In addition, these results highlight the importance of the skin barrier function and host-pathogen interaction in the development of asthma and allergies.

10. SUMMARY AND CONCLUSIONS

The prevalence of atopy and asthma has increased over the past decades in conjunction with improved hygiene status in the western countries, suggesting that environmental factors are playing a role in this trend. Microbial recognition and the initiation of innate immune responses are mediated by the TLRs present as the first line of host defence, such as macrophages, mucosal epithelial cells and dendritic cells, binding bacterial and viral components. The present thesis focused on exploring how treatment with different TLR ligands could modulate the asthmatic response in mice. First, a murine model of asthma was established by elucidating the most effective way to induce Th2-type responses using natural NRL as an antigen.

By using different routes of NRL exposure, the variable effects on airway inflammation and airway reactivity were demonstrated in mice. Whereas i.c. and i.p. sensitization and subsequent allergen challenge with NRL induced a strong Th2-type immunity, airway inflammation and AHR, allergen administration via respiratory route produced more of an immunosuppressive state by inducing the expression of tolerogenic cytokines IL-10 and TGF-β. In addition, sensitization via the skin resulted in markedly higher numbers of eosinophils in addition to increased levels of IL-5 in the lungs compared to i.p. or i.n. routes of exposure. These results indicate that allergen exposure through the epidermis could induce a strong Th2-type immune response, suggesting that the skin is a potent site for primary antigen sensitization predisposing to the later development of airway diseases.

Administration of immunostimulatory CpG molecules present in bacterial DNA effectively reduced all the main Th2-type inflammatory markers such as Th2-type cytokines, lung eosinophils, mucus production and IgE antibodies in the serum at the same time induced Th1-type cytokines IFN-γ and IL-12 in the lungs. In addition to the beneficial effects, CpG also induced airway hyperreactivity to metacholine. By using different knock-out mouse strains and IFN-γ neutralizing antibody, the present study showed that the effect was dependent on Th1 cells and enhanced production of IFN-γ by CD8+ T cells in the airways. These results suggest that intradermal CpG administration may not only have beneficial influences on asthma

which should be taken into account when using CpG nucleotides as immunomodulatory agents in treating asthma.

Both LPS (ligand for TLR4) and Pam_3Cys (ligand for TLR2) efficiently downmodulated asthmatic responses, i.e. BAL eosinophilia, Th2-type cytokines, mucus formation and IgE levels in the murine model. Poly(I:C) (ligand for TLR3), on the other hand, had no clear effect on the local asthma responses but achieved a reduction of IgE antibodies in the serum. Further analysis revealed that LPS was the only ligand able to shift the cytokine balance towards Th1 dominance. In addition, LPS-induced IFN-γ production was related to the reduced airway inflammation and Th2-type cytokines in the lungs. These results are in line with the hygiene hypothesis which postulates that bacterial endotoxins such as LPS present in the environment favour the skewing from Th2- to Th1-type immunity, thus providing protection from the later development of asthma. Furthermore, the present results emphasize the importance of microbe-host interactions in the skin and their relation to the development of asthma and other allergies.

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Cutaneous, But Not Airway, Latex Exposure Induces Allergic Lung Inflammation and Airway Hyperreactivity in Mice

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As respiratory symptoms are common in addition to skin reactions in natural rubber latex allergy, we investigated the significance of different allergen exposure routes in the development of lung inflammation and airway hyperreactivity (AHR). Both intracutaneous (IC) and intraperitoneal (IP) exposure followed by airway challenge with latex proteins induced an influx of mononuclear cells and eosinophils to the lungs. AHR and lung mucus production increased significantly after IC and IP but not after intranasal (IN) exposure. Infiltration of inflammatory cells was associated with the induction of T-helper type 2 (Th2) cytokines and several CC chemokines. Only a marginal induction of these mediators was found after IN exposure. On the contrary, increased levels of transforming growth factor-β1 and forkhead box 3 mRNA, markers of regulatory activities, were found in the lungs after IN but not after IC exposure. Finally, IC and IP, but not IN, latex exposure induced a striking increase in specific immunoglobulin E (IgE) levels. Cutaneous latex exposure in the absence of adjuvant followed by airway challenge induces a local Th2-dominated lung inflammation and a systemic IgE response. Cutaneous exposure to proteins eluting from latex products may therefore profoundly contribute to the development of asthma in latex allergy.

Key words: asthma/chemokines/cytokines/latex hypersensitivity
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Natural rubber latex (NRL) allergy has been recognized as a major cause of occupational contact urticaria, rhinitis, and asthma in health care workers (Ahmed *et al*, 2003; Reunala *et al*, 2004). People in other occupations requiring the use of latex gloves or who are frequently in contact with other NRL products are also at risk of becoming sensitized to NRL (Turjanmaa and Makinen-Kiljunen, 2002; Ahmed *et al*, 2003; Sparta *et al*, 2004). Although skin is the most frequently reported site of allergic reactions from latex gloves, airway symptoms are also common (Fish, 2002; Nolte *et al*, 2002). In a recent study, one-fourth of the latex-allergic health care workers were reported to suffer from rhinitis and asthma and several of these asthmatic individuals were forced to change their jobs (Bernstein *et al*, 2003). Sensitization routes and pathomechanisms of NRL-induced rhinitis and asthma, however, are still poorly understood.

Sensitization to NRL proteins may occur through various exposure routes (Weissman and Lewis, 2002). Skin is an obvious target for exposure to latex proteins in people using latex gloves (Bernstein *et al*, 2003) but NRL allergens also become easily airborne in association with glove powder, thereby permitting exposure and sensitization via the airways (Charous *et al*, 2002). On the other hand, children with spina bifida are thought to become sensitized through mucous membranes to NRL proteins eluting from surgeon's gloves during operations (Weissman and Lewis, 2002). Today, little is known on how these different exposure routes interact during the development of NRL allergy or what is their importance in the elicitation of clinical symptoms.

In this study, we used mouse models of latex allergy to investigate the significance of different exposure routes, i.e. intracutaneous (IC), intraperitoneal (IP), and intranasal (IN) routes, to the development of lung inflammation, airway hyperreactivity (AHR), and antibody production to NRL allergens.

