

**Impact of Asthma, Environmental Exposures and
Ethnicity on Functional Responsiveness to Toll-like
Receptor Stimulation in Children**

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ABSTRACT

TLRs play a key role in initiating innate immunity and in regulating the nature of the adaptive immune response that subsequently develops. Alterations in TLR responsiveness in distinct individuals as a result of differences in environmental exposures and/or ethnicity may influence their likelihood of exhibiting allergic diseases such as asthma. We hypothesized that functional responsiveness to TLR stimulation differs in (i) clinically (allergic asthmatic vs. non-atopic); (ii) environmentally (exposed vs. unexposed to environmental tobacco smoke (ETS)); (iii) ethnically (healthy First Nation vs. Caucasian) distinct pediatric populations. We employed a novel approach of using “threshold” concentrations of TLR ligands (more closely approximate natural environmental levels) than widely utilized “maximal” stimulation conditions.

PBMC obtained from 272 children at 8-9 years of age were stimulated with a panel of TLR ligands: lipopolysaccharide/TLR4L, peptidoglycan/TLR2L, poly(I:C)/TLR3L, 3M-011/TLR8L, CpG ODN/TLR9L. Levels of pro- and anti-inflammatory, Th1- and Th2-associated cytokines/chemokines were quantified by ELISA.

We demonstrate that use of threshold concentrations of both TLR4 and TLR2 ligands reveal striking differences in cytokine responses between asthmatic and non-atopic children. Specifically, non-atopic controls produce higher levels of pro-inflammatory cytokines IL-1beta and TNF-alpha, whereas asthmatics exhibit markedly

increased anti-inflammatory IL-10 responses. In contrast, commonly used maximal ligand concentrations elicit strong, indistinguishable patterns of cytokine production.

In asthmatic population, ETS exposures augment chemokine responses to TLR stimulation. In particular, asthmatic children exposed to ETS demonstrated greatly elevated levels of CCL2 and CCL22 chemokines relative to non-ETS exposed asthmatics and controls.

We identified remarkable differences in immunoregulatory responses between ethnically distinct populations. Specifically, First Nation children favor anti-inflammatory IL-10 responses to threshold TLR2 stimulation. Conversely, Caucasian population respond to TLR 2, 4, 8 or 9 activation by production of more robust pro-inflammatory and Th1 biased cytokine and chemokine responses.

Thus, this novel strategy of using threshold levels of TLR stimulation provides markedly increased sensitivity to reveal functional diversity in TLR responsiveness that associate with clinical status and ethnicity.

1. INTRODUCTION

1.1 Toll-like receptors (TLRs): newly discovered and key pathogen recognition molecules in innate immunity

1.1.1 Discovery of TLRs

All vertebrates are constantly exposed to microorganisms that are present in the environment. They have all evolved systems of immune defense to eradicate invading microbial pathogens. The mammalian immune system consists of innate and adaptive immunity.

Adaptive immune responses are mediated by antigen-specific T and B cells, both of which express highly diverse antigen receptors that are generated via gene rearrangement and are thereby able to detect non-self through recognition of a broad spectrum of potential antigens. Adaptive immune responses are involved in elimination of pathogens in the late phase of infection and in generation of immunological memory. This is a highly sophisticated system of antigen recognition and is found only in vertebrates (Akira, Takeda, 2004).

The innate immune system is phylogenetically conserved and is present in almost all multicellular organisms. Innate immune responses are the first line of defense against pathogens. Important components include macrophages/monocytes, neutrophils, dendritic cells, natural killer cells, the complement system (Janeway, Medzhitov, 2002; Takeda e.a., 2003).

Innate immunity was first described more than a century ago (Mechnikoff, from Silverstein AM, 2003). However, while the system of adaptive immunity has been the field of intense investigation over the past century; innate immunity has been less well appreciated. Therefore, until quite recently the mechanism by which innate immunity recognizes non-self has remained unclear (Takeda, Akira, 2001).

The spectacular discoveries of the past few years have sparked great interest in research of innate immunity. Recent studies have yielded substantial insight into innate immune recognition and function.

In 1989, Charles Janeway proposed that innate immune recognition relies on a set of germ-line encoded, non-clonal receptors (Janeway, 1989). These receptors, termed pattern-recognition receptors (PRRs), have evolved to detect specific conserved structures shared by a large group of microorganisms referred to as pathogen-associated molecular patterns (PAMPs). These molecular structures are essential for replication and/or survival of the microorganism, and were not believed to be ever expressed by the host. Recognition of PAMPs allows the innate immune system to distinguish infectious non-self (pathogen) from noninfectious self. (Janeway, Medzhitov, 2002). In the late 1990s, Toll-like receptors, a major class of pattern-recognition receptors, were discovered.

First, in 1996, the *Drosophila melanogaster* Toll protein, identified originally as a receptor responsible for dorso-ventral axis formation in embryogenesis (Anderson e.a., 1984), was found to be critical for host defense responses against fungal infection with *Aspergillus fumigatus* in adult flies, which only have innate immunity (Lemaitre e.a., 1996).

One year later, R. Medzhitov, P. Preston-Hurlburt and C.A. Janeway (1997) discovered the first homologue of Toll in humans, now known as Toll-like receptor 4. They demonstrated that human Toll was able to activate the transcription factor NF- κ B leading to gene expression of proinflammatory cytokines and up-regulate co-stimulatory molecules (Medzhitov e.a., 1997).

In 1998, the group of B. Beutler (Poltorak e.a., 1998) identified a point mutation in the intracellular region of the Tlr4 gene. C3H/HeJ mice carrying this mutation were unresponsive to LPS challenge and highly susceptible to Gram-negative bacterial sepsis, indicating essential role of TLR4 for the LPS recognition.

This breakthrough led the way to identification of other pattern recognition receptors and their agonists, and as a consequence the TLR repertoire has been steadily growing. At present, TLR family is known to consist of a minimum of 11 members: with each TLR having its intrinsic signaling pathway and eliciting specific immune responses against microorganisms. Among PRRs, TLRs are highlighted as the key recognition structures of the innate immunity (Uematsu e.a., 2006; Akira e.a., 2006).

1.1.2 Structure of TLRs

The TLRs are type I integral membrane glycoproteins, and on the basis of considerable homology in the cytoplasmic region, they are members of a larger Interleukin-1 receptor (IL-1R) superfamily. (Akira, Takeda, 2004)

Structurally, all TLRs contain extracellular leucine-rich repeat (LRR) domains, which participate in the recognition of PAMPs, and a cytoplasmic domain known as the Toll/IL-1R (TIR) domain, which associates with intracellular TIR-domain-containing

adaptor proteins and is responsible for initiating signaling pathways via protein-protein interactions.

TIR domain is a conserved region of ~ 200 amino acids (Akira, Takeda, 2004). Y. Xu e.a. (2000) analyzed the crystal structures of the TIR domains of human TLR1 and TLR2 to elucidate the molecular basis of TIR domain signaling. They found that the structures contain a central five-stranded parallel beta-sheet that is surrounded by five alpha-helices on both sides. These two secondary structural elements are connected by loops. Within the TIR domain, the regions of homology comprise three conserved boxes. These conserved boxes and loops are adjacent and may mediate interactions with the downstream MyD88 adapter molecule. Thus, TIR domain is crucial for signal transduction by Toll-like receptors.

The extracellular LRR domain is a tandem repeat of a 20-29 residue sequence motif, L(X₂)LXL(X₂)NXL(X₂)L(X₇)L(X₂), (in which X is any amino acid). J.K. Bell e.a. (2005) have described the crystal structure of the TLR-3-pathogen-binding ectodomain using a baculovirus expression system. Its structure is a horseshoe-shaped solenoid that contains a large beta-sheet on its concave surface. The TLR ectodomain may provide a scaffold that can accommodate a variety of PAMPs-binding insertion. Thus, LLR domain of TLRs is involved directly in the recognition of various pathogens and mediates ligand-binding specificity.

1.1.3 Roles of TLRs in pathogen recognition. Toll-like receptor ligands

Numerous studies have identified a wide variety of exogenous (pathogen-derived) and endogenous (generated by the host) ligands for TLRs. These ligands, molecular

patterns recognized by TLRs, are associated with a broad range of pathogens including bacteria, viruses, protozoa, and fungi (Pandey, Agrawal, 2006; Uematsu e.a., 2006; West e.a., 2006).

The main TLR ligands are summarized in Table 1.

Table 1. Toll-like receptors and their ligands

| Receptor | Ligand | Origin of ligand | References |
|----------|----------------------------|----------------------------------|---|
| TLR1 | Tryacyl lipopeptides | Bacteria and mycobacteria | Takeuchi e.a., 2002 |
| | Soluble factors | <i>Neisseria meningitides</i> | Wyllie e.a., 2002 |
| TLR2 | Peptidoglycan | Gram-positive bacteria | Takeuchi e.a., 1999; Schwadner e.a., 1999 |
| | Lipoprotein/ Lipopeptides | Various pathogens | Aliprantis e.a., 1999 |
| | Lipoteichoic acid | Gram-positive bacteria | Schwadner e.a., 1999 |
| | Lipoarabinomannan | Mycobacteria | Means e.a., 1999 |
| | Phenol-soluble modulin | <i>Staphylococcus epidemidis</i> | Hajjar e.a., 2001 |
| | Glycoinositolphospholipids | <i>Tripanosoma cruzi</i> | Coelho e.a., 2002 |

| | | | |
|------|-----------------------------------|----------------------------------|--|
| | Glycolipids | <i>Treponema maltophilum</i> | Opitz e.a., 2001 |
| | Porins | <i>Neisseria</i> | Massari e.a., 2002 |
| | Atypical LPS | <i>Leptospira interrogans</i> | Werts e.a., 2001 |
| | Atypical LPS | <i>Porphyromonas gingivalis</i> | Hirschfeld e.a., 2001 |
| | Zymosan | Fungi | Underhill e.a., 1999 |
| | Heat-shock protein 70* | Host | Asea e.a., 2002 |
| TLR3 | Double-stranded RNA/ poly(I:C) | Viruses | Alexopou pou e.a., 2001 |
| TLR4 | Lipopolysaccharide | Gram-negative bacteria | Poltorak e.a., 1998 |
| | Taxol | Plants | Kawasaki e.a., 2000 |
| | Fusion protein | Respiratory syncytial virus | Kurt-Jones e.a., 2000 |
| | Envelope protein | <i>Mouse mammary-tumor virus</i> | Rassa e.a., 2002 |
| | Heat-shock protein 60* | <i>Chlamydia pneumoniae</i> | Bulut e.a., 2002; Ohashi e.a., 2000 |
| | Heat-shock protein 70* | Host | Vabulas e.a., 2002 |

| | | | |
|------|--|---------------------------|--|
| | Type III repeat extra domain A of fibronectin | Host | Okamura e.a., 2001 |
| | Oligosaccharides of hyaluronic acid | Host | Termeer e.a., 2002 |
| | Polysaccharide fragments of heparin sulfate | Host | Johnson e.a., 2002 |
| | Fibrinogen | Host | Smiley e.a., 2001 |
| | beta-Defensin 2 | Host | Biragyn e.a., 2002 |
| TLR5 | Flagellin | Bacteria | Hayashi e.a., 2001 |
| TLR6 | Diacyl lipopeptides | <i>Mycoplasma</i> | Takeuchi e.a., 2001 |
| | Lipoteichoic acid | Gram-positive bacteria | Schwadner e.a., 1999 |
| | Zymosan | Fungi | Ozinsky e.a., 2000 |
| TLR7 | Imidazoquinoline | Synthetic compounds | Hemmi e.a., 2002 |
| | Loxoribine | Synthetic compounds | Heil e.a., 2003 |
| | Bropirimine | Synthetic compounds | Heil e.a., 2003 |
| | Single-stranded RNA | Viruses | Heil e.a., 2004; Diebold e.a., 2004 |
| TLR8 | Imidazoquinoline | Synthetic compounds | Jurk e.a., 2002 |
| | Single-stranded RNA | Viruses | Heil e.a., 2002 |
| TLR9 | CpG DNA | Bacteria | Hemmi e.a., 2000 |

| | | | |
|---|--|------------------------------------|-------------------------------|
| | CpG DNA | Viruses | Lund e.a., 2003 |
| | GpG ODNs | Synthetic compounds | Klinman, 2004; Krieg, 2006 |
| | Haemozoin | Protozoa | Coban e.a., 2005 |
| TLR10 | N.D. | N.D. | - |
| TLR11 | UPEC protein (component of uropathogenic bacteria) | Uropathogenic bacteria | Zhang e.a., 2004 |
| | Profilin-like protein | Protozoa/ <i>Toxoplasma gondii</i> | Yarovinsky e.a., 2005 |
| * It is possible that these ligand preparations, particularly those of endogenous origin, were contaminated with LPS and/or other potent microbial components, so more precise analysis is required to conclude that TLRs recognize these endogenous ligands.N.D., not determined | | | |

1.1.3.1 TLR2 and TLR1, TLR6: pathogen recognition

Several lines of evidence support the view that TLR2 has a capacity to bind a wide variety of microbial structures of bacterial, fungal, protozoan, and viral origin (Akira e.a., 2006; Fournier e.a., 2005; Uematsu e.a., 2006)

TLR2 is a principal receptor implicated in recognizing of Gram-positive bacteria.

Peptidoglycan (PGN) is an essential and major component of Gram-positive bacterial cell walls, in which it accounts for approximately half of their mass (Dziarski e.a., 2004).

TLR2 was originally identified as a receptor for PGN using TLR2-transfected HEK293 and CHO cells (Schwandner e.a., 1999; Yoshimura e.a., 1999), TLR2-deficient

macrophages (Takeuchi e.a., 2000), and TLR2-knockout mice (Takeuchi e.a., 1999). Since then, this observation has been supported by almost 200 publications (Dziarski e.a., 2005).

It has been shown that TLR2 directly binds PGN (Iwaki e.a., 2002). A sequence of 25 amino acids (Ser40–Ile64) in its extracellular domain is required for the PGN-elicited cell activation (Mitsuzawa e.a., 2001), suggesting that this sequence may constitute or be a part of the TLR2 binding site for PGN (Dziarski e.a., 2003).