Results

IC exposure and challenge with NRL induces a vigorous influx of eosinophils into the lungs H&E staining of lung tissues revealed enhanced peribronchial and perivascular cell infiltrates consisting primarily of lymphocytes and eosinophils after IC and IP exposure (Fig 1*A* and S1). Only a slight, mostly perivascular, inflammation was observed after IN exposure. The number of infiltrating eosinophils both in perivascular and in peribronchial areas of lung tissues showed a dramatic increase in intracutaneously

Abbreviations: AHR, airway hyperreactivity; BAL, bronchoalveolar lavage; Foxp3, forkhead box 3; Hev b6.01, prohevein; IC, intracutaneous; IgE, immunoglobulin E; IL, interleukin; IN, intranasal; IP, intraperitoneal; MCh, methacholine; NRL, natural rubber latex; PAS, periodic acid-Schiff; PBS, phosphate-buffered saline; Penh, enhanced pause; RU, relative units; TGF-β1, transforming growth factor-β1; Th2, T-helper type 2

[1]These authors contributed equally to this work.

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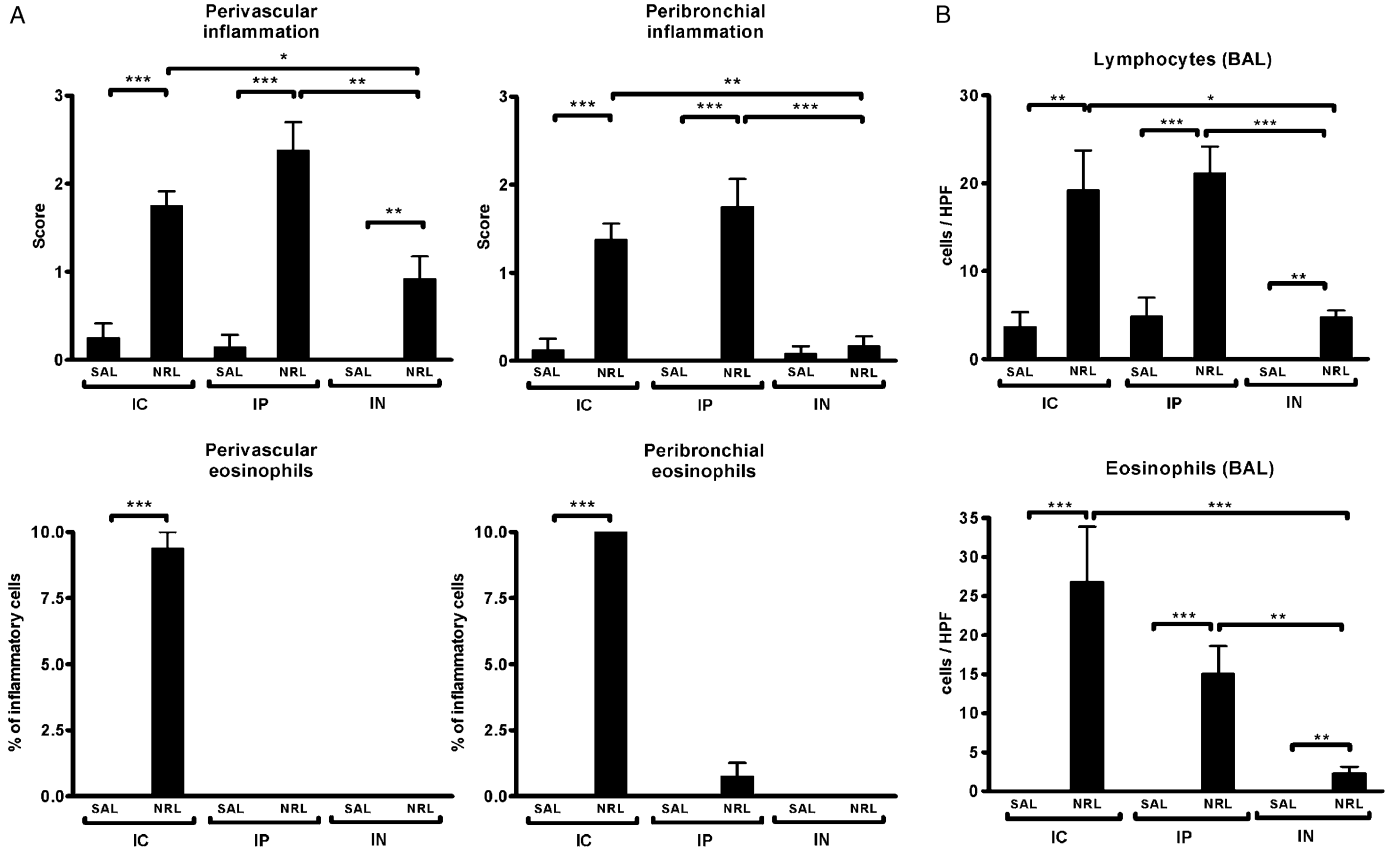

Figure 1
Lung inflammation. (*A*) Inflammation was expressed as score values and eosinophils as percentages of inflammatory cells (n = 7–12 mice per group). (*B*) Lymphocytes and eosinophils in bronchoalveolar lavage (BAL) fluids were counted per high-power field (n = 12–16 mice per group). The columns and error bars represent mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001.

exposed (p < 0.001) but not in intraperitoneally or intranasally exposed mice (Fig 1*A* and S1).

Differential cell counts of bronchoalveolar lavage (BAL) fluid samples indicated a significant eosinophil and lymphocyte recruitment into the lungs of IC and IP NRL-exposed mice as compared with phosphate-buffered saline (PBS)-treated mice (Fig 1*B*). In contrast, eosinophils were virtually absent from the BAL of the mice treated intranasally with NRL, and only a few lymphocytes were detectable (Fig 1*B*).

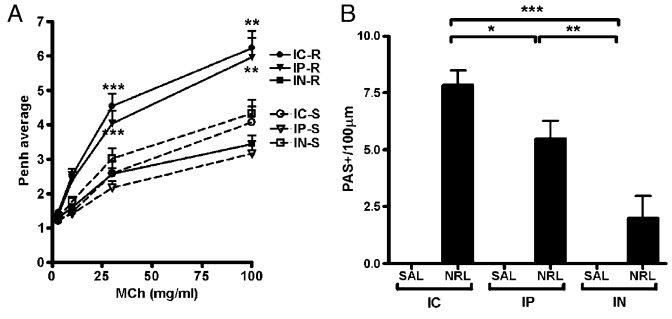

Figure 2
Airway hyperresponsiveness and lung mucus production. (*A*) Airway hyperreactivity was expressed by average enhanced pause (Penh) values in relation to increasing doses of aerosolized methacholine. Data represents mean ± SEM. (*B*) The number of mucus-producing cells was represented as periodic acid-Schiff (PAS)-positive cells. The columns and error bars represent mean ± SEM; ns, non-significant, *p < 0.05, **p < 0.01, ***p < 0.001; n = 12–16 mice per group.

We rarely detected any neutrophils or any significant changes in the number of macrophages in the BAL in any group of mice investigated (data not shown).

IC exposure and challenge with NRL induces marked AHR and lung mucus production AHR to inhaled methacholine (MCh) was analyzed in order to evaluate changes in airway resistance in the response to allergen exposure and challenge. IC and IP NRL exposure followed by airway challenge induced a strong and significant increase in airway reactivity to inhaled MCh (Fig 2*A*). In contrast, IN NRL exposure failed to induce AHR, whereas control mice showed a slightly elevated but statistically non-significant response to inhaled MCh.

Airway mucus overproduction contributes significantly to the pathophysiologic changes in asthma (Maddox and Schwartz, 2002). Lung sections were stained with periodic acid-Schiff (PAS) to quantify the amount of mucus-producing cells around the bronchioles. As shown in Fig 2*B* and S2, animals treated intracutaneously or intraperitoneally with NRL displayed a significant increase in mucus production, as seen in the high amount of goblet cells around the airway lumen. IN NRL exposure, on the other hand, induced only a minor and insignificant increase in mucus production in the airways.

Expression of several CC chemokine mRNA is strongly upregulated in lung tissue after IC and IP exposure to

NRL We analyzed several CC chemokines known to play an important role in allergic airway inflammation (Lukacs, 2001; Bisset and Schmid-Grendelmeier, 2005). Following IC, IP, and also IN NRL exposure and challenge, significant increases in CCL1, CCL8, CCL11, CCL17, and CCL24 mRNA expressions were detected in the lungs relative to PBS-treated mice (Fig 3). The level of CCL3 mRNA was significantly elevated in the lungs after IC and IP NRL exposures, whereas IN exposure did not elicit induction of this chemokine (Fig 3). Expression levels of most of the chemokines were significantly higher after IC latex exposure (CCL1, CCL3, CCL8, CCL11, and CCL24) and IP exposure (CCL1, CCL3, CCL8, CCL17, and CCL24) compared with intranasally exposed mice. CCL3 mRNA levels were significantly higher in intraperitoneally exposed mice compared with intracutaneously exposed mice. In addition, mRNA expression levels of chemokine receptors corresponding to the investigated chemokine ligands are shown in Fig S3.

IC NRL exposure induces strong expression of T-helper type 2 (Th2)-type cytokine mRNA, whereas IN exposure elicits marked induction of transforming growth factor-β1 (TGF-β1) and forkhead box 3 (Foxp3) mRNA in the airways Elevated expression of Th2-type cytokines interleukin (IL)-4, IL-5, and IL-13 in lung tissues is a characteristic feature of the pulmonary allergic response (Oettgen and Geha, 2001). IC and IP exposure to NRL elicited clear increases in the expression levels of IL-4 and IL-13 mRNA, but only IC exposure increased expression of IL-5 mRNA significantly in lung tissue compared with PBS-treated controls (Fig 4*A*). Moreover, an 8-fold enhancement in the level of IL-13 mRNA in IN NRL-exposed mice was observed in comparison with controls. IC exposure elicited significantly higher levels of IL-4, IL-5, and IL-13 mRNA compared with the intranasally exposed group. In addition, IL-4 mRNA levels were significantly elevated in intraperitoneally exposed mice compared with intranasally exposed mice.

A significant enhancement in the mRNA levels of regulatory cytokine IL-10 was observed in all NRL-exposed groups, especially in intranasally treated mice (Fig 4*B*). Interestingly, a significant elevation in the levels of TGF-β1 mRNA was observed after IN and IP NRL exposure ($p < 0.001$ and $p < 0.01$, respectively) but not after IC exposure. The TGF-β1 mRNA levels were significantly higher in IN and IP mice when compared with intracutaneously exposed mice (Fig 4*B*). In addition, Foxp3 transcription factor mRNA expression also enhanced significantly after IP and IN NRL administration but not after IC exposure (Fig 4*B*). Expression increased most significantly after IN latex exposure.

Total and Hev b 6.01-specific immunoglobulin E (IgE) levels are strongly elevated after IC and IP NRL exposure Allergen-specific IgE antibodies are the hallmark of an immediate allergic reaction (Geha *et al*, 2003). Both IC and IP exposure to NRL elicited the strong induction of total IgE antibodies (Fig 5). On the contrary, total serum IgE remained in the baseline level after IN NRL exposure. IC and IP exposure induced high levels of IgE antibodies against major