PGN is composed of long linear sugar chains of alternating N-acetyl glucosamine (GlcNac) and N-acetyl muramic acid (MurNac) that are interlinked by peptide bridges to form a large macromolecular structure (Akira e.a., 2006). The exact PGN structure recognized by TLR2 is not known. However, it is well established that high molecular weight polymeric PGN, but not low molecular weight soluble fragments, act as TLR2 activators (Dziarski e.a., 2005; Underhill e.a., 1999; Ozinsky e.a., 2000; Xu e.a., 2001).

TLR2 has also been demonstrated to recognize a broad spectrum of other bacterial components that are mainly present in the bacterial cell membrane. These include lipoproteins/lipopeptides from various pathogens such as mycoplasmas, mycobacteria, spirochetes; lipoteichoic acid from Gram-positive bacteria (e.g. *S. pneumoniae*), mycobacterial lipoarabinomannan, a phenol-soluble modulin from *Staphylococcus epidermis*, glycolipids of *Treponema maltophilum*, and porins present in the outer membrane of *Neisseria*.

Initial reports have suggested that TLR2 might be involved in the recognition of several atypical LPS from non-enterobacteria such as *Leptospira interrogans*, *Porphyromonas gingivalis* and *Helicobacter pylori* (Hirschfeld e.a., 2001; Werts e.a.,

2001; Smith e.a., 2003). Their structures are different from the typical LPS of Gram-negative bacteria detected by TLR4 in the number of acyl chains in the lipid A component, which presumably confers differential recognition (Netea e.a., 2002). However, a recent study have shown that activation of TLR2 is mediated by lipoproteins contaminating LPS preparation from *Porphyromonas gingivalis* and atypical LPS itself has poor activity for TLR2 stimulation (Hashimoto e.a., 2004). Therefore, careful reassessment will be needed to conclude that some types of LPS are recognized by TLR2, but not TLR4 (Takeda, Akira, 2005).

TLR2 has been shown to form heterophilic dimers with either TLR1 or TLR6, both of which are structurally related to TLR2 (Ozinsky e.a., 2000; Wyllie e.a., 2000; Akira e.a., 2004). These heterodimers appear to be involved in the discrimination of subtle changes in the lipid portion of lipopeptides. It has been demonstrated that TLR2/TLR1 complex recognizes triacylated lipopeptides which found in a large number of bacterial outer membranes (e.g. mycobacteria, meningococci, *Borelia burgdorferi*) (Alexopoulou e.a., 2002; Takeuchi e.a., 2002; Wetzler e.a., 2003), whereas TLR2/TLR6 complex can function as a receptor for diacylated lipopeptides (e.g. MALP2 from *Mycoplasma pneumonia*) (Takeuchi e.a., 2001). This ability of TLR2 to associate physically and functionally with TLR1 and TLR6 might explain why it could recognize a broad spectrum of microbial structures. In contrast to lipoproteins, PGN is detected by TLR2 independently of TLR1 and TLR6 (Takeuchi e.a., 2001; Henneke e.a., 2006)

It has also been found that TLR2 is involved in the recognition of fungal pathogens (Netea e.a., 2006). Underhill e.a.(1999) and Ozinsky e.a. (200) have shown that zymosan, a structure derived from *Sacchomyces cerevisiae*, activates immune cells

via TLR2/TLR6 heterodimer (Underhill e.a., 1999; Ozinsky e.a., 2000). TLR2 has been demonstrated to be important for the recognition of *Candida albicans* yeasts or hyphae (Villamon e.a., 2004; Jouault e.a., 2003) as well as *Aspergillus fumigatus* conidia or hyphae (Netea e.a., 2003). Recently, Biondo e.a. (2005) reported that TLR2 is required for host defense against *Cryptococcus neoformans*.

Protozoan components are also sensed by TLR2. Glycosylphosphatidylinositol (GPI) anchors derived from *Trypanosoma cruzi* (Campos e.a., 2001), *Toxoplasma gondii* (Debierre-Grockiego e.a., 2003), *Leishmania major* (Becker e.a., 2003), and *Plasmodium falciparum* (Krishnegowda e.a., 2005) have been shown to trigger immune cell activation through TLR2, suggesting that GPI is a major class of PAMPs (Gazzinelli, Denkers, 2006).

TLR2 has been implicated in responding to viruses and viral components (Bowie, 2007). Bieback e.a. (2002) found that hemagglutinin protein of measles virus stimulates murine and human cells via TLR2. Varicella zoster virus can also induce the production of proinflammatory cytokines by human monocytes and macrophages through TLR2-dependent pathway (Wang e.a., 2005). TLR2 was shown to be responsible for the recognition of envelope glycoproteins, gB and gH, of human cytomegalovirus (Boehme e.a., 2006), and be able to mediate cytokine responses to herpes simplex virus (Kurt-Jones e.a., 2005) and vaccinia virus (Zhu e.a., 2007).

Thus, TLR2 represents a highly versatile PRR that recognizes a wide range of pathogens and their products.

1.1.3.2 TLR3: pathogen recognition

TLR3 is involved in the recognition of viral double-stranded RNA and polyinosinic-polycytidylic acid (poly(I:C), a synthetic analogue of dsRNA widely used to mimic viral infection).

Double-stranded RNA is known to be generated by most viruses during infection as a replication intermediate for ssRNA viruses or as a by-product of symmetrical transcription in DNA viruses (Akira e.a., 2006).

Mammalian TLR3 as a PRR for dsRNA was discovered in 2001 by the group of R.A. Flavell (Alexopoulou e.a., 2001). They demonstrated that expression of human TLR3 confers responsiveness to purified dsRNA and poly(I:C) in HEK293 cells, and TLR3-deficient mice display reduced cytokine responses to these ligands. Signaling via TLR3 results in the activation of interferon regulatory factor 3 and NF- κ B, leading to the production of type I interferons (IFN-alpha/beta).

In 2005, the human TLR3 ectodomain structure has been described (Choe e.a., 2005; Bell e.a., 2005). It represents a large horseshoe-shaped solenoid comprising 23 leucine-rich repeats. More recently, J.K. Bell e.a. (2006), using mutational analysis, found that glycan-free surface of TLR3 extracellular domain appears to be the site of ligand binding, and also identified two amino acids (H529, N541) critical for its function, providing first molecular description of how TLRs bind their ligands.

While TLR3 is clearly a receptor for dsRNA/poly(I:C), its particular role in anti-viral innate immunity in vivo is as yet undefined (Bowie, 2007).

K. Tabeta e.a. (2004) reported that TLR3-deficient mice are susceptible to mouse cytomegalovirus (MCMV). On the other hand, K.H. Edelmann e.a. (2004) failed to demonstrate the requirement of TLR3 for the generation of effective anti-viral responses

against MCMV and three other viruses (lymphocytic choriomeningitis virus, vesicular stomatitis virus, reovirus) in mice. Moreover, T. Wang e.a. (2004) showed that TLR3-deficient mice are more resistant to lethal West Nile virus (WNV) infection, suggesting that WNV benefits from its interaction with TLR3.

In contrast to these inconclusive data described above, recent study of Schulz e.a. (2005) has provided evidence for a clear specific role of TLR3 in the anti-viral responses. They observed that immunization with virus-infected cells or cells containing poly(I:C) leads to striking increase in CTL cross-priming against cell-associated antigens, which is dependent on TLR3 expression by antigen-presenting cells. Thus, TLR3 may have evolved to promote cross-priming against viruses.

1.1.3.3 TLR4: pathogen recognition

TLR4 is known to recognize a broad spectrum of microbial products associated with bacteria, fungi, protozoa, and viruses (Akira e.a., 2006).

One of the most remarkable aspects of TLR4 is its capacity to detect lipopolysaccharide (LPS), a glycolipid component of the outer membrane of Gram-negative bacteria.

Bacterial lipopolysaccharides typically consist of three distinct parts: a distal polysaccharide or “O-antigen”, a non-repeating “core” oligosaccharide, and a hydrophobic lipid portion known as “lipid A” or endotoxin (Miller e.a., 2005; Raetz, Whitfield, 2002). Lipid A is the only region of LPS to be recognized by the innate immune system of mammals and exhibits highly potent immunostimulatory activity, even at low concentrations (Miller e.a., 2005; Miyake, 2004).

It is well established that recognition of LPS largely occurs through the TLR4/MD2/CD14 complex, and TLR4 is central part of it (Fitzgerald e.a., 2004).

LPS released from Gram-negative bacteria first associated with LPS-binding protein (LBP), an acute-phase protein present in the bloodstream. This accessory protein converts oligomeric micelles of LPS to monomer for delivery to CD14, which is a glycosyl phosphatidylinositol (GPI)-anchored, high-affinity membrane protein that can also circulate in a soluble form. CD14 concentrates LPS for binding to the TLR4/MD2 complex. LPS is transferred to MD2, which associates with the extracellular portion of TLR4, followed by oligomerization of TLR4, a key molecule of LPS signaling (Akira e.a., 2006; Shimazu e.a., 1999; Uematsu e.a., 2006).

An essential role of TLR4 in LPS recognition is highlighted by the facts that inbred mice having either deletions in TLR4 (C57BL/10ScCr) or mutations in this gene (C3H/HeJ) (Poltorak e.a., 1998) as well as genetically engineered, TLR4-deficient mice (Hoshino e.a., 1999), are unresponsive to systemic LPS; and in humans, a polymorphism of TLR4 (Asp299Gly) is associated with a blunted response to LPS in vitro and also with diminished airway obstruction after inhaled endotoxin (Arbour e.a., 2000).

Additionally, TLR4 has been shown to be involved in the detection of mycobacterial phosphatidylinositol mannoside (Abel e.a., 2001) and taxol, a diterpene purified from the bark of the western yew (*Taxus brevifolia*) (Byrd-Leifer e.a., 2001; Kawasaki e.a., 2000).

Several components of fungi have also been reported as potential ligands for TLR4. Mannan derived from *Candida albicans* (Tada e.a., 2002), heat-killed *Candida albicans* blastospores (van der Graaf e.a., 2005), and *Aspergillus* conidia (Netea e.a.,

2003) are recognized by TLR4. In addition, TLR4 interacts with glucuronoxylomannan, the major capsular polysaccharide of *Cryptococcus neoformans* (Shoham e.a., 2001).

TLR4 appears to sense the products of protozoan parasites (Gazzinelli, Denkers, 2006). Glycoinositolphospholipids (GIPLs) containing ceramide of *Trypanosoma cruzi* (Oliveira ea., 2004), GIPLs of *Toxoplasma gondii* (Debierre–Grokiego e.a., 2003), GPI anchors derived from *P. falciparum* merozoites (Krishnegowda e.a., 2005) have been demonstrated to activate TLR4.

Some viral envelope proteins have been reported to be recognized by TLR4.

E.A. Kurt-Jones e.a. (2000) showed that the fusion (F) protein from respiratory syncytial virus (RSV) stimulated IL-6 production by wild-type macrophages, but not by those isolated from TLR4-deficient mice. In addition, TLR4-deficient mice have higher levels of infectious virus in the lungs and reduced rate of viral clearance than control mice, leading authors to conclude that innate immune response to RSV is mediated by TLR4. However, another study demonstrated no significant role for TLR4 in RSV infection in mice (Ehl e.a., 2004), and therefore the importance of F protein-induced cytokine secretion via TLR4 remains unclear.

Other envelope protein (Env) of mouse mammary tumor virus has also been found to activate murine B lymphocytes through TLR4 (Rassa e.a., 2002).

It seems plausible that viral detection by TLR4 leads to inflammation rather than specific anti-viral innate immune responses.

Furthermore, TLR4 has been shown to be involved in the recognition of non-microbial endogenous ligands, such as heat shock proteins HSP60 and HSP70 (Ohashi e.a., 2000; Vabulas e.a., 2002), the type III repeat extra domain A of fibronectin

(Okamura e.a., 2001), oligosaccharides of hyaluronic acid (Termeer e.a., 2002), polysaccharide fragments of heparan sulfate (Johnson e.a., 2002), fibrinogen (Smiley e.a., 2001), and beta-defensin 2 (Biragyn e.a., 2002). Thus, in contrast to the original hypothesis of C. Janeway (Janeway, 1989), TLR-mediated activation is not restricted to solely microbial ligands.

However, it needs to be noted that all these endogenous products require very high concentrations to stimulate TLR4. Moreover, B. Gao and M.F. Tsan (2003) demonstrated that contamination of endotoxin in the HSP70 confers its ability to mediate TLR4 activation. LPS is a highly potent immuno-stimulant, and consequently, TLR4 can be activated by a very small amount of LPS, contaminating these preparations of exogenous ligands (Takeda e.a., 2006). Therefore, more precise analysis will be required to conclude that TLR4 detects these endogenous ligands.

1.1.3.4 TLR5: pathogen recognition

TLR5 is responsible for the detection of flagellin from both Gram-negative and Gram-positive bacteria (Hayashi e.a., 2001). This is the major structural component of bacterial flagella and is a highly conserved protein among bacterial pathogens (Honko, Mizel, 2005).

TLR5 has further been demonstrated to specifically recognize via close physical interaction an evolutionary conserved site/domain of flagellin that is essential for profilament formation and bacterial motility (Smith e.a., 2003). This recognition site is buried in the flagellar filament, indicating that TLR5 senses only monomeric flagellin (Smith e.a., 2003). Moreover, flagellin has been shown to bind directly to TLR5

extracellular domain at residues 386-407 and that the NH₂-terminal 358 amino acids of TLR5 play an important role in its signaling activity (Mizel e.a., 2003).

A.T. Gewirtz e.a. (2001) found that TLR5 is expressed on the basolateral, but not the apical side of intestinal epithelial cells, suggesting that TLR5 could be activated by pathogenic, but not commensal, bacteria that translocate flagellin across epithelia. Also, TLR5 is highly expressed in the lung and flagellin has been shown to be a principal stimulant of proinflammatory cytokine production in lung epithelial cells (Hawn e.a., 2003). These findings point to a key role of TLR5 in bacterial recognition at the mucosal surface. Thus, TLR5 appears to have evolved to recognize flagellated bacterial pathogens.