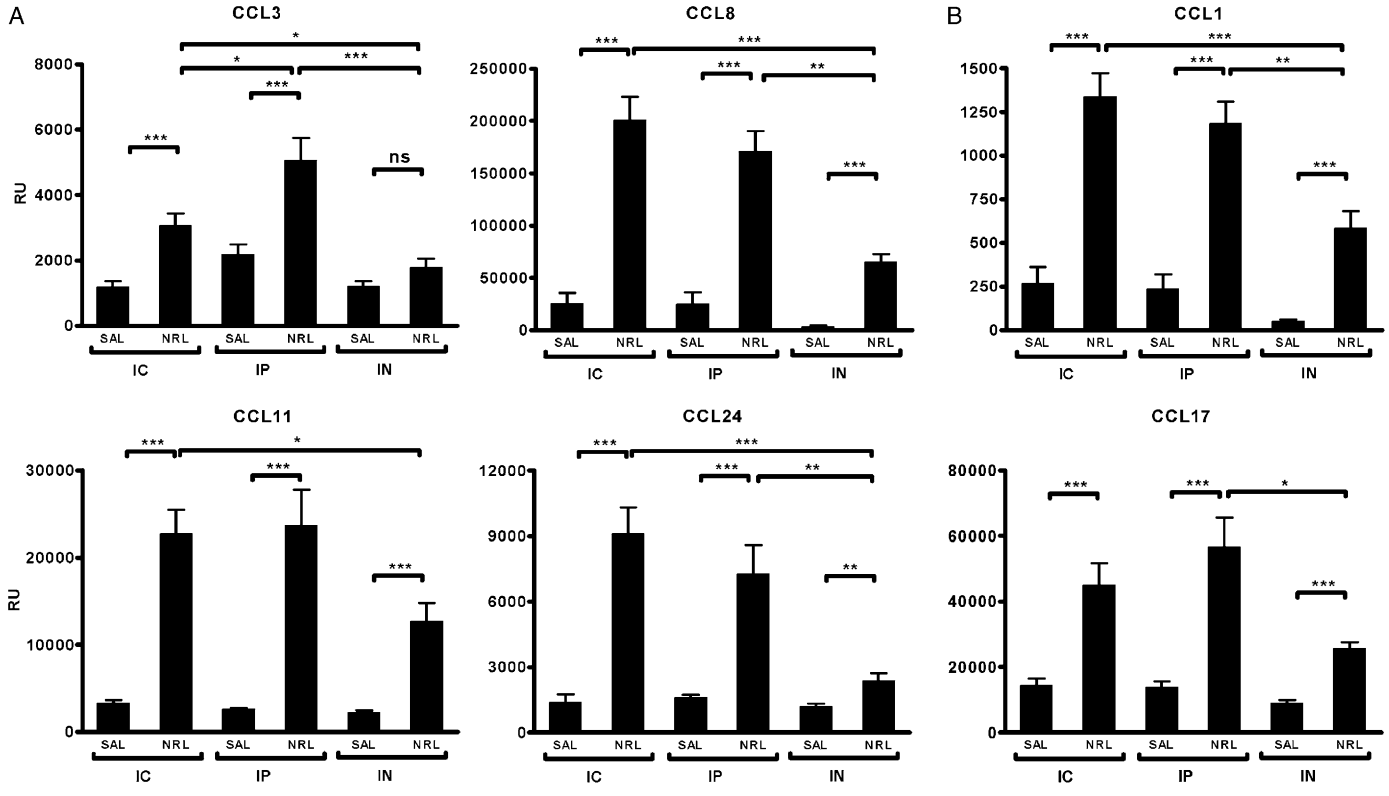

Figure 3
Chemokine mRNA exposure. mRNA expression of chemokines, which attract especially eosinophils (*A*) or T-helper type 2 cells (*B*) in lung samples after exposures to vehicle (saline (SAL)) or natural rubber latex (NRL). Relative units (RU) are relative differences compared with the calibrator. The columns and error bars represent mean ± SEM; ns, non-significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; $n = 12$–15 mice per group.

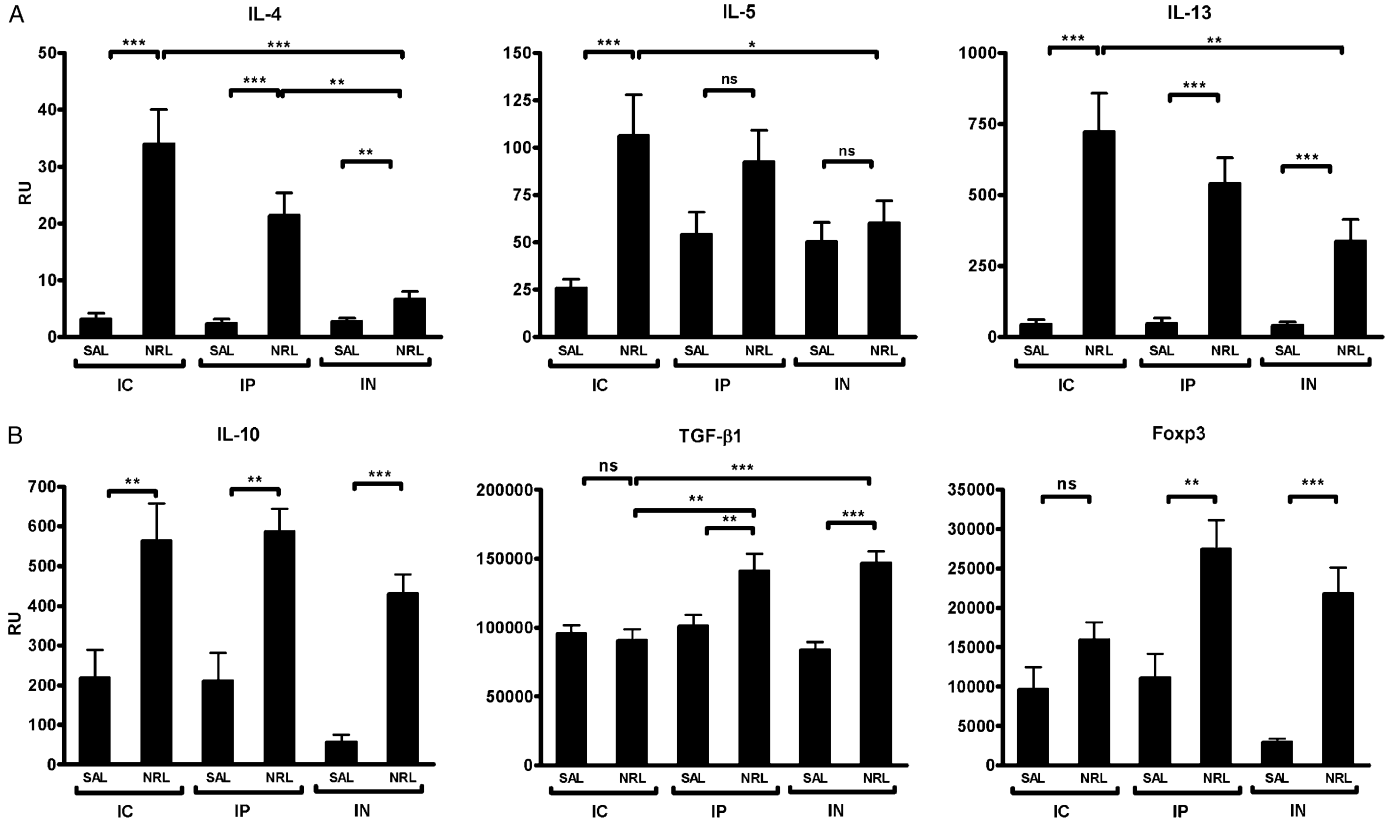

Figure 4
Expression levels of cytokines and Foxp3 mRNAs. T-helper type 2 cytokine mRNA (*A*) and regulatory cytokine and forkhead box 3 (Foxp3) mRNA (*B*) expression in lung samples after exposures to vehicle (saline (SAL)) or natural rubber latex (NRL). The columns and error bars represent mean ± SEM; ns, non-significant, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$; n = 12–15 mice per group.

NRL allergen, Hev b 6.01 (Fig 5). In contrast, IN NRL exposure did not induce detectable levels of prohevein (Hev b 6.01)-specific IgE antibodies.

Discussion

Sensitization to NRL products has been one of the leading causes of occupational asthma during the last several years (Fish, 2002). The sensitization routes and pathomechanisms of NRL-induced rhinitis and asthma are, however, not fully understood. As sensitization to NRL proteins may take place through various exposure routes, we investigated their significance in the development of allergic asthma in mouse models. Our findings demonstrate that cutaneous, but not airway, NRL administration in the absence of adjuvant induces a local Th2-dominated lung inflammation and an intense AHR after airway NRL challenge.

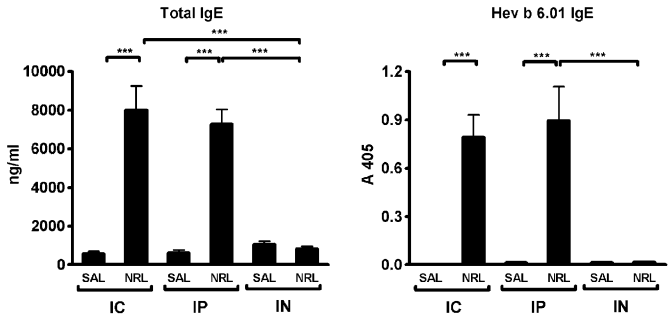

Figure 5
Immunoglobulin E (IgE) levels. Total serum and prohevein (Hev b 6.01)-specific IgE levels in mice after exposures to vehicle (saline (SAL)) or natural rubber latex (NRL) were measured by ELISA. Specific IgE levels of the serum are expressed as absorbance units (405 nm). The columns and error bars represent mean ± SEM; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$; n = 12–15 mice per group.

Chronic airway inflammation, an integral feature of allergic asthma, is characterized by the accumulation of inflammatory cells, such as eosinophils (Herrick and Bottomly, 2003). In this study, lung eosinophilia was significantly stronger after IC exposure compared with IN or IP exposures, demonstrating that repeated cutaneous NRL exposure without an external adjuvant is highly efficient in inducing eosinophilic lung inflammation after airway NRL challenge. AHR and airway mucus overproduction contribute significantly to the pathophysiological events in allergic asthma (Herrick and Bottomly, 2003). Both IC and IP exposure, but not IN exposure, followed by NRL airway challenge induced a major increase in AHR to inhaled MCh. Intracutaneously and intraperitoneally exposed mice also showed a significant increase in mucus production. Only a minor increase in mucus production was seen in the airways after IN NRL exposure. In agreement with these findings, Howell *et al* (2002) recently reported that repeated topical NRL exposure to tape-stripped skin elicited an increase in AHR to inhaled MCh, but repeated intratracheal exposure of NRL was unable to induce AHR to inhaled MCh (Howell

et al, 2004). On the other hand, Woolhiser *et al* (2000) previously demonstrated IgE response and AHR following IN exposure of 50 mg of NRL administered 5 d per wk for 10 wk. Our results, however, clearly demonstrate that dermal route exposure using much lower allergen dosage and length of exposure leads to vigorous IgE response, AHR, and airway inflammation after airway challenge.

In order to investigate the mechanisms of leukocyte recruitment to the airways, in detail we studied several CC chemokines in lung tissue. Eosinophils are known to respond to CCL3, CCL5, CCL8, CCL11, and CCL24 (Zimmermann *et al*, 2003). In this study, expressions of CCL8, CCL11, and CCL24 mRNA were significantly higher in intracutaneously exposed mice compared with intranasally exposed mice with marked airway eosinophilia after cutaneous exposure. These chemokines were, however, equally expressed in intraperitoneally and intracutaneously exposed groups although lung eosinophilia was significantly more intense in the intracutaneously exposed mice. Expression levels of CCL1 and CCL17, which are known to attract Th2 cells (Panina-Bordignon *et al*, 2001), were also higher after IC and IP exposure compared with IN exposure. Taken together, our results demonstrate that cutaneous and IP NRL exposure elicits induction of various chemokines in lung tissue, which in turn may recruit Th2-type inflammatory cells, a characteristic of allergic airway inflammation.

A consensus exists that IL-4 and IL-13 are key regulators of IgE class switching (Geha *et al*, 2003). In addition, it has been shown that IL-13 contributes to AHR and mucus overproduction (Kuperman *et al*, 2002). Expression levels of IL-4 and IL-13 mRNA were significantly enhanced after all routes of exposure with NRL in lung tissue in this study. But increases were markedly higher after IC exposure and to a lesser extent after IP exposure, in comparison with IN exposure. The expression level of IL-5, a key cytokine regulating eosinophil recruitment and survival (Hamelmann and Gelfand, 2001), was significantly elevated only after IC NRL exposure. Thus, the greater number of eosinophils in the lungs of intracutaneously exposed mice may be because of an increased eosinophil survival rate induced by increased levels of IL-5 in the airways. On the other hand, CCL24 and IL-5 have been shown to cooperatively promote eosinophil accumulation into airways and to increase AHR to inhaled MCh (Yang *et al*, 2003). Only a few studies exist in the literature describing cytokine expression in lungs after NRL administration. Hardy *et al* (2003) recently reported that mice sensitized intraperitoneally with NRL allergens, and with NRL glove extract demonstrated elevated levels of IL-5 protein in the BAL fluid. They found no significant differences in the levels of IL-4 protein between controls and latex-sensitized groups. Our results clearly indicate that repeated cutaneous NRL exposure induces Th2-dominated cytokine expression in the lungs after airway challenge.

Increased levels of total and allergen-specific IgE antibodies in the patient serum are characteristic of allergic asthma (Busse and Lemanske, 2001; Lemanske and Busse, 2003). In this study, both IC and IP NRL exposure elicited a significant elevation of total serum IgE levels. As an example of allergen-specific IgE response, we investigated antibody responses against Hev b 6.01, which is a major NRL allergen (Alenius *et al*, 1996; Wagner and Breiteneder, 2005). The sera from intracutaneously or intraperitonelly exposed mice contained high levels of IgE antibodies to Hev b 6.01. On the contrary, IN exposure failed to induce elevation of total and specific IgE antibodies. In agreement with these findings, Hufnagl *et al* (2003) also reported increased NRL allergen-specific IgE levels after IP immunization, and a recent study by Woolhiser *et al* (2000) demonstrated that different exposure routes can induce differences in NRL-specific IgE profiles in mice.