1.1.3.5 TLR7 and TLR8: pathogen recognition

TLR7 and TLR8 are highly homologous and play important roles in the recognition of viruses. The first evidence for the potential anti-viral role for TLR7 was obtained by the group of S. Akira in 2002 (Hemmi e.a., 2002). They observed that the low-molecular-weight synthetic imidazoquinoline compounds such as imiquimod (R-837) and resiquimod (R-848), which was known to possess potent anti-viral properties, have a capacity to activate murine macrophages via TLR7.

After a short while, M. Jurk e.a. (2002) revealed that human TLR7 and TLR8 and murine TLR7, but not TLR8, could independently confer cells with responsiveness to R-848. In addition, TLR7 has been found to detect another synthetic compound, loxoribine, which also has anti-viral activities (Heil e.a., 2003).

Both imidazoquinoline and loxoribine are structurally related to guanosine nucleoside. Therefore, it has been anticipated that a nucleic acid-like structure of the virus might be a natural ligand for TLR7 and TLR8. This expectation came true in 2004, when three groups independently discovered that TLR7 and TLR8 recognize viral single-stranded RNA (ssRNA).

F. Heil e.a. (2004) have demonstrated that guanosine- and uridine-rich ssRNA oligonucleotides derived from human immunodeficiency virus-1 (HIV-1) were shown to stimulate dendritic cells and macrophages to secrete IFN-alpha and proinflammatory cytokines via human TLR8 and murine TLR7. S.S. Diebold e.a. (2004) found that the production of vast amounts of IFN-alpha by murine plasmacytoid dendritic cells in response to wild-type influenza virus required endosomal recognition of influenza genomic RNA and signalling through TLR7.

Additionally, it has been shown that another ssRNA virus, vesicular stomatitis virus (VSV), activates plasmacytoid dendritic cell and B lymphocyte responses via TLR7 in mice (Lund e.a., 2004). K. Triantafilou e.a (2005) demonstrated that mainly TLR8 and also TLR7 act as the host sensors for human parechovirus 1. Coxsackie B virus (Triantafilou e.a, 2005) and paramyxovirus (Melchjorsen e.a., 2005) have been reported to interact with TLR7 and TLR8

Recently, S.S. Diebold e.a. (2006) found that uridine and ribose, the two defining features of RNA, are both necessary and sufficient for TLR7 activation and that short ssRNA act as TLR7 agonists for mouse and human cells in a sequence-independent manner. Thus, molecular basis for the recognition of ssRNA by TLR7 has been clarified.

1.1.3.6 TLR9: pathogen recognition

It has long been known that bacterial DNA has potent stimulatory effects on the mammalian immune system, which depend on the presence at high frequency of unmethylated CpG motifs in DNA from bacteria (Klinman e.a., 1996; Krieg e.a., 1995). In contrast, mammalian DNA has low frequency and high rate of methylation of CpG dinucleotides, which leads to abrogation of the immunostimulatory activity.

In 2000, group of S. Akira (Hemmi e.a., 2000) first found that TLR9 is responsible for the recognition of these immunostimulatory unmethylated CpG motifs that abundantly present on bacterial DNA. They observed that TLR9-deficient mice did not respond to CpG DNA challenge, including proliferation of splenocytes, production of proinflammatory cytokines, maturation of dendritic cells (Hemmi e.a., 2003). A year later, it has been shown that TLR9 mediates CpG DNA recognition in humans (Bauer e.a., 2001; Takeshita e.a., 2001).

To date, a number of synthetic immunostimulatory sequence oligonucleotides (ISS-ODN) containing unmethylated CpG dinucleotides (also known as CpG-ODN), which mimic the activity of bacterial DNA, have been designed (Krieg, 2006). There are at least two classes of CpG ODNs, termed A/D-class CpG ODN and B/K-class CpG ODN, that are structurally and phenotypically distinct (Klinman D.M., 2004; Krieg A.M., 2006). The B/K-class CpG ODNs (e.g. ODN 2006) has been demonstrated to be potent stimulators of B-cell activation and TNF-alpha, IL-12 responses, but have less ability to trigger IFN-alpha production (Kerkmann e.a., 2003; Krieg A.M., 2002). On the contrary, A/D-class CpG ODNs (e.g. ODN 2216) are strong inducers of IFN-alpha secretion by

plasmacytoid dendritic cells, but only weakly stimulate B lymphocytes and production of IL-12 (Krug e.a., 2001; Verthelyi e.a., 2001).

CpG DNA dinucleotides are also presented in abundance in genomes of some DNA viruses. Recent work has demonstrated the involvement of TLR9 in sensing viral DNA. CpG DNA derived from herpes simplex virus type 1 (Krug e.a., 2004) and type 2 (Lund e.a., 2003) has been shown to induce type I IFN secretion via TLR9 which is expressed on plasmacytoid dendritic cells. Furthermore, Tabeta e.a. (2004) reported that TLR9 contributes to innate immune defense against mouse cytomegalovirus infection, due presumably to detecting viral DNA.

In addition, TLR9 has been observed to sense genomic DNA derived from protozoan parasites such as *Trypanosoma cruzi* (Bafica e.a., 2006) and *Trypanosoma brucei* (Drennan e.a., 2005). C. Coban e.a. (2005) found that the non-DNA ligand called haemozoin, which is a hydrophobic heme polymer produced by *Plasmodium falciparum*, is able to activate immune cells via TLR9.

Thus, TLR9 has been demonstrated to recognize unmethylated CpG motifs that are found in bacterial DNA and also in genomic DNA of some DNA viruses and parasites.

1.1.3.7 TLR10

The natural ligand(s) for TLR10 remains unknown.

The mouse Tlr10 gene is substituted to non-productive sequence, indicating that mouse TLR10 is non-functional (Hasan e.a., 2005; Takeda e.a., 2006).

The human TLR10 gene is tightly clustered with TLR1 and TLR6, both of which are known to function as co-receptors for TLR2. Human TLR10 is presumably functional receptor and has been shown to heterodimerase with either TLR1 or TLR2 and directly associate with MyD88 (Hasan e.a., 2005).

1.1.3.8 TLR11: pathogen recognition

TLR11, the most recently discovered TLR (Zhang e.a., 2004), is functional in mice and expressed in abundance in the kidney and bladder epithelial cells. In humans, however, Tlr11 gene contains a premature stop codon and therefore encodes a non-functional form of TLR11 (Zhang e.a., 2004).

Gene-targeting studies by group of S. Ghosh demonstrated that mice lacking TLR11 are highly susceptible to uropathogenic *E. coli* infection (Zhang e.a., 2004). While the bacterial ligand for TLR11 has not yet been identified, these findings indicate a potentially important role for TLR11 in preventing infection of urogenital system.

The first chemically defined ligand for murine TLR11 has recently been discovered as a profilin-like protein from the protozoan parasite *Toxoplasma gondii* (Yarovinsky e.a., 2005). It has been shown that this molecule activates mouse dendritic cells via TLR11, and that TLR11-deficient mice have increased susceptibility to infection with *T. gondii* (Yarovinsky e.a., 2005).

A profilin-like protein from *T. gondii* is present as a relatively conserved molecule in a number of apicomplexan parasites, suggesting that these proteins might serve as another broad class of protozoan PAMPs (Gazzalini, Denkers, 2006).

1.1.4 Expression of TLRs in humans

TLRs can be expressed extracellularly and/or intracellularly depending on the receptor examined. TLR1, TLR2, TLR4, TLR5 and TLR6 are localized on the cell surface for recognition of extracellular, mostly bacterial, pathogens, while TLR3, TLR7, TLR8 and TLR9 are found almost exclusively in intracellular compartments (Heil e.a., 2003; Lee e.a., 2003; Matsumoto e.a., 2003), such as endosomes. They detect viral and bacterial nucleic acids in late endosomes-lysosomes (Diebold e.a., 2004; Lund e.a., 2004; Lund e.a., 2003). Thus, the localization of different TLRs correlates to certain extent with the molecular patterns of their respective ligands.

Toll like receptors are widely expressed in many different cell types, including both myeloid and lymphoid cells, and on various epithelial surfaces (Azuma e.a., 2006, Chaudhuri e.a., 2005, Iwasaki, Medzhitov, 2004). All human immune cells such as monocytes/macrophages, neutrophils, dendritic cells (DCs), B cells, T-lymphocytes, eosinophils, basophils, NK cells, mast cells express unique sets of TLRs (Table 2).

What complicates matters is that the activation and differentiation state also influences TLR expression levels. For example, monocytes express a broad repertoire of TLRs, namely TLR1, TLR2, TLR4, TLR5, TLR6, and TLR8 (Hornung e.a., 2002). The expression of these receptors is progressively reduced as they differentiate into immature dendritic cells in vitro in the presence of GM-CSF and IL-4. These cells instead acquire the expression of TLR3 (Visintin e.a., 2001).

Table 2. Expression of TLRs on human immune cells of peripheral blood

| Cell type/TLR | TLR 1 | TLR 2 | TLR 3 | TLR 4 | TLR 5 | TLR 6 | TLR 7 | TLR 8 | TLR 9 | TLR 10 |
|---------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|--------|
| Monocyte | ++ | ++ | - | ++ | ++ | ++ | +/- | ++ | +/- | +/- |
| Neutrophils | ++ | ++ | - | ++ | + | ++ | + | ++ | + | + |
| mDC | ++ | ++ | ++ | - | + | ++ | +/- | ++ | - | + |
| pDC | + | - | - | - | - | ++ | ++ | - | ++ | ++ |
| mDC in vitro | ++ | ++ | ++ | ++ | +/- | ++ | - | ++ | - | n/d |
| CD4+T cell | ++ | ++ | + | + | ++ | + | + | + | + | - |
| CD8+T cell | + | + | + | - | + | + | - | - | + | + |
| CD4+CD25+ | n/d | n/d | n/d | +/- | ++ | n/d | - | ++ | n/d | n/d |
| B cell | ++ | + | - | + | - | ++ | ++ | +/- | ++ | ++ |
| Eosinophils | + | + | - | + | - | + | ++ | - | + | + |
| Basophils | - | + | - | + | - | + | - | - | + | + |
| NK cells | + | + | ++ | + | + | + | + | ++ | ++ | + |
| Mast cells | + | ++ | + | ++ | + | + | + | +/- | + | - |

+, ++ and - indicate the relative mRNA expression of each TLR by the cells. +/- indicate conflicting results: some studies found expression (+), whereas others found no expression (-), of a given TLR on a particular cell type.

mDC in vitro indicates in vitro differentiated DC in the presence of IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF).

References: Heil e.a., 2003; Matsumoto e.a., 2003; Hornung e.a., 2002; Jarrossay e.a., 2001; Kadowaki e.a., 2001; Krug e.a., 2001; Ito e.a., 2002; Visintin e.a., 2001; Muzio e.a., 2000, Iwasaki, Medzhitov, 2004; Becker e.a., 2003; Bourke e.a., 2003; Bernasconi e.a., 2003; Nagase e.a., 2003; Saikh e.a., 2003, Schmidt e.a., 2004; Komai-Koma e.a., 2004; Sivori e.a., 2004; Kulka e.a., 2004; Suttmuller e.a., 2006; Crellin e.a., 2005; Peng e.a., 2005; Kabelitz, 2007; Komiya e.a., 2006; Sabroe e.a., 2002; Bieneman e.a., 2005

Neutrophils express a variety of TLRs: mRNA for TLR1 through TLR10, but not for TLR3 (Sabroe e.a., 2002; Komiya e.a., 2006)

Studies of DCs showed that human myeloid (mDC) and plasmacytoid DC (pDC) express distinct TLR expression patterns (Jarrossay e.a., 2001; Kadowaki e.a., 2001; Krug e.a., 2001; Ito e.a., 2002). Freshly isolated mDC express TLR1, TLR2, TLR3, TLR5, TLR6, TLR8 and respond to bacteria, fungi, viruses by producing proinflammatory cytokines, whereas pDC express TLR7 and TLR9 and can recognize viral pathogens and secrete vast amounts of type I IFN, especially IFN-alpha.

Nearly all TLR-encoding mRNAs have been detectable in human blood T cells, although at varying levels with relatively strong expression of TLR1, TLR2, TLR3, TLR5, TLR9 (Hornung e.a., 2002; Zarembler e.a., 2002). Little is known about the expression patterns and functions of TLRs on different T-cell subsets. Of interest, Mansson e.a. (2006), examining the expression profile of TLRs in human tonsil T cells, showed that the TLR expression on CD4⁺ T cells is generally higher than on CD8⁺ T cells.

CD25^{high}CD4⁺ human regulatory T cells (Treg) but not CD25⁻ naïve CD4⁺ T cells were demonstrated to express TLR8 (Peng e.a., 2005), and Treg also expressed TLR5 mRNA at high levels (Crellin e.a., 2005). Expression of TLR1, TLR2, TLR4, TLR6, TLR7, TLR9, TLR10 mRNA has been observed in B cells (Bourke e.a., 2003). Eosinophils express transcripts of TLR1, TLR2, TLR4, TLR6, TLR7, TLR9, TLR10 (Nagase e.a., 2003), and mRNA for TLR2, TLR4, TLR6, TLR9, TLR10 has been detected in basophils (Komiya e.a., 2006). High levels of TLR3, TLR8, and TLR9 were expressed in NK cells (Hornung e.a., 2002; Sivori e.a., 2004). Human cultured mast cells

have been shown to express mRNA and protein for all TLRs except TLR8 (Kulka e.a., 2004). TLRs also expressed on epithelial cells (Gulliot e.a., 2005; Hauber e.a., 2005; Platz e.a., 2004), endothelium (Andonegui e.a., 2003), fibroblasts (Chaudhuri e.a., 2005), vascular smooth muscle (Sasu e.a., 2001), astrocytes, microglial and neural cells (Sioud e.a., 2006). The expression of TLR in diverse cell types illustrates their distinct functional roles in innate as well as adaptive immune responses.

It should be noted that although the analysis of TLR expression provides some valuable information, the interpretation of these results must be approached with caution. A caveat to keep in mind is that the expression of TLRs either at mRNA or protein levels cannot always be extrapolated to a functional phenotype. For example, eosinophils constitutively express TLR2, TLR4, TLR6, TLR7, TLR9, TLR10, however, these cells only respond to stimulation with R-848 via TLR7, which leads to their activation and prolonged survival (Nagase e.a., 2003). Similarly, NK cells express TLR9 mRNA, but are not activated by CpG ODNs (Horning e.a., 2002).

These findings demonstrate the importance of functional studies to investigate TLR responsiveness to their corresponding ligands.

1.1.5 Toll-like receptors link innate and adaptive immunity and regulate adaptive immune responses

1.1.5.1 Innate immune recognition by TLRs

It is now well established that Toll-like receptors are a key component of the innate immune system. They constitute a first line of host defense against a variety of

pathogens and play a major role in innate immune recognition and initiation of innate immune responses (Janeway, Medzhitov, 2002).

TLR detection of microbial infection (specifically, molecular patterns associated with microbes) leads to the activation of signaling pathways that generate rapid and robust anti-microbial and inflammatory responses characterized by the production of pro- and anti-inflammatory cytokines and chemokines (Iwasaki, Medzhitov, 2004), induction of anti-microbial intermediates (Brightbill e.a., 1999) and phagocytosis (Aderem e.a., 1999). Chemokines/cytokines trigger the recruitment of the inflammatory cells to the sites of infection. Recruited macrophages and neutrophils are activated, ingest invading pathogens, and subsequently kill them by producing nitric oxide, radical oxygen species, or defensins.

Thus, TLRs activate multiple steps in the inflammatory reactions that help to eliminate the invading pathogens (Kaisho e.a., 2006)

1.1.5.2 Regulation of adaptive immunity by TLRs

TLR activation induces not only innate immune responses but also initiates and orchestrates the adaptive immunity that subsequently develops. TLRs influence several steps of the development of adaptive immune responses.

First, TLR signaling results in the activation and maturation of DC. The recognition and uptake of microorganism via TLRs by DC lead to up-regulation of the expression of MHC class II bearing pathogen-derived peptides (antigen-specific signal 1) as well as increased expression of co-stimulatory molecules such as CD80 and CD86 on the surface of DC (co-stimulatory signal 2) (Kapsenberg, 2003). Induction of co-

stimulatory molecules is a crucial step in the initiation of adaptive immunity (Janeway, Medzhitov, 2002). TLR-mediated program of DC maturation endows the cells with the ability to stimulate naïve T lymphocytes, and DCs provide the naïve T cells with these two signals required for their activation.

After activation of T cells, a particularly important step in regulating of adaptive immunity is the differentiation of naïve T cells into either Th1-like or Th2-like cells.

A key signal (polarizing signal 3) responsible for Th differentiation is the type of cytokine profile which present at the time of T-cell activation. In general, Th1-polarizing profile represented by IL-12 family members and IFN-alpha while Th2-polarizing cytokine(s) is IL-4 (and IL-10 in mice) (de Jong e.a., 2005; Trinchieri e.a., 2003)

More recently, it has been realized that the expression of a specific cytokine profile is greatly dependent on and imprinted by the recognition of microbial pathogens by specific TLRs (de Jong e.a., 2005; Iwasaki, Medzhitov, 2004; Netea, 2006; Pulendran, 2004)

However, there is still a question whether TLR signaling, in general, favors a type 1 or a type 2 immune responses?

Many initial studies have demonstrated that TLR signals induce antigen-presenting cells to produce IL-12, which plays a pivotal role in Th1 cell differentiation (Trinchieri e.a., 2003). This is believed by many to constitute the main mechanism by which they favor Th1-type adaptive immune response.

This global view is strongly supported by studies conducted on MyD88-deficient mice. Schnare e.a. (2001) showed that MyD88-deficient mice immunized with OVA in

CFA were unable to generate Th1-type immune responses. In contrast, Th2 responses (in the presence of alum) were completely preserved.

It has been postulated that signaling via TLRs is required for Th1 responses, but not for Th2 priming. (Barton, Medzhitov, 2002). Indeed, there is extensive evidence for an important role of TLRs in driving strong Th1 responses (Dabbagh e.a, 2003; Netea e.a., 2006).

A number of studies have shown that agonists of TLR2, TLR4, TLR5, TLR7/8, TLR9 have the ability to direct adaptive immune responses to the Th1 type. Specifically, Pam3Cys, a synthetic lipopeptide activator TLR2, has been found to be capable of generating Th1 cytokine responses: it stimulates human PBMC to produce IFN-gamma, but not detectable IL-4 (Sieling, 2003), and induces IL-12, but not IL-10, production from monocyte-derived DCs in a TLR2-dependent manner (Thoma-Uszynski e.a, 2000), suggesting that Th1 response promoted by TLR2 activation.

B. Pulendran e.a. (2001), using a murine vaccination model, found that co-injections of *E.coli* LPS, a TLR4 agonist, with OVA triggers a Th1-like response with high levels of IFN-gamma, but little or no IL-4, IL-5, and IL-13. Activation of human immature DC (Re e.a., 2001) or murine DC (Qi e.a., 2003) by LPS has been demonstrated to promote the secretion of the IL-12p70, a Th1 cytokine, and induce low levels of IL-10. S. Agrawal e.a. (2003) reported that *E.coli* LPS and flagellin, TLR4 and TLR5 ligands, respectively, instruct monocyte-derived DCs to stimulate Th1 responses via IL-12p70 production.

T. Ito e.a. (2002) showed that activation of human pDCs and mDCs by imiquimod (R-837) via TLR7 results in the induction of Th1-type allogenic T cell responses through

secretion of IFN- α and IL-12, respectively. The study of T.L. Wagner e.a. (1999) revealed that imiquimod (R-837) and resiquimod (R-848) could modulate the Th1/Th2 responses *in vitro* by inducing the production of IFN- γ and IL-12, while inhibiting the Th2 cytokines, IL-4 and IL-5, in both human PBMC and mouse splenic cultures. In experiments *in vivo*, J.P. Vasilakos e.a. (2000) observed that administration of resiquimod (R-848) with OVA plus alum reduces IgE levels while enhancing IgG2a levels, thus, indicating that this TLR7/8 agonist redirect immunity away from Th2 (IgE) toward Th1(IgG2a) Ab responses, acting as Th1 adjuvant.

It has long been known that unmethylated GpC motifs, TLR9 stimulants, act as potent vaccine adjuvants by skewing the host's immune milieu in favor of Th1-cell responses, which is dominated by cytokines such as IFN- γ , IL-12, IL-18 (Wagner e.a., 2004; Klinman, 2004). *In vivo*, CpG ODNs can exacerbate pathologies in disease models that are Th1-dependent, such as murine experimental autoimmune encephalomyelitis and colitis (Ichikawa e.a., 2002; Obermeier e.a., 2002). On the other hand, there is ample evidence for the ability of CpG ODNs to convert the Th2-mediated allergic responses to a dominant Th1 phenotype (Kline e.a., 1998; Broide e.a., 1998; Sur e.a., 1999; Shirota e.a., 2000).

All these data are consistent with a view (Iwasaki, Medzhitov, 2004) that TLRs inherently direct developing adaptive responses towards Th1 type.

However, more recent studies demonstrated that triggering of particular TLRs may also result in Th2 responses (Pulendran, 2004).

T. Kaisho e.a. (2002) reported that LPS stimulation of MyD88-deficient DCs leads to Th2-type allogenic response, suggesting the involvement of a MyD88-

independent pathway in Th2 differentiation. Dabbagh e.a. (2002) found a decrease of Th2 responses in TLR4-deficient mice sensitized to inhaled allergen, showing that TLR4 is required for optimal development of Th2 immunity.

F. Re e.a. (2001) observed that Pam3Cys, a synthetic TLR2 ligand, failed to induce IL-12p70 and IP-10 cytokines from human immature DCs in vitro, but trigger the release of the IL-12 inhibiting p40 homodimer, producing conditions that favor Th2 responses. S. Agrawal e.a. (2003) revealed that TLR2 stimulation with Pam3Cys results in high IL-10 levels, but little IL-12, in human monocyte-derived DCs, thus, skewing Th responses toward Th2 cytokine profile.

Similarly, in the mouse system, S. Dillon e.a. (2004), employing vaccination model in vivo, demonstrated that injection of Pam3Cys plus OVA elicits weak IL-12p70, but strong IL-10 production, and directs Th2 and T cytotoxic 2 responses. Also, murine DCs have been shown to secrete low levels of IL-12 but high levels of IL-10 after exposure to PGN in vitro, suggesting TLR2 signaling might favor a Th2 bias (Qi e.a., 2003). In addition, another ligand, a TLR5 agonist, flagellin has been found to drive Th2-type immunity in mice by promoting the production of IL-4 and IL-13 in antigen-specific CD4⁺ T cells as well as IgG1 responses in a MyD88-dependent manner (Didierlaurent e.a., 2004)

Thus, the relationship between TLRs and Th1/Th2 polarization is more complex than it was originally postulated by G.M. Barton and R. Medzhitov in 2002. Currently, available data provide support for the notion that signaling via distinct TLRs elicits qualitatively different cytokine profiles (Pulendran, 2005; Netea, 2006). There is compelling evidence that TLR4, TLR7/8, TLR9 are capable of triggering a Th1-type

responses. Several studies have shown that TLR2, and possibly TLR5 might be able to induce a skew towards Th2 differentiation (Pulendran, 2004). The physiological importance of TLR2-mediated Th2 responses needs to be clarified, because microbial pathogens that recognized by TLR2, such as Gram-positive bacteria, do not generally induce Th2 responses in vivo (Iwasaki, Medzhitov, 2004). Therefore, the role of specific TLRs in Th polarization is still very much under debate.

In conclusion, TLRs are key pattern recognition receptors that recognize molecular patterns presented on various classes of microbes, resulting in the production of cytokines/chemokines, induction of anti-microbial mechanisms, activation and maturation of DCs, and T-cell activation and differentiation into either Th1 or Th2 cells. Thus, TLRs represent a crucial link between innate and adaptive immunity and regulate the nature of adaptive immune responses and ultimately clinical outcomes.

1.1.6 Toll-like receptors and human diseases. Potential involvement of TLRs in the pathogenesis of allergic diseases

Many groups have investigated the possible relationship of differential polymorphisms, expression, function of TLRs to clinical phenotype. Currently, several lines of evidence indicate that TLRs are implicated in the development of different human conditions (Akashi-Takamura, Miyake, 2006; Cristofaro, Opal, 2006; Lee, Hwang, 2006; Pandey, Agrawal, 2006)

These include:

1. Infectious diseases: tuberculosis (Bhatt, Salgame, 2007; Doherty, Ardit, 2004; Jo e.a., 2007), leprosy (Kang e.a., 2004); Legionnaire's disease (Hawn e.a., 2003), RSV bronchiolitis (Gargo e.a., 2004);

2. Inflammatory diseases and disorders: sepsis or systemic inflammatory response syndrome (SIRS) (Agnese e.a., 2002; Lorenz e.a., 2002; Romics e.a., 2006), inflammatory bowel disease (Cario, Podolsky, 2006; Lakatos e.a., 2005; Torok e.a., 2004), primary biliary cirrhosis (Riordan e.a., 2003), acute respiratory distress syndrome (Ramirez Cruz e.a., 2004), atherosclerosis (Edfeldt e.a., 2002; Kiechl e.a., 2002; Michelsen, Ardit, 2006), psoriasis (Baker e.a., 2003; McInturff e.a., 2005), Lyme disease (Salazar e.a., 2003; Singh, Girschick, 2006)

3. Autoimmune diseases: rheumatoid arthritis (Kyburz e.a., 2003; Pierer e.a., 2004; Radstake e.a., 2005), systemic lupus erythematosus (Hawn e.a., 2005; Rahman, Eisenberg, 2006)

4. Cancer (Chen e.a., 2004; Zheng e.a., 2004)

5. Allograft rejection (Chandra e.a., 2007; Goldstein e.a., 2006; Palmer e.a., 2003)

Some studies have addressed the potential involvement of TLRs in the pathogenesis of allergic diseases.

R.P. Lauener e.a. (2002) analyzed expression of TLR2 and TLR4 at the mRNA level with RT-PCR in blood cells taken from 25 farmers' and 71 non-farmers' children of the ALEX Study. The expression of TLR2 was found to be markedly higher in farmers' children compared with controls. While very suggestive that differential TLR expression

is associated with distinct populations, they stratified subjects based on place of residence (farming vs. urban), not on clinical phenotype.

Employing in-situ hybridization and immunochemistry, M. Tulic e.a (2004) examined the expression of TLR4 in the nasal mucosa after exposure to LPS in atopic children and atopic adults. The authors found much higher numbers of TLR4-positive cells in children compared to adults. However, they did not observe any difference between atopic and non-atopic groups in TLR4 expression. Although this investigation provides evidence for an important relation of differential TLR expression to the age (children vs adults), study participants were grouped on the basis of atopic, but not clinical status.

N. Nagata e.a. (2003) reported the upregulation of mRNA for TLR2, TLR4, TLR6 on CD14+ cells in the blood of individuals with atopic dermatitis relative to healthy controls. Of note, all studied patients were treated with topical corticosteroids, which might substantially influence expression patterns. Another limitation of this study is marked age difference between compared groups: 10 year old children with atopic dermatitis were compared with non-atopic adults aged 37 years. Consequently, it is possible that “upregulation” of TLRs might be associated with younger age of patients but not with the disease. This is in line with abovementioned observations by M. Tulic e.a. (2004). Thus, the results of the study of N. Nagata e.a. (2003) should be interpreted with caution.

Recently, M. Fransson e.a. (2005) have quantified the expression of TLR2, TLR3 and TLR4 by PT-PCR in the nasal mucosa of adult subjects with seasonal allergic rhinitis. One group of patients sampled before and the other during pollen season. A

significant increase of mRNA for TLR3, but not for TLR2 and TLR4, has been found among individuals with ongoing symptomatic allergic rhinitis during pollen season.

Thus, available data on the possible involvement of TLRs in allergic diseases are very limited and inconclusive. The contribution of TLRs to the development and/or maintenance of human asthma or protective immunity to environmental antigens remains poorly understood.

All relevant studies analyzed the expression of TLRs, mostly at the mRNA level. Even though this strategy gives some precious information, the expression would not necessarily be indicative of functional responsiveness. For instance, while eosinophils express transcripts for TLR4, exposure to LPS has been shown has no effect on several functional parameters of these cells, such as adhesion molecule expression, survival, and superoxide generation (Nagase e.a., 2003).