It is of interest that airway inflammation, AHR, and IgE levels were substantially lower after IN exposure compared with IC exposure. To address the role of regulatory cytokines in the induction of airway inflammation, we analyzed the mRNA levels of IL-10 and TGF-β1, both of which are known to play important roles in the downregulation of immune responses (Terui *et al*, 2001; Akbari *et al*, 2003; Nagler-Anderson *et al*, 2004). Proportional increase (allergen-exposed mice *vs* PBS-exposed control) in the expression of IL-10 mRNA was markedly more prominent in intranasally exposed mice (7.4-fold increase) compared with intracutaneously (2.6-fold increase) and intraperitoneally exposed (2.8-fold increase) mice. In line with these results, Akbari *et al* (2001) have reported that respiratory exposure to allergens can induce T cell tolerance, which appears to be mediated by IL-10 production. On the other hand, we found significant induction of TGF-β1 mRNA only after IN and IP NRL exposure but not after IC exposure. Furthermore, proportional expression of transcription factor Foxp3, which is expressed predominantly by CD4 + CD25 + T regulatory cells (Fontenot *et al*, 2003; Khattri *et al*, 2003), was significantly upregulated after IN exposure (7.3-fold) but to a lesser extent after IP exposure (2.5-fold) and IC exposure (1.6-fold). Thus, it is possible that increased levels of IL-10 and TGF-β1 as well as Foxp3 + regulatory T cells in the lungs of intranasally exposed mice suppress the inflammatory responses, resulting in diminished airway inflammation. The link between TGF-β and regulatory T cells is supported by the finding that TGF-β is able to promote differentiation of T cells into Foxp3 + regulatory T cells (Chen *et al*, 2003; Fantini *et al*, 2004).

These findings underline the role of cutaneous route allergen exposure in the elicitation of allergic airway inflammation and AHR. Instead of epicutaneous (Spergel *et al*, 1998; Lehto *et al*, 2003) allergen application, we used IC allergen exposure assuring that the actual allergen dose entering the body was the same in all exposure routes. The present observations are of importance when considering prevention of airway hypersensitivity to NRL allergens. Glove powder is the most important carrier of NRL allergens to airways, and thus primary prevention has mainly focused on the use of non-powdered latex gloves (Charous *et al*, 2002). As the exposure via the cutaneous route could be an important way for sensitizing airways, attention will need to be paid to the use of low- or non-allergen NRL gloves instead of using only non-powdered gloves.

Materials and Methods

Allergens NRL and Hev b 6.01 were purified as earlier described (Lehto *et al*, 2003).

Mice exposure protocols and AHR Six- to eight-week-old female BALB/cJBom mice were obtained from Taconic M&B A/S (Ry, Denmark), Social and Health Services of Finland; Provincial Office of Southern Finland approved all experimental protocols.

Exposure protocols are shown in Fig 6. Mice were exposed for 4 wk before airway challenge. In saline (SAL) groups exposures were made with PBS and with NRL in NRL groups. Mice were anesthetized with isoflurane (Abbott Laboratories Ltd, Queenborough, UK) for IC and IN exposure. The backs of the mice were shaved and tape-stripped (Tegaderm, 3M Health Care, St Paul, Minnesota) four times before IC exposure to remove loose hair and to induce standardized skin injury mimicking the scratching that is a characteristic feature in patients with atopic dermatitis. In IC exposure, mice were injected once a week with 40 μg of NRL in 100 μL of PBS or with 100 μL PBS as control. IN exposure was also made once a week with the same amounts of NRL in 50 μL PBS. In IP exposure, mice were injected on days 1 and 14 with 80 μg NRL together with 2 mg alum (Pierce, Rockford, Illinois) in 100 μL PBS. Mice were challenged after exposures via the respiratory route using an ultrasonic nebulizator (Aerogen Ltd, Galway, Ireland) with 0.5% NRL (in PBS) for 20 min on days 28, 29, and 30. On day 31, AHR to MCh (Sigma-Aldrich Co, St Louis, Missouri) was measured by using whole-body plethysmography (Buxco Electronics Inc., Sharon, Connecticut) as previously described (Hamelmann *et al*, 1997) and monitored by average Penh (enhanced pause = pause × (peak inspiratory box flow/peak expiratory box flow)) values. MCh concentrations were 3, 10, 30, and 100 mg per mL. The mice were then sacrificed with carbon dioxide, and samples (BAL fluids, lung biopsies, and blood) were taken for subsequent analysis. The experiment was repeated two times, and the combined results are presented.

BAL and lung histology Lungs were lavaged with 0.8 mL of PBS, and BAL specimens were cytocentrifuged (Shandon Scientific Ltd, Runcorn, UK) at 150 × *g* for 5 min. Cells were stained with May–Grünwald–Giemsa and counted blinded in 15–20 high-power fields at × 1000 under light microscopy.

Formalin-fixed lungs were embedded in paraffin, and lung sections (4 μm) were stained with H&E or with PAS solution. Inflammation in different lung areas was expressed after H&E staining as score values 1–5 (1, slightly inflamed tissue; 2, inflammation of the area over 20%; 3, inflammation of the area over 50%; 4, inflammation of the area over 75%; and 5, entire tissue inflamed). The number of mucus-producing cells was determined after PAS staining by choosing three bronchial sections randomly and counting PAS-positive cells per 100 μm. The slides were analyzed blinded with a Nikon EclipseE800 microscope (Leica Microsystems GmbH, Wetzlar, Germany).

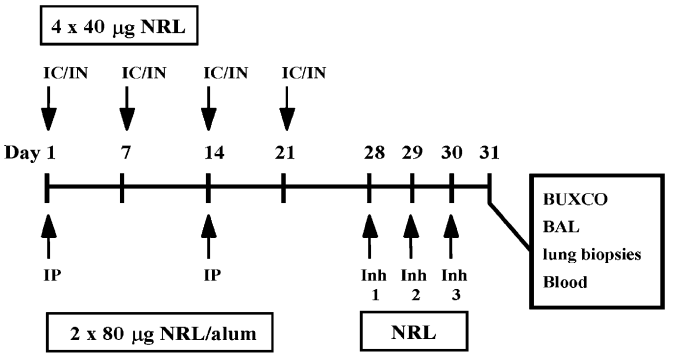

Figure 6
Intracutaneous (IC), intraperitoneal (IP), and intranasal (IN) exposure schedules. Mice were exposed for 4 wk before airway challenge. In saline (SAL) groups exposures were made with phosphate-buffered saline and with natural rubber latex (NRL) in NRL groups. Airway challenges were made by NRL. Airway hyperreactivity to methacholine was measured 24 h after airway challenges and different samples were taken for subsequent analysis.