Therefore, functional studies, examining the immune responses to TLR stimuli, will be required to advance our understanding of the role of TLRs in human asthma. Thus, we hypothesized that functional responsiveness to TLR stimulation differs in allergic asthmatic versus non-atopic control children.

1.1.7 The role of LPS and other TLR ligands in asthma and atopy

The prevalence of asthma and other atopic disorders has increased dramatically over the last 30 years in developed and many developing countries. At present, asthma is

the most frequent chronic disease in children in the Western world (Eder e.a., 2006; Keil e.a., 2006).

It is well known that asthma and atopy are the result of complex interactions between genetic and environmental factors (Upham, Holt, 2005). In spite of the importance of genetic predisposition, the limited time frame over which the incidence of allergic diseases has been rising makes it unlikely that this could be due to changes in genetic constitution. This emphasizes a great consequence of environmental influences that have changed over the last few decades in industrialized countries as a result of modernization (Koppelman, Postma, 2003; Wills-Karp e.a., 2001).

As a potential explanation for the ongoing epidemic trend in childhood allergy in Western societies, the “hygiene hypothesis” was proposed by David Strachan in 1989. Having observed a strong inverse association between hay fever and number of siblings in his cohort of children in the United Kingdom, he formulated a thought-provoking hypothesis that infections in early childhood transmitted by “unhygienic contact with older siblings” may actually prevent allergic diseases (Strachan, 1989).

This initial observation has been confirmed in a number of epidemiological surveys around the world (Karmaus, Botezan, 2002; Ponsonby e.a, 1999; Svanes e.a., 2002).

Similar protective effects have been found for early day-care attendance during the first years of life (Ball e.a., 2000; Kramer e.a. 1999); childhood residence on a farm with livestock (Ernst, Cormier, 2000; Riedler e.a., 2000; von Ehrenstein e.a., 2000); pet ownership (Gern e.a., 2004; Hesselmar e.a., 1999; Nafstad e.a., 2001).

Several cross-sectional studies to identify infections as the explanatory protective factor for the sibling effect have demonstrated an inverse relationship between the prevalence of atopic diseases and serological markers of previous infections with food-borne and oro-fecal pathogens as hepatitis A (Matricardi e.a., 1997), *Toxoplasma gondii* (Matricardi e.a., 2000), *Helicobacter pylori* (Kosunen e.a., 2002).

Over time, the “hygiene hypothesis” have evolved such that infections itself might be not as crucial as exposure to microbial burden (Song, Liu, 2003).

Among a variety of potential microbial products that might modify the risk for development of allergic asthma and atopic disorders, bacterial LPS, the endotoxin of Gram-negative bacteria, a prototypical TLR4 ligand, attracted the greatest interest and has been the subject of intense investigation over the last decade. In fact, there is little conclusive evidence of which TLRs are most important in pathogenesis or protection from allergic disorders.

Evidence suggests that exposure to endotoxin, which ubiquitously present in the environment, has a double-edged effect: it can exacerbate preexisting asthma and, conversely, may be protective against the development of allergic asthmatic phenotype.

1.1.7.1 Exposure to endotoxin might exacerbate established asthma in humans

Endotoxin has strong pro-inflammatory properties and when inhaled, even by non-asthmatic individuals, it can directly elicit features of asthma such as airway inflammation, bronchoconstriction with a decrease in pulmonary function, and an increase in bronchial reactivity (Rylander e.a., 1989; Michel e.a., 1992). Consequently, it

was not surprising that several investigators have found a positive association between endotoxin exposures and exacerbations of asthma in patients with established disease.

In a cross-sectional study by O. Michel e.a. (1996), the high concentration of endotoxin in house dust was correlated with airflow obstruction, daily need for oral and inhaled corticosteroids, inhaled beta2-agonists, and xanthines among 49 adults with asthma sensitized to house dust mite allergen. Clinical severity of asthma was also positively associated with endotoxin exposure. M.C. Rizzo e.a. (1997) have demonstrated that the levels of house dust endotoxin are correlated with clinical symptom scores in asthmatic children, suggesting that exposure to indoor endotoxin increases the severity of childhood asthma.

Two recent epidemiological studies sustain these initial observations.

In the National Survey of Endotoxin in the United States, P.S. Thorne e.a. (2005), assaying of 2,552 house dust samples and evaluating of 2,456 residents, demonstrated the relationships between increasing endotoxin levels and diagnosed asthma, recent asthma symptoms, current use of asthma medications, and wheezing. This led authors to conclude that household endotoxin exposure is a significant risk factor for increased asthma prevalence. One limitation of this study is that it was not designed or powered to explore specific hypotheses regarding endotoxin and asthma exacerbation in young children.

G. Tavernier e.a. (2006) investigated the indoor environment of 200 children living in South Manchester, U.K., in IPEADAM survey. The asthmatic children were found to be exposed to higher levels of endotoxin than the non-asthmatic matched control subjects, suggesting that endotoxin exposures might be predictive factor for asthma.

Interestingly, B. Boehlecke e.a. (2003) showed that pre-exposure to low-dose airborne endotoxin that may be encountered in domestic settings enhances bronchial responsiveness to inhaled allergen in allergic asthmatics. M.W. Eldridge and D.B. Peden (2000) revealed that allergen challenge augments subsequent LPS-induced nasal inflammation in subjects with allergic asthma.

Therefore, among allergic asthmatic individuals, endotoxin exposure might potentiate the response to allergen and vice versa.

1.1.7.2 Exposure to endotoxin early in life might protect from developing atopy and allergic asthma in humans

In contrast to its deleterious effect on asthma, exposure to endotoxin in early childhood may, paradoxically, prevent the development of allergic sensitization and asthma.

Several well-performed cross-sectional epidemiological studies provide evidence for a protective association between early endotoxin exposures and allergic diseases.

In the United States, J.E. Gereda e.a. (2000) studied a cohort of 61 infants with repeated wheezing episodes who considered of being at high risk of developing of asthma. They found that the homes of allergen-sensitized infants contain significantly lower levels of house dust endotoxin than those of non-sensitized infants. Furthermore, higher concentrations of house dust endotoxin correlated with increased proportions of IFN-gamma-producing CD4+T cells, but not with proportions of cells that secrete IL-4, IL-5, IL-13 in the peripheral blood. This study was the first to show directly that indoor

exposure to LPS early in life may protect from allergen sensitization by promoting Th1 immune response.

These findings by J.E. Gereda e.a. (2000) have gained further support by European studies. In a larger survey of 740 schoolchildren from Germany, U. Gehring e.a. (2002) found an inverse association between exposure to house dust endotoxin and having an elevated IgE level to two or more allergens. The protective effect was strengthened with increasing degree of sensitization. They concluded that exposure to higher levels of house dust endotoxin is associated with lower prevalence of allergic sensitization in children.

C. Braun-Fahrlander e.a. (2002) examined environmental exposure to endotoxin and its relation to asthma and allergy in 812 school-age children in multinational ALEX Study in Europe. They demonstrated that mattress endotoxin levels were inversely associated with the occurrence of hay fever, atopic sensitization, atopic wheeze, atopic asthma, and the capacity of blood leukocyte to produce cytokines (TNF-alpha, IFN-gamma, IL-12, IL-10) after stimulation with LPS in both children from farming and non-farming households.

Thus, from these studies, it appears that early environmental exposures to LPS might have a protective effect on the development of childhood atopy and asthma later in life.

1.1.7.3 A potential role of other TLR ligands in asthma and allergic sensitization

Of note, the majority of epidemiological studies on possible relationships between exposure to microbial products and allergic diseases have focused on LPS, in part because detection assays for other TLR agonists have not been standardized and are not readily available. Yet endotoxin represents only a small part of the indoor microbial exposure. A variety of other microbial cell components, including ligands of additional TLRs, are also present at biologically significant levels in living environments and have immuno-modulatory properties (Horner, Raz, 2003; Horner, 2006).

A series of experimental studies provide evidence for the role of TLRs in modulating allergic responses and airway inflammation in animal models and humans.

G. Velasco e.a. (2005), examining the effect of pulmonary exposures of TLR2 ligands (PGN, Pam3Cys) in a model of allergic sensitization in mice, demonstrated that both TLR agonists decrease allergic parameters of inflammation: pulmonary eosinophilia, bronchoalveolar IL-13 levels, total serum IgE and airway hyperresponsiveness.

On the contrary, V. Redecke e.a. (2004) found that exposures to TLR2 ligand, Pam3Cys, aggravate experimental asthma in mice by both increasing eosinophil recruitment to the lungs and Th2 cytokine production. Similarly, intranasal challenge with PGN resulted in an enhancement of airway hyperresponsiveness, higher Th2 cytokine synthesis and lung eosinophilia in murine model of allergic sensitization (Chisholm e.a., 2004).

TLR7/8 ligand (resiquimod/S-28463) has been shown to be capable of preventing allergen-induced airway hyperresponsiveness, lung eosinophilia and of abolishing the elevation of serum IgE as well as the induction of Th2 cytokines by OVA challenge in a model of acute allergic asthma in mice (Moisan e.a., 2007). Moreover, continued

treatment with this compound can prevent the development of chronic airway remodeling including goblet cell and airway smooth muscle hyperplasia in rat model of chronic asthma (Camateros e.a., 2007).

TLR9 agonist, immunostimulatory oligodeoxynucleotide (ISS, a synthetic analogue of microbial DNA) has been demonstrated to be a potent Th1 adjuvant effective in both preventing and reversing Th2-biased immune deviation and the asthmatic phenotype in mice: early- and late-phase allergic responses such as airway hyperresponsiveness to inhaled metacholine, lung and BALF eosinophilia, and serum IgE levels (Broide e.a., 1998; Chisholm e.a., 2004; Hayashi, Raz, 2006; Kline e.a , 1998; Takabayashi e.a , 2003)

Human studies of ISS (TLR9 agonist) for allergic diseases have been promising.

In the first randomized, placebo-controlled trial, F.E.R. Simons e.a. (2004) treated patients allergic to ragweed with Amb a 1-ISS conjugate (AIC). They demonstrated that after 6 weekly injections of AIC, ex vivo ragweed-specific Th2 responses were redirected toward Th1 cytokine production, with decreases in IL-5, CCL17, CCL22 and increases in IFN-g, CXCL9, CXCL10.

Another study by M. Tulic e.a. (2004) showed that a short course immunotherapy with AIC led to a reduction in nasal inflammation in ragweed-sensitive patients with allergic rhinitis by decreasing Th2 cytokine responses (IL-4 mRNA-positive cells) and eosinophilia and increasing Th1 cytokines (IFN-g mRNA-positive cells). This was followed by a decrease in chest and nasal symptoms in patients treated with AIC.

Taken together, these findings highlight the importance of studying of potential effects of different endogenous or potentially therapeutic TLR ligands on the developing or pre-established allergic phenotype.

1.1.7.4 Factors that might influence the impact of TLR ligands on immune responses and subsequent development of asthma

The available evidence indicates that the relation between endotoxin exposure and asthma is complex, not direct or linear. LPS appears to have dual opposing effect: both exacerbation and protection have been observed. Whether immune modulation by LPS will result in allergic phenotype might be dependent not only on the endotoxin exposure itself, but also on the following determinants (Liu, Leung, 2006; Vercelli, 2006)

1. Dosage of exposure. S.C. Eisenbarth et al. (2002) elegantly delineated a critical role of LPS dose effect in a mouse model of allergic sensitization. They found that mice exposed to OVA depleted of endotoxin did not develop airways inflammation. Low doses of inhaled LPS combined with allergen induce eosinophilic Th2 responses. In contrast, inhalation of high levels of LPS with allergen results in neutrophilic Th1 responses. These data suggest that the dose of LPS exposure during allergen sensitization determined Th2-pro-asthmatic versus Th1-asthma-protective response.

2. Timing of exposure. This was clearly shown in a rat model of allergic sensitization by M. Tulic e.a. (2000). Administration of LPS before or during early sensitization process reduced OVA-specific IgE levels and prevented lung inflammation, eosinophilia and airway hyperresponsiveness. In marked contrast, LPS exposure after sensitization resulted in exacerbation of allergic inflammation.

3. Routes of exposure. D. Rodriguez e.a. (2003) showed that administration of LPS intravenously totally blocks OVA-induced lung inflammation in mice, while intranasal LPS challenge elicits a strong airway inflammation with predominance of neutrophils.

4. Genetic predisposition. M. Fageras Bottcher e.a. (2004) have shown that the TLR4 (Asp299Gly) polymorphism was associated with a 4-fold higher prevalence of asthma in school-aged children.

Thus, the effect of LPS, and, possibly, other TLR ligands, on allergic sensitization and asthma appears to be influenced by the levels of exposure, time at and route by which it occurs, and genetic background of individuals exposed.

The underlying immunological mechanisms for the observed relationship between exposure to TLR ligands and asthma are still poorly understood and need further elucidation.

1.1.8 Levels of TLR ligands in natural environments are much below those commonly used experimentally

To date, numerous studies have examined immune responses of cells to TLR stimulation in vitro. Our analysis of 25 randomly selected research reports published over last 10 years revealed usage of a broad range of LPS concentrations to stimulate cytokine production by human PBMC (Table 3). The greatest number of publications that were

analyzed employed a single dose of TLR4 agonist. Most remarkable is that the vast majority, over 90%, of the sampled studies utilized LPS at very high pharmacologic concentrations, i.e. 100 ng/ml and higher.

Table 3. Analysis of 25 randomly selected research studies on cytokine responses by human PBMC to LPS/TLR4 stimulation in vitro published between 1995 and 2006

| LPS dose / per ml | 10 ug | 1 ug | 100 ng | 50 ng | 10 ng |
|---|-------|------|--------|-------|-------|
| Number of studies | 5 | 8 | 10 | 1 | 1 |
| References: Arbour e.a., 1996; Borish e.a., 1996; Burkart e.a., 2002; El-Mezzein e.a., 2001; Gabriel e.a., 2002; Giovannini e.a., 2001; Hasegawa e.a., 2000; Hew e.a., 2006; Jansky e.a., 2003; Kallio e.a., 2002; Krakauer, 2002; Laufer e.a., 2002; Literat e.a., 2001; Manigold e.a., 2000; Mayringer e.a., 2000; Noma e.a., 1998; Pohl e.a., 1997; Porreca e.a., 1999; Siedlar e.a., 2005; van der Kleij e.a., 2004; Vowels e.a., 1995; Weber, Levitz, 2000; Yagoob, Calder, 1998; Yagoob e.a., 1999; Zeidel e.a., 2000 | | | | | |

It should be noted that potential caveat of this experimental approach exist.