Total and specific IgE antibodies in serum Total IgE levels were measured as previously described (Lehto *et al*, 2003). A specific ELISA for purified Hev b 6.01 allergen was slightly modified. Plates were first coated for 3 h at +20°C and then overnight at +4°C. The blocking time was 1 h at +20°C and 1:10 diluted samples were incubated for 2 h at +20°C. Biotin-labeled anti-mouse isotype-specific antibody was added for 2 h at +20°C. Streptavidin-conjugated alkaline phosphatase (Zymed, San Francisco, California, diluted 1:1000) was incubated for 30 min at +20°C, color substrate (*p*-nitrophenyl phosphate, Sigma-Aldrich Co) was added, and absorption at 405 nm was read. The sera, biotinylated anti-mouse antibodies, and streptavidin-conjugated phosphatase were diluted in 0.2% BSA/0.05% Tween/PBS.

Expression of mRNA in lungs RNA extraction and synthesis of cDNA was carried out as described earlier (Lehto *et al*, 2003). Real-time quantitative PCR was performed with an AbiPrism 7700 Sequence Detector System (SDS) (Applied Biosystems, Foster City, California) as previously described (Lehto *et al*, 2003). PCR primers and probes were from Applied Biosystems as predeveloped reagents (18S ribosomal RNA, IL-4, IL-5, IL-10, IL-13, CCL1, CCL3, and Foxp3) or were self-designed. The self-designed sequences of CCL11, CCL17, and CCL24 have been described earlier (Lehto *et al*, 2003), and others are as follows: TGF-β1 (forward 5′-CAA GGG CTA CCA TGC CAA CTT-3′, probe 5′-CAC ACA GTA CAG CAA GGT CCT TGC CCT CT-3′, and reverse 5′-ATG GGC AGT GGC TCC AAA G-3′), CCL8 (forward 5′-CCC TTC GGG TGC TGC AAA G-3′, probe 5′-TAC GAG AGA ATC AAC AAT ATC CAG TGC CCC-3′, and reverse 5′-TCT GGA AAA CCA CAG CTT CCA-3′). The results are expressed as relative units (RU), which were calculated by the comparative C_T method (Lehto *et al*, 2003).

Statistics Statistical tests were performed using GraphPad Prism Version 4 (GraphPad Software Inc.). Single group comparisons were conducted by the non-parametric Mann–Whitney *U* test. A p-value of less than 0.05 was considered to be statistically significant.

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Supplementary Material

The following material is available online for this article.
Figure S1 Histology of H & E stained penvascular using tissues.
Figure S2 Histology of PAS-stained peribronchial lung sections.
Figure S3 Chemokine receptor mRNA expression.

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Supplementary Figures (Lehto *et al.* 2005)

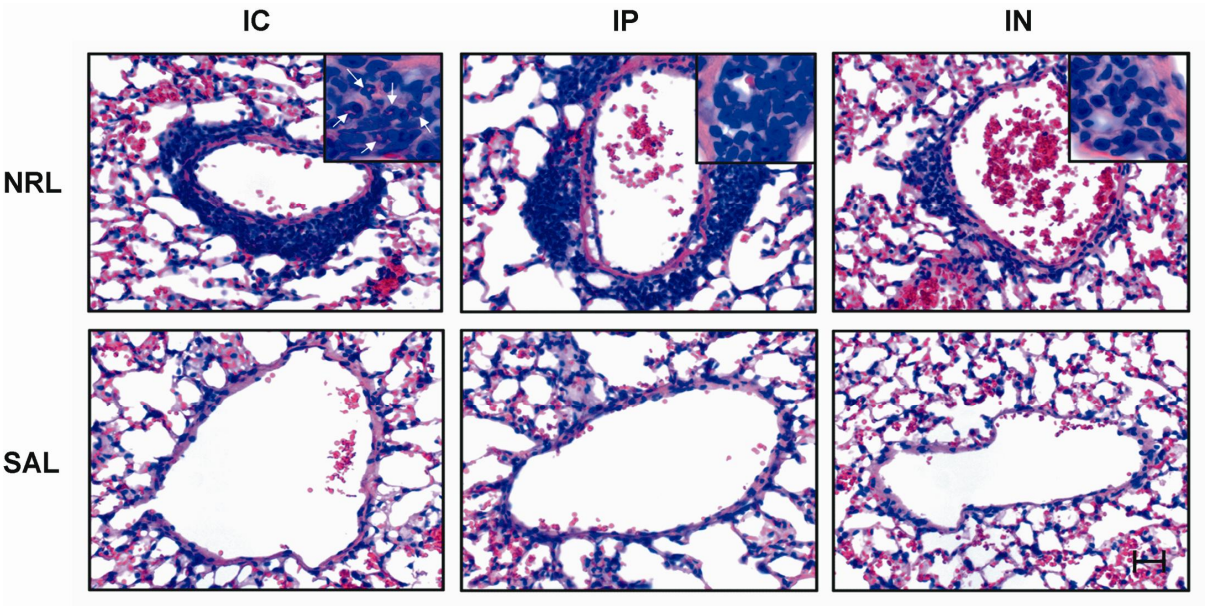

Figure S1. Representative micrographs from perivascular lung tissues after i.c., i.n. and i.p. exposures (H&E staining, magnification x400 and x1000 in inserts). Arrows in the insert of i.c. NRL exposure photograph point to eosinophils. Scale bar = 20 µm.