First, humans have evolved to detect very low levels of LPS to sense microbes. Experimentally, N.P. O’Grady e.a. (2001), investigating the response of 34 healthy volunteers to direct bronchial instillation of endotoxin, demonstrated that a dose as low as 4 ng/kg was sufficient to initiate local airway inflammation composed of neutrophil influx with elevated levels of cytokines (TNF-alpha, IL-1beta, IL-6, G-CSF) in bronchoalveolar lavage. Similarly, a small 4 ng/kg dose of LPS administered intravenously to healthy volunteers was observed to be able to induce a systemic syndrome similar to clinical sepsis characterized by chills, fever, tachycardia, myalgia, headache, nausea, vomiting as well as leukopenia followed by leukocytosis, increased

levels of serum C-reactive protein and proinflammatory cytokines such as TNF-alpha and IL-6 (Lynn e.a., 2003).

D.B. Peden e.a. (1999) reported that nasal challenge with 0.1ug LPS results in a rise of GM-CSF levels and 1 ug of LPS causes an influx of eosinophils and neutrophils in nasal airways of atopic subjects, suggesting that exposure to low-dose endotoxin may increase allergen-induced bronchial inflammation in asthmatics. Several inhalation human studies have shown that asthmatics are more sensitive to the bronchoconstrictive effects of aerosolized LPS relative to healthy individuals (Michel e.a., 1989; Boehlecke e.a., 2003).

An environmental study of J.P. Zock e.a (1998), examining the relationship between acute lung function changes and personal endotoxin exposure in 61 workers of potato processing industry, showed that shortness of breath, chest tightness and acute airway obstruction are already apparent at low endotoxin levels of 4.5 ng/m³ corresponding to a total endotoxin exposure of 21 ng over 8 hours.

The guidelines by R. Rylander (1997) suggested that the threshold level of environmental airborne endotoxin for inducing local airway inflammation in healthy adults is as low as 10 ng/m³, and for systemic inflammatory responses is 100 ng/m³.

Thus, these studies provide evidence that extremely low levels of LPS can elicit inflammatory and pathophysiologic responses in humans.

Secondly, LPS concentrations seen in the natural environment are much below those that are widely used in analysis of immune capacity in vitro.

Until recently, endotoxin content in household dust has been the main test to assess environmental endotoxin exposure (Liu, 2002). However, there was a question

whether the quantity of endotoxin in settled family-room house dust truly reflect personal exposure?

Horick e.a. (2006) measured both the levels of airborne household endotoxin and endotoxin in the house dust to re-examine the relationship between wheeze and home endotoxin exposure in a cohort of 404 children in the Boston metropolitan area. They clearly demonstrated that the amount of endotoxin present in a household's air rather than in settled dust better characterizes its inhabitants' exposure to endotoxin.

Several environmental field studies analyzed the levels of airborne endotoxin in home settings. The reported data are summarized in Table 4.

Platts-Mills e.a. (2005) analyzed air sampled in 71 homes in Central Virginia, USA, and found the median endotoxin concentration of 0.01 ng/m³. Study by Bouillard e.a. (2005) documented that the median level of endotoxin is 0.239 EU/m³ in inhalable particles of airborne samples collected from urban homes in Brussels, Belgium. The median endotoxin amount was 0.18 ng/ml in the air of 100 dwellings in the Strasburg metropolitan area in France (Sohy e.a., 2006). Diette e.a. (2005), monitoring air of the child's bedrooms in 300 homes in Baltimore, USA, found the median endotoxin level of 0.03 ng/m³. Analysis of airborne endotoxin in living-rooms of 404 families in Boston, USA, revealed the median of 0.81 EU/m³ (Horick e.a., 2006). Wan e.a. (1999) reported that the median values of indoor airborne endotoxin were 0.065 ng/m³ and 0.156 ng/m³ in home settings and daycare centers, respectively, in Taipei, Taiwan.

Rabinovitch e.a. (2005) employed personal monitoring approach to accurately quantify levels of endotoxin exposure in school-age children in Denver, USA. They found that median amounts of endotoxin inhaled over 24 hours were 0.046 ng/day for

PM2.5 exposures and 0.22 ng/day for PM10 exposures (measured at a particulate matter size range $\leq 2.5 \mu\text{m}$ and $\leq 10 \mu\text{m}$ in diameter, respectively).

Table 4. Airborne endotoxin levels and calculated amounts of endotoxin inhaled by child in home settings

| Median airborne endotoxin levels (ng/m ³) | Median daily amount of endotoxin inhaled by child (ng/day) # | Location (reference) |
|---|--|--|
| 0.01 | 0.06 | Homes, Central Virginia, USA (Platts-Mills e.a., 2005) |
| 0.024* | 0.14 | Homes, Brussels, Belgium (Bouillard e.a., 2005) |
| 0.18 | 1.04 | Strasburg, France (Sohy e.a., 2006) |
| 0.03 | 0.17 | Homes, Baltimore, USA (Diette e.a., 2005) |
| 0.08* | 0.46 | Homes, Boston, USA (Horick e.a., 2006) |
| 0.008 (PM2.5) – 0.037 (PM10)** | 0.046 (PM2.5) – 0.22 (PM10) | Homes, Denver, USA (Rabinovitch e.a., 2005) |
| 0.065 | 0.37 | Homes, Taipei, Taiwan (Wan, Li, 1999) |
| 0.157 | 0.90 | Day care centers, Taipei, Taiwan (Wan, Li, 1999) |
| <p># median daily amount of endotoxin inhaled by child (ng/day) is calculated as an equivalent of median airborne endotoxin level (ng/m³) multiplied by respiratory ventilation rate (m³/min) and multiplied by the duration (min) (Liu, 2002) assuming that children typically have respiratory ventilation rate of 4 L/min or 0.004 m³/min (Chatburn e.a., 1990).</p> <p>* median airborne endotoxin levels expressed in original reports as EU/m³ were converted to ng/m³ on the commonly used basis of 10 EU = 1 ng of endotoxin</p> <p>** PM2.5 and PM10 show that endotoxin is measured at a particulate matter size range $\leq 2.5 \mu\text{m}$ and $\leq 10 \mu\text{m}$ in diameter, respectively.</p> | | |

Taken together, these observations indicate that airborne endotoxin levels to which children typically exposed are dramatically low, namely in the range of 0.008 - 0.18 ng/m³. Assuming that children have a mean ventilation rate of 4 L/min (Chatburn e.a., 1990), these concentrations correspond to a daily personal cumulated endotoxin

exposure of 0.18 to 1.04 ng/day. Thus, it might be concluded that very low levels of LPS reflect true environmental airway exposure in humans.

On the contrary, the great majority of published studies used LPS stimulation *in vitro* to mimic environmental exposures at concentrations ~ 1,000 -10,000 fold higher than LPS levels, which may be encountered in natural domestic environment.

We speculate that using low physiological concentrations of LPS and other TLR ligands that more closely reflect environmental levels rather than typically utilized “maximal” stimulation doses would provide greatly enhanced sensitivity to detect differences in responsiveness between clinically different populations.

In summary, Toll-like receptors are key regulators of both innate and adaptive immune responses and has been shown to be involved in the development of several human infectious, inflammatory, autoimmune diseases (Akashi-Takamura, Miyake, 2006; Brentano e.a., 2005; Pandey, Agrawal, 2006). However, the contributions of TLRs to human asthma remain poorly understood.

While a few investigations published to date provide valuable information on differential TLR expression among distinct populations, they stratified their study participants on the basis of domicile (farming vs. urban children) (Lauener e.a., 2002) or atopic status (Tulic e.a., 2004), but not clinical phenotype. In our study we will provide a direct comparison of how TLR functions differ in asthmatic vs. non-atopic control children.

Although a large number of clinical studies on the immune responses of cells to TLR stimulation *in vitro* are available, the vast majority of them utilized LPS, a TLR4 ligand, at greatly high pharmacologic concentrations. In contrast, levels of LPS observed

in common environments (Rabinovitch e.a., 2005) are many orders of magnitude lower than those widely used in vitro.

Reasoning that using “threshold” (low) rather than “maximal” (high) concentrations of TLR ligands would provide increased sensitivity to differentiate responsiveness between clinically distinct populations, we have developed the hypothesis to test that functional responsiveness to TLR stimulation by threshold concentrations of ligands differs in asthmatic and non-atopic children.

1.2 Environmental tobacco smoke and allergy

1.2.1 Health effects associated with cigarette smoking

In the early 1950s Sir Richard Doll provided the first accepted evidence on a causal association between cigarette smoking and lung cancer (Doll, 1953). Since these important findings, cigarette smoking (and exposure to environmental tobacco smoke, ETS) has been demonstrated to cause a broad array of diseases and adverse health outcomes (US Department of Health and Human Services, CDC. The Health Consequences of Smoking: A Report of the Surgeon General. Atlanta, GA; 2006; Gilliland e.a., 2006)

Currently, it is recognized that cigarette smoking is a worldwide leading preventable cause of morbidity and mortality. It accounts for approximately 431,000 deaths annually in the United States (Sopori, 2002). About 20% of all deaths (over

100,000) each year in the United Kingdom are caused by active smoking (Britton, 2005). Worldwide the annual death total is close to 5 million (Ezzati, Lopez, 2003).

Tobacco use adversely affects multiple organ systems, but especially the lungs. It is well documented that cigarette smoke is the major risk factor for chronic obstructive pulmonary disease and lung carcinoma (Barnes e.a., 2003; Brody, Spira, 2006; Burns, 2003). In addition, smoking has been shown to increase the incidence and/or severity of a wide spectrum of other respiratory illnesses, ranging from the common cold, influenza to pulmonary hemorrhage, spontaneous pneumothorax, and various interstitial lung diseases (Murin e.a., 2000).

Cigarette smoking is also associated with a higher incidence of cardiovascular diseases such as atherosclerosis, myocardial infarction, fatal coronary artery disease (Ambrose, Barua, 2004; Sopori, 2002); cancers of various sites, e.g. oral cavity, larynx, pharynx, nasal cavity and sinuses, esophagus, pancreas, kidney, bladder, uterine cervix (Howe e.a., 2006; International Agency for Research on Cancer, 2004); rheumatoid arthritis (Majka, Holers, 2006; Saag e.a., 1997), systemic lupus erythematosus (Freemer e.a., 2006), and Crohn's disease (Mahid e.a., 2006).

Interestingly, while harmful effects of tobacco use are well established, there is increasing evidence that cigarette smoking might also limit the risk of several inflammatory and neurodegenerative diseases. Epidemiological studies demonstrated that tobacco smokers have a lower incidence and/or severity of hypersensitivity pneumonitis (Arima e.a., 1992; Baldwin e.a., 1998), sarcoidosis (Peros-Golubicic, Ljubic, 1995; Valeyre e.a., 1988), ulcerative colitis (Mahid e.a., 2006), Sjögren's syndrome (Manthorpe e.a., 2000), Parkinson's disease, and, perhaps, Alzheimer's disease (Fratiglioni, Wang,

2000). Similarly, some experimental data showed that nicotine exposure diminished the development of hypersensitivity pneumonitis (Blanchet e.a., 2004) and ulcerative colitis (Sykes e.a., 2000) in mice.

Thus, cigarette smoking has multiple effects on human health. It is well-documented that tobacco use increases the risk for a number of diseases, whilst it also appears to act as a protective factor for some illnesses.

1.2.2 Adverse health effects associated with exposure to ETS

Since the early 1980s, there has been growing scientific and public health concern that involuntary exposure to environmental tobacco smoke might have the harmful impact on the health of non-smokers.

Exposure to environmental tobacco smoke - also known as “passive smoking”, “involuntary smoking” or “exposure to secondhand smoke” – is defined as the exposure of a non-smoking person to tobacco-combustion products from smoking by others (Jaakkola e.a., 1997; Sopori, 2002)

ETS is the complex mixture composed of the escaping smoke of a tobacco product (sidestream tobacco smoke) and smoke exhaled by the smoker (exhaled mainstream tobacco smoke) (Report of California Environmental Protection Agency, 2005).

Despite quantitative differences, the chemical composition of ETS is very similar to mainstream tobacco smoke encountered by smokers (Dye, Adler, 1994; Sopori, Kozak, 1998).

Tobacco smoke contains more than 4,500 chemicals. Many of them are human toxic, irritant and/or carcinogenic agents, including carbon monoxide, carbon dioxide, nitrogen oxides, sulfur dioxide, formaldehyde, hydrogen cyanide, nicotine, acrolein, hydroquinone, phenol, ammonia, inorganic compounds (eg, cadmium, lead, nickel); benzopyrenes, nitrosonornicotine, dimethylnitrosamine) (Kum-Nji e.a., 2006; Report of Royal College of Physicians, 2005).

It is particularly important for this thesis that a high content of biologically active endotoxin is found in cigarette smoke particles (Hasday e.a., 1999). Exposure to ETS has been shown to result in inhalation of amounts of endotoxin that are dramatically, more than 120 times, greater than those existing in smoke-free air (Larsson e.a., 2004).

These pathogenic substances in tobacco smoke produce irritant, inflammatory and immunologic effects. It therefore is not surprising that exposure to ETS might contribute to the development, maintenance and/or aggravation of various diseases and disorders.

Children are particularly vulnerable to secondhand smoke as their respiratory and immune systems are not fully developed; they have higher ventilation rates and inhale greater levels of ETS than adults for the same level of external exposure (Chan-Yeung, Dimich-Ward, 2003).

Numerous epidemiological studies have shown that passive smoking might adversely affect the health of children. Recent comprehensive reviews of an extensive literature published over the last decade (Report of California Environmental Protection

Agency, 2005; US Department of Health and Human Services, CDC. The Health Consequences of Smoking: A Report of the Surgeon General. Atlanta, GA; 2006) concluded that ETS exposure causes a variety of adverse health outcomes in children. These include sudden infant death syndrome, decrease in birth weight, acute lower respiratory tract infections (e.g., bronchitis and pneumonia); chronic respiratory symptoms (e.g. cough, phlegm, wheeze, and breathlessness), middle ear infections, and asthma. There are several effects of ETS for which evidence for casual association is suggestive, but further research is needed for confirmation, including decreased lung function growth, brain cancer and lymphomas/leukemias, adverse impact on cognition and behavior of children.

1.2.3 ETS and asthma

A large number of epidemiological studies have examined the association between ETS and asthma. Much of the literature has been reviewed periodically and analyzed in meta-analyses or systematic quantitative reviews.

In 1997-1998 D.P. Strachan and D.G. Cook published a series of meta-analyses of the effects of ETS on children's respiratory health. They found that the pooled odds ratio (OR) for increased likelihood of asthma prevalence from 14 case control studies was 1.37 (95% CI, 1.15 to 1.64) if either parent smoked (Strachan, Cook, 1998). Meta-analysis based on 25 cross-sectional studies gave a pooled OR 1.21 (95% CI, 1.10 to 1.34) for asthma prevalence among school age children with smoking parents (Cook, Strachan, 1997).

These findings have recently been supplemented by the comprehensive 2005 Report of the California Environmental Protection Agency that included a meta-analysis of 37 studies carried out between 1996 and 2003. The pooled OR for new-onset asthma of 1.32 (95% CI, 1.24-1.41) and a causal role of ETS in childhood asthma induction and severity of disease was documented.

More recent the PATY (Pollution And The Young) project has been conducted a pooled analysis of data on ETS exposure on respiratory health for 53,879 children at 6-12 yrs of age from 12 cross-sectional studies carried out in Europe and North America. Effect estimates for asthma were found to be positive with mean ORs of 1.09 (95% CI, 1.01 to 1.19) for the current passive smoking, of 1.10 (95% CI, 1.00 to 1.21) for passive smoking during the first two years of life, and of 1.18 (95% CI, 1.08 to 1.28) for maternal smoking during pregnancy (Pattenden e.a., 2006). This large population-based study provides additional data linking ETS to childhood asthma.

Thus, epidemiological evidence is sufficient to infer a casual association between parental smoking and increased prevalence and severity of asthma in children

1.2.3.1 ETS and allergic and non-allergic asthma

Some studies reported that the effects of ETS on childhood asthma appeared to be stronger in children with non-allergic asthma vs allergic asthmatics. Ronmark e.a. (1999), evaluating the impact of ETS on asthma in a sample of 2,545 Swedish children aged 7-8 years, found the effect estimate for ETS was greater for non-allergic (OR=1.67; 95% CI 1.04-2.68) than allergic asthma (OR=1.17; 95% CI 0.68-2.01)

Tariq e.a. (2000) followed-up the Isle of Wight birth cohort of 1,218 children to the age 4 years. Although they observed a strong association between exposure to maternal tobacco smoke and asthmatic symptoms at ages 1 (OR=2.5; 95% CI 1.7 to 3.7, $p<0.001$) and 2 years (OR=2.2, 95% CI 1.5 to 3.4, $p<0.001$), this was lost by 4 years (OR=1.2, 95% CI 0.3 to 2.7, $p>0.05$). There was, however, a link between maternal smoking and asthma in a subgroup of non-allergic asthmatic children (those with asthmatic symptoms, but negative skin prick test) even at 4 years (OR=1.4, 95% CI 0.6 to 2.1, $p>0.05$)

However, the available literature on this issue is limited: Additional investigations are required to examine the effect of ETS exposures on different asthmatic phenotypes (allergic and non-allergic asthma).

1.2.3.2 ETS and atopy

Several earlier reports noted that parental smoking was linked with markers of atopy in children, including positive skin prick tests (Martinez e.a., 1988; Lindfors e.a., 1999), levels of serum IgE (Kulig e.a., 1999; Ronchetti e.a., 1990; Wjst e.a., 1994), and eosinophilia (Halonen e.a., 1991; Ronchetti e.a., 1990). Increased risk of atopic disorders in children related to ETS exposure was demonstrated by studies in Italy (Ronchetti e.a., 1992), Sweden (Norrman e.a., 1994), Estonia (Braback e.a., 1995). However, other studies in Germany (von Mutius e.a., 1994), Norway (Soyseth e.a., 1995), Poland (Braback e.a., 1995) found a slightly decreased risk of the development of atopy among children of smoking parents.

A systematic review of 36 studies on the effects of ETS on skin prick test positivity, IgE levels, and allergic rhinitis or eczema in children conducted by D.P. Strachan and D.G. Cook (1998) did not find any conclusive associations. It has been concluded that parental smoking during pregnancy or early childhood is unlikely to increase substantially the risk of allergic sensitization.

Similarly, a large survey of 7,798 schoolchildren in France failed to observe any association between skin prick test positivity, atopic disorders and exposure to parental smoke in utero or during childhood (Raheison e.a, 2007). The PATY project, investigating the relationship between ETS exposures at different times of a child's life and atopy in a pooled analysis based on 9 cross-sectional studies, documented predominantly negative effect estimates with mean ORs of all around 0.9 for hay fever and the lack of associations for "sensitivity to inhaled allergens" (Pattenden e.a., 2006)

Thus, available evidence concerning the effect of passive smoking on the development of atopy is mixed/ inadequate. While earlier studies reported a higher risk of sensitization in children exposed to secondhand smoke, recent population-based surveys did not find consistent relationship between ETS and atopy.

Despite the overwhelming epidemiological data, mechanisms underlying effects of parental smoking on asthma and of atopy remains poorly understood.

1.2.4 Effects of cigarette smoke on the immune system of humans

It has long been proposed that the increased susceptibility of smokers to respiratory diseases and cancers might, in part, be due to consequences of chronic inhalation of cigarette smoke on the immune system (Holt, Keast, 1977). At present, a considerable body of literature exists on the impact of tobacco smoke on both innate and adaptive immunity (Sopori, 2002). These are reviewed below.

1.2.4.1 Smoking and innate immunity

Many reports indicated that cigarette smoking substantially (by several fold) increases the number of alveolar macrophages in bronchoalveolar lavage fluid (Adesina e.a., 1991; Bosken e.a., 1992; Reynolds, 1987; Sopori, 2002). Moreover, smokers' alveolar macrophages have been demonstrated to exhibit a number of morphological and functional alterations (Floreani, Rennard, 1999). These include increased chemotactic activity, such as higher release of chemotactic factors and greater motility to chemotactic stimuli (Hunninghake, Crystal, 1983; Koyama e.a., 1991; Thompson e.a., 1989); enhanced secretion of lysosomal enzymes and proteases; elevated oxygen-radicals production and myeloperoxidase activity (Sopori, 2002). However, notwithstanding this "active state" alveolar macrophages from smokers have diminished phagocytic and bactericidal activity (King e.a., 1988; Plowman, 1982) and decreased cell-surface expression of CD11/CD18 adhesion molecule complex (Hoogsteden e.a., 1991)

Several groups, investigating the effects of tobacco use on the function of natural killer (NK) cells, also demonstrated reduced NK cell cytotoxic activity in cigarette smokers (Ferson e.a., 1979; Nair e.a., 1990; Zeidel e.a., 2002).

1.2.4.1.1 Effects of cigarette smoking on pro-inflammatory and anti-inflammatory cytokine responses in humans

Cytokines exhibit a wide variety of biological effects in humans. Pro-inflammatory cytokines play a key role in initiation and regulation of inflammation, in innate immune responses to pathogens, in host defense against pathogens, and can mediate tissue damage (Arend e.a., 2002; Clark, 2007; Gabay, 2006; Rosenwasser, 1998).

Several groups have examined the effects of cigarette smoking on proinflammatory cytokine responses in humans, but available data are conflicting.

Some studies have provided evidence of increased proinflammatory cytokine synthesis in asymptomatic smokers. W.G. Kushner e.a. (1996) found that smokers had higher levels of IL-1 beta, IL-6, CCL2, IL-8 in BAL fluid compared to non-smokers. Similarly, C. Gessner e.a. (2005) observed elevated concentrations of TNF-alpha, IL-6, IL-8, IL-12p70 in exhaled breath condensate (EBC) obtained from smokers. Increased levels of IL-8 in BAL fluid (Mio e.a., 1997; Ohta e.a., 1998), sputum (Wang e.a., 2000) and IL-6 in EBC (Carpagnano e.a., 2003) of smokers were also reported by other investigators.

Also, several ex vivo studies showed that cigarette smoking enhanced LPS-induced production of TNF-alpha (Lim e.a., 2000) and IL-8 (Koch e.a., 2004; Morrison e.a., 1998) by alveolar macrophages. A. Zeidel e.a. (2002) demonstrated an increase in IL-1 beta, TNF-alpha, IL-6 production by PBMC to LPS or PHA stimulation in individuals who smoke compared to non-smokers. Correspondingly, mononuclear cells derived from smokers which were then exposed to cigarette smoke in vitro produce more IL-1 beta than those from non-smokers (Ryder e.a., 2002). In addition, it has been shown

that cigarette smoking to upregulate the expression of mRNA for IL-8, ICAM-1 in airway epithelial cells (Takizawa e.a., 2000) and increase NF-kB activity in lung tissue (Szulakowski e.a., 2006) and mononuclear cells (van den Berg e.a., 2000).

On the contrary, a number of studies reported a reduced capacity of alveolar macrophages of smokers to produce IL-1 beta (Brown e.a., 1989; Yamaguchi e.a., 1989; Soliman e.a., 1992), TNF-alpha (Yamaguchi e.a., 1993; McCrea e.a., 1994), IL-6 (Soliman e.a., 1992; McCrea e.a., 1994; Mikuniya e.a., 1999), IL-8 (Ohta e.a., 1998) *in vitro* in response to LPS. Similarly, A.E. Gordon e.a. (2002) found that smokers exhibited a diminished release of IL-1 beta by LPS-stimulated whole blood cultures. Also, levels of IL-6 were demonstrated to be reduced in BAL fluid of smokers compare to non-smokers (McCrea e.a., 1994).

Of note, some studies revealed an equivalent production of proinflammatory cytokines among smokers and non-smokers. It has been demonstrated that cigarette smoking had no effect on the levels of IL- beta, TNF-alpha, IL-6, IL-12p70 and IL-8 in exhaled breath condensate (Sack e.a., 2006) and on mRNA expression for these cytokines in lung tissues (Szulakowski e.a., 2006). Similar quantities of TNF-alpha released by sputum and blood cells in response to LPS (Dentener e.a., 2005) and its comparable levels in BAL fluid were found in smoking and non-smoking subjects. Smokers have also been shown to exhibit equal synthesis of IL-1 beta by alveolar macrophages in response to PMA (Bardelli e.a., 2005) and by bronchial epithelial cells exposed to cigarette smoke *in vitro* (Rusznak e.a., 2000) relative to non-smokers. T. Ohta e.a. (1998) reported that PBMC derived from individuals who smoke have the same ability to secrete IL-8 to LPS stimulation as those from non-smoking volunteers.

IL-10 is a pleiotropic cytokine with a broad spectrum of anti-inflammatory and immunoregulatory properties (Asadullah e.a., 2003; Moore e.a., 2001). Data on the relationship between cigarette smoking and IL-10 synthesis is limited and also inconclusive. Lim e.a. (2000) found increased production of IL-10 by LPS-stimulated alveolar macrophages of smokers compared to non-smokers. Study by C. Gessner e.a. (2005) showed that smoking subjects had elevated levels of IL-10 in exhaled breath condensate.

In contrast, S. Takanashi e.a. (1999) reported that cigarette smoking reduced the levels of IL-10 and the percentage of IL-10-positive cells in sputum. A.E. Gordon e.a. (2002) demonstrated diminished release of IL-10 in whole blood cultures on LPS stimulation of individuals who smoke.

As seen for the pro-inflammatory cytokines reviewed above, some studies did not observe any difference in IL-10 levels among smoking and non-smoking groups. A. Zeidel e.a. (2002) showed that alveolar macrophages of smokers have an equal capacity to produce IL-10 in response to LPS or PHA as those from non-smokers. Similar levels of IL-10 have also been found in plasma (Whetzel e.a., 2007) and exhaled breath condensate (Sack e.a., 2006) of smoking and non-smoking volunteers.

Thus, conflicting results have been reported by human studies regarding both pro- and anti-inflammatory cytokine responses in asymptomatic smokers. The reason for this discrepancy is currently not clear. A possible explanation is that resultant effect of cigarette smoking on immune responses might reflect the cumulative effect of both immunostimulatory and immunosuppressive compounds of cigarette smoke. In addition,

these effects might depend on duration and dose of exposures (Sopori, 2002) or the experimental conditions used to elicit cytokine production by immune cells.

1.2.4.1.2 Effects of cigarette smoke on cytokine responses in vitro

In addition to effects in vivo, cigarette smoke has also been shown to alter the release of pro-inflammatory cytokines from various human cell types in vitro. Some studies have demonstrated that short-term direct exposure to cigarette smoke extract (CSE) induces cytokine responses in cell cultures.

M.J. Walters e.a. (2005) found that CSE added to culture stimulates monocytes and alveolar macrophages to produce IL-8. More recently, similar findings have been reported for TNF-alpha, IL-6 and IL-8 secretion by monocyte-derived macrophages in response to CS medium (Karimi e.a., 2006). Other studies have demonstrated an increase in TNF-a production by human monocytic-like cells (U937) (Demirijan e.a., 2006) and in IL-8 by macrophage-like cells (MonoMac6) (Yang e.a., 2006) exposed to cigarette smoke.

T. Mio e.a. (1997) showed that CSE augmented IL-8 release from human bronchial epithelial cells. Increased secretion of IL-8, G-CSF, MCP-1 has also been observed for human alveolar epithelial-like cell line exposed to CSE (Masubuchi e.a., 1998). Moreover, both human bronchial and alveolar epithelial cells has been demonstrated to upregulate the expression of IL-1beta, IL-8, GM-CSF, sICAM-1 genes in response to CS condensate (Hellermann e.a., 2002). Exposure to CSE induced mRNA expression and protein production of IL-8, GM-CSF and MCP-1 by cultured human fetal

lung fibroblasts (Sato e.a., 1999), and IL-8 release by human airway smooth muscle cells (Oltmanns e.a, 2005)

On the contrary, a number of groups have reported an inhibitory effect of cigarette smoke (or of some its components) on the release of pro-inflammatory cytokines/chemokines by human cells in vitro.