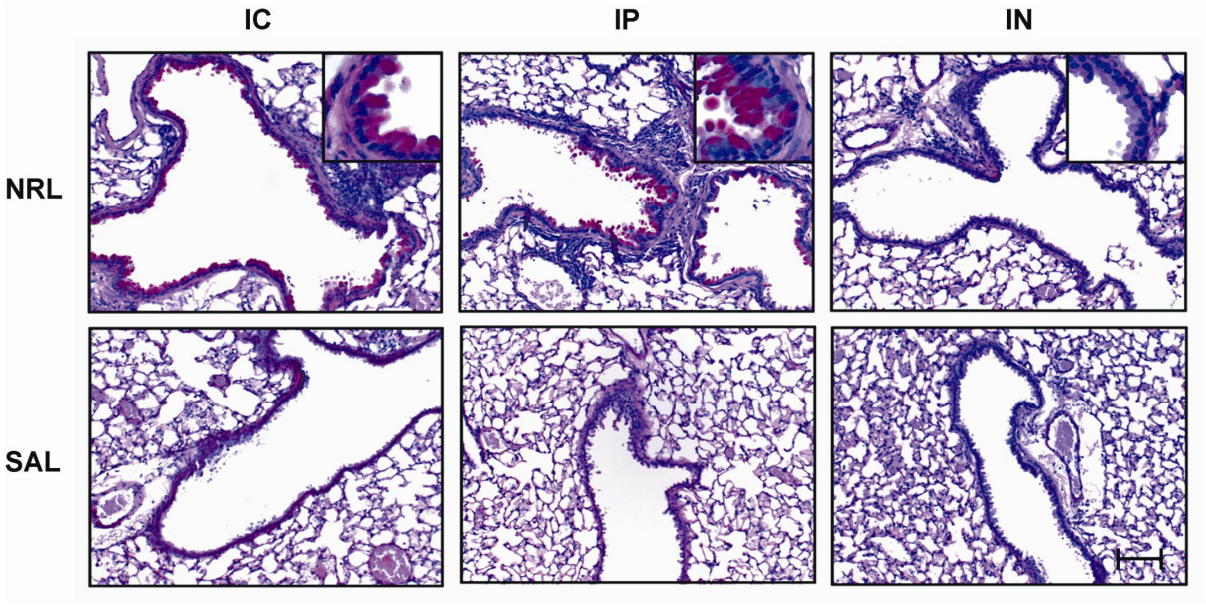

Figure S2. Histological features of PAS-stained peribronchial lung sections (magnification x200 and x400 in inserts). Scale bar = 100µm.

Figure S3.

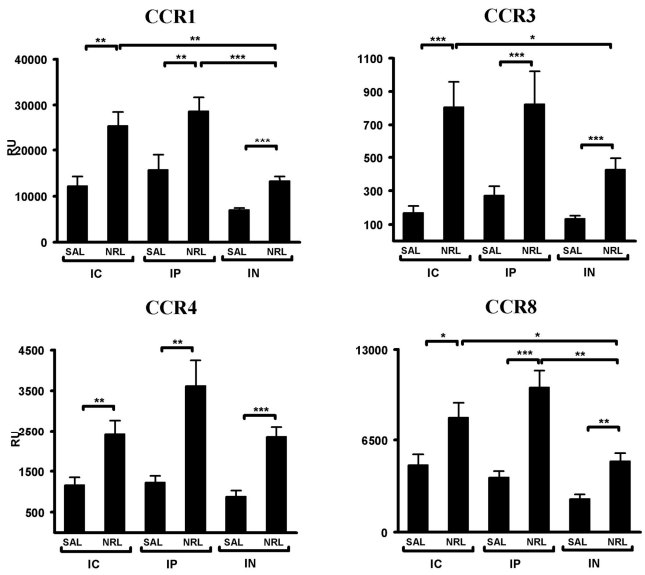

Figure S3. Chemokine receptor mRNA expression levels in lung samples after exposures to vehicle (SAL) or NRL. RU values are expressed as relative differences to the calibrator. The columns and error bars represent mean ± SEM; *p<0.05, **p<0.01, ***p<0.001; n = 12-15 mice/group.

Intradermal Cytosine-Phosphate-Guanosine Treatment Reduces Lung Inflammation but Induces IFN-γ–Mediated Airway Hyperreactivity in a Murine Model of Natural Rubber Latex Allergy

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Abstract

Asthma and other allergic diseases are continuously increasing, causing considerable economic and sociologic burden to society. The hygiene hypothesis proposes that lack of microbial T helper (Th) 1–like stimulation during early childhood leads to increased Th2-driven allergic disorders later in life. Immunostimulatory cytosine-phosphate-guanosine (CpG)–oligodeoxynucleotide motifs are candidate molecules for immunotherapeutic studies, as they have been shown to shift the Th2 response toward the Th1 direction and reduce allergic symptoms. Using natural rubber latex (NRL)–induced murine model of asthma, we demonstrated that intradermal CpG administration with allergen reduced pulmonary eosinophilia, mucus production, and Th2-type cytokines, but unexpectedly induced airway hyperreactivity (AHR) to inhaled methacholine, one of the hallmarks of asthma. We found that induction in AHR was dependent on STAT4, but independent of STAT6 signaling. CpG treatment increased production of IFN-γ in the airways and shifted the ratio of $CD4^+$:$CD8^+$ T cells toward $CD8^+$ dominance. By blocking soluble IFN-γ with neutralizing antibody, AHR diminished and the $CD4^+$:$CD8^+$ ratio returned to $CD4^+$ dominance. These results indicate that increased production of IFN-γ in the lungs may lead to severe side effects, such as enhancement of bronchial hyperreactivity to inhaled allergen. This finding should be taken into consideration when planning prophylaxis treatment of asthma with intradermal CpG injections.

Link to article:

http://ajrcmb.atsjournals.org/content/44/5/639.short

Toll-Like Receptor Activation during Cutaneous Allergen Sensitization Blocks Development of Asthma through IFN-Gamma-Dependent Mechanisms

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Abstract

Toll-like receptors (TLRs) are pattern-recognition receptors that have a pivotal role as primary sensors of microbial products and as initiators of innate and adaptive immune responses. We investigated the role of TLR2, TLR3, and TLR4 activation during cutaneous allergen sensitization in the modulation of allergic asthma. The results show that dermal exposure to TLR4 ligand lipopolysaccharide (LPS) or TLR2 ligand Pam_3Cys suppresses asthmatic responses by reducing airway hyperreactivity, mucus production, Th2-type inflammation in the lungs, and IgE antibodies in serum in a dose-dependent manner. In contrast, TLR3 ligand Poly(I:C) did not protect the mice from asthmatic symptoms but reduced IgE and induced IgG2a in serum. LPS (especially) and Pam_3Cys enhanced the activation of dermal dendritic cell (DCs) by increasing the expression of CD80 and CD86 but decreased DC numbers in draining lymph nodes at early time points. Later, these changes in DCs led to an increased number of $CD8^+$ T cells and enhanced the production of IFN-γ in bronchoalveolar lavage fluid. In conclusion, dermal exposure to LPS during sensitization modulates the asthmatic response by skewing the Th1/Th2 balance toward Th1 by stimulating the production of IFN-γ. These findings support the hygiene hypothesis and pinpoint the importance of dermal microbiome in the development of allergy and asthma.

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