Studies of Y. Ouyang e.a. (2000) and C. Lambert e.a. (2005) demonstrated that short-term exposure of PBMC derived from healthy non-smoking volunteers to CSE followed by activation with anti-CD3 plus PMA resulted in inhibition of IL-1beta, TNF-alpha, IFN-gamma, IL-6, IL-8, GM-CSF and IL-10 production. Tobacco smoke has also been shown to suppress the LPS-induced TNF-alpha synthesis by monocytes (Vayssier e.a., 1998) and decrease the production of IL-6, TNF-alpha by alveolar macrophages (Dubar e.a., 1993).

I.R. Witherden e.a. (2004), examined the effect of CSE on the release of chemokines, including MCP-1, IL-8, GRO-alpha, MIP-1alpha, RANTES, from primary human alveolar type II epithelial cells. CSE (5%) reduced the levels of all these chemokines. However, the effect of 1% CSE was not detectable. Similarly, M. Laan e.a. (2004) demonstrated that in vitro CSE exposure inhibits the LPS-elicited IL-8 and GM-CSF protein secretion and mRNA expression in human bronchial epithelial cells.

B.K. Nordskog e.a. (2005) investigated both gene expression and protein release of 17 different cytokines and chemokines in human aortic endothelial cells (HAECs) and THP-1 monocyte/macrophages in response to CS condensate exposure. They found that CSC treatment resulted in the down-regulation of IL-6, IL-1beta, MIP-1beta, MCP-1 in THP-1 and MCP-1 in HAECs, while IL-8 and IL-4 were stimulated in HAECs.

Thus, available data on the pro-inflammatory cytokine/chemokine responses by cultured human cells in vitro to cigarette smoke exposure are somewhat contradictory. Cigarette smoke extracts added to culture have been reported to activate or inhibit the activation of cytokine synthesis in vitro. This discrepancy may be attributed, at least in part, to the variations in the composition of cigarette smoke extracts and the culture conditions under which it used (doses, timing of exposure).

Notably, a study by C. Rusznak e.a (2001) demonstrated that short-term exposure (20 min) to cigarette smoke in combination with house dust mite allergen increases the release of IL-1beta, IL-8 in human bronchial epithelial cell cultures, whereas longer exposure (1-6 h) reduced it.

1.2.4.1.3 Effects of cigarette smoking on the expression of Toll-like receptors

It is well established that Toll-like receptors play a crucial role in the initiation of innate immune and inflammatory responses and in the regulation of adaptive immune responses that subsequently develops (Iwasaki, Medzhitov, 2004; Lee, Hwang, 2006)

Recently, K. Karimi e.a. (2006) have shown that in vitro release of IL-8 by human monocyte-derived macrophages in response to cigarette smoke containing medium could be mediated via TLR4. These findings raise the possibility that tobacco use might affect the function of TLRs in humans.

However, available data is scarce. So far, only one study by D. Droeman e.a. (2005) has addressed the effects of cigarette smoking on TLRs, demonstrating a reduced TLR2 surface expression on alveolar macrophages of patients with COPD and healthy

smokers relative to non-smokers. Expression of TLR4 and CD14 were equivalent among the groups.

These results are in contrast to those seen on cigarette smoke exposure models in mice. T. Maes e.a. (2006) observed an increased TLR4 and TLR2 protein expression in macrophages in lung tissue sections of cigarette smoke-exposed mice compared to air-exposed animals. In addition, they demonstrated protective effect of TLR4 mutation against cigarette smoke-induced recruitment of neutrophils, lymphocytes and dendritic cells to the lungs. This suggests that TLR4 signaling plays a role in pulmonary inflammatory response towards cigarette smoke.

R. Vlahos e.a. (2006) showed that in experimental mice subchronic exposure to cigarette smoke resulted in an upregulation of mRNA for TLR2 in the BAL cells whereas expression of TLR4, 3, 9 were not markedly increased.

This striking contradiction in results of human and murine studies may be partially explained by differences in the regulation of TLR expression among both species resulting from species variations in a genomic organization of TLR genes (Rehli e.a., 2002). Thus, future research is awaited for evaluating the impact of cigarette smoking on Toll-like receptors.

1.2.4.2 Smoking and adaptive immunity

Most studies examining the effects of cigarette smoking on lymphocytes in humans have documented a leukocytosis (Corre e.a., 1971; Sopori, Kozak, 1998) with a greater numbers of T- and B-lymphocytes (Hughes e.a., 1985; Smart e.a, 1986).

Also, increased numbers of both CD4+ and CD8+ T cell subpopulations (Smart e.a., 1986) have been shown in smokers with reduced CD4+/CD8+ ratios (Costabel e.a., 1986; Mattoli e.a., 1997). Cigarette smoking decreases the serum levels of antibodies and total IgG, IgA, IgM, IgD (Andersen e.a., 1982; Ferson e.a., 1979; Gerrard e.a., 1980; McSharry e.a., 1984), except IgE, which might be elevated in smokers (Burrows e.a., 1981; Sherrill e.a., 1994). This suggests that tobacco use can impact humoral immune responses in humans.

Conflicting results have been reported regarding the effects of cigarette smoking on T-cell responses. While some authors have observed a reduced ability of T cells to proliferate in response to mitogens in smokers (Holt, 1987), other studies demonstrated enhanced mitogen-induced T-cell proliferative responsiveness (Zeidel e.a., 2002) or did not find any difference between smoking and non-smoking individuals (Hughes e.a., 1985).

1.2.4.2.1 Effects of cigarette smoking on the balance of Th1/Th2 cytokines in humans

The polarized T helper cell subsets Th1 and Th2 are responsible for orchestrating appropriate immune responses to a wide variety of pathogens. Excessive synthesis of Th1 cytokines has been implicated in the pathogenesis of autoimmune diseases (Szabo e.a., 2003), whereas Th2 cytokines play critical roles in atopy and asthma (Georas e.a., 2005)

It is conceivable that cigarette smoke might affect Th1/Th2 cytokine equilibrium resulting in altered immune responses. An early report by K.A. Byron e.a. (1994) demonstrated elevated production of IL-4 by PBMC in response to PHA among smokers

relative to non-smokers. W. Cozen e.a (2004) compared cytokine synthesis by PHA-stimulated PBMC among monozygotic twins with varying levels of cigarette consumption. They found an association between cigarette smoking and increased IL-13 responses; but no difference in IL-4, IL-5 levels between the groups. Both studies (Byron e.a., 1994; Cozen e.a., 2004), however, observed equivalent production of IFN-gamma among smoking and non-smoking individuals.

E. Hagiwara e.a. (2001) evaluated the effect of cigarette smoking on BAL cells secreting Th1 and Th2 cytokines. While IL-4-secreting cells were undetectable, smokers exhibited a decreased number and frequency of cells secreting IFN-gamma compare to non-smokers, leading to conclusion that cigarette smoking depletes Th1 cytokine-secreting cells in human airways. Similarly, A. McKay e.a. (2004) reported that cigarette smoking reduced levels of IL-18 in sputum in asthmatic and normal subjects. This was more pronounced in asthmatic smokers and was associated with a decrease in IL-18 mRNA expression. In contrast, C.A. Whetzel e.a. (2007) found an increased IFN-gamma concentrations in plasma of smoking group.

M. Majori e.a. (1999), examining IFN-gamma and IL-4 expression in peripheral blood CD4+ and CD8+ T cells, did not observe any difference in T cell cytokine profile between smokers and non-smokers.

Thus, some observations suggest that cigarette smoking might skew the immune system toward a Th2 pattern. It is not clear whether tobacco use has an influence on IFN-gamma synthesis: while some studies showed altered IFN-gamma production in smokers, others did not observe any effect.

1.2.5 Effects of ETS exposures on the immune system of humans

1.2.5.1 ETS exposure and innate immunity

Whether ETS might have impact on innate immunity is not clear so far. Available data on this issue is scarce. To our knowledge, there are only three reports that have been published.

1.2.5.1.1 Effects of ETS exposures on pro-inflammatory and anti-inflammatory cytokine responses in humans

S. Hockertz e.a. (1994) evaluated acute effects of exposure to ETS on the immune system under controlled laboratory conditions. Five non-smoking volunteers were exposed (2 days, 8 hours/day) to ETS generated by the smokers. These experimental exposures induced a slight increase in CD16+ and CD56+ cells in peripheral blood; however, did not alter other immunological parameters examined which include concentrations of IL-1 beta, IL-6, soluble CD14, PGE2 in serum, numbers of blood CD3+, CD4+, CD8+, CD19+ cells, and in vitro stimulated production of reactive oxygen intermediates by granulocytes. Authors concluded that exposure to experimental ETS did not result in an impairment of cellular immune responses. It should be noted that while this study reported original valuable data concerning possible immunological effects of ETS in humans, experimental conditions used do not accurately reflect natural, real-life

exposures to second-hand smoke (duration, dose of exposure). Future well-designed studies are needed to clarify this issue.

D. Gentile e.a. (2005) examined IL-10 synthesis by LPS-stimulated myeloid DC obtained from 16 healthy infants exposed to ETS and 21 controls with no history of ETS exposure. They found an association between ETS and diminished IL-10 production in healthy infants. Authors reported that these results are preliminary and other larger, well-designed studies are required to confirm these observations.

1.2.5.1.2 Effects of ETS exposures on the expression of Toll-like receptors

A. Ricci e.a. (2005) evaluated the expression of TLR2 and TLR4 in adenoidal CD4+ and CD8+ T cells by flow cytometry of 8 children exposed to ETS. They observed a decrease in the percentage of TLR4-positive CD8+ cytotoxic T cells.

Although obtained in a small number of subjects, these results raise the possibility that ETS might alter the expression of TLR in humans. However, the effect of parental smoking on TLR function remains unknown.

1.2.5.2 ETS exposure and adaptive immunity

1.2.5.2.1 Effects of ETS exposures on the balance of Th1/Th2 cytokines in humans

Few studies have addressed the effect of secondhand smoke exposures on Th1/Th2 balance in humans. A. El-Nawawy e.a. (1996), assessing IL-4 concentrations in serum of 70 randomly selected children of smoking parents, demonstrated that parental

smoking leads to an elevation of IL-4 and correlated with a higher frequency of respiratory symptoms. A reduction in the percentage of IFN-gamma-producing CD4+ and CD8+Tcells on adenoids of children exposed to secondhand smoke has been observed by M.A. Avanzini e.e (2006).

W. Feleszco e.a. (2005) investigated the effects of ETS on the expression of cytokines, including IFN-gamma and IL-5, IL-13, in nasopharyngeal secretions obtained from allergic asthmatic and healthy control schoolchildren. They found higher levels of IL-13 in subjects with asthma exposed to ETS relative to non-ETS-exposed asthmatics and controls. Elevated concentrations of IL-13 were also seen in ETS exposed control children in comparison to unexposed controls. Levels of IFN-gamma and IL-5 were similar among the groups. Authors concluded that ETS is associated with augmented secretion of IL-13 in allergic asthmatic children.

D. Diaz-Sanchez e.a. (2006) used a human in vivo model to investigate the role that ETS might play in exacerbations of allergic airway diseases. Nineteen non-smoking volunteers with allergic rhinitis were randomly exposed to ETS or air followed by nasal challenge with allergen or placebo. ETS exposure alone had no detectable effect on the local cytokine release. Exposure to ETS plus allergen resulted in an increase in levels of IL-4, IL-5, IL-13 and a decrease in IFN-gamma levels in nasal washes. This suggests that short-term exposure to ETS in combination with allergen might promote the local formation of a Th2-type cytokine milieu.

Collectively, these findings support the notion that passive smoking might favor a Th2 bias. However, available literature is limited and this conclusion remains to be confirmed.

To summarize, epidemiological evidence supports a casual role of environmental tobacco smoke in the development and exacerbation of asthma in children. However, the mechanism underlying these adverse effects of ETS is still poorly understood. Alterations in Toll-like receptor responsiveness in different individuals as a result of differences in environmental exposures might influence their likelihood of exhibiting allergic diseases such as asthma. In the research presented below, we hypothesized that ETS exposures affect TLR responsiveness in asthmatic children.

1.3 Ethnicity and Toll-like receptor responsiveness: First Nation versus Caucasian populations

1.3.1 The influence of ethnicity on susceptibility to certain infectious and allergic diseases

1.3.1.1 Epidemiology of tuberculosis among Aboriginal population in Canada

Tuberculosis remains a major public health problem for many Aboriginal people in Canada. In 1999, the incidence rate among Aboriginal Canadians (34.5 per 100,000 population) was twenty times higher than that of Canadian-born people of non-Aboriginal descent (1.5 per 100,000 population) and more than five times the national rate (6.5 per 100,000 population) (Long, 2000).

A similar epidemiologic profile of tuberculosis is seen in Manitoba. In that province, treaty (registered) Aboriginal Canadians are at the highest risk of acquiring tuberculosis. In 1999, the incidence rate among them (48.4 per 100,000 population) was remarkably higher than both those of Canadian-born non-treaty people (3.3 per 100,000), and of population of the province as a whole (9.2 per 100,000) by fifteen and five times, respectively (Al-Azem e.a., 2000; Al-Azem e.a., 2000). Of the new active cases of tuberculosis, 44% occurred among treaty Aboriginal people, despite the fact that they represent only 8.9% of Manitoba's population (Blackwood e.a, 2003).

Incidence rates tend to be highest in certain northern First Nation communities elevating up to 496.3 per 100,000, more than fifty times higher than provincial rate (9.2 per 100,000) (Blackwood e.a., 2003; FitzGerald e.a., 2000).

Notably, new cases of tuberculosis in the Aboriginal population often occur in young individuals: 30% of cases were in people less than 15 years as compared to only 9% in non-Aboriginals (Special Report of the Canadian Tuberculosis Committee, Tuberculosis in Canadian-born Aboriginal Peoples, Public Health Agency of Canada, 1999). Thus, the prevalence and incidence rates for Canadian Aboriginal population remain greatly higher than those of non-Aboriginal people. First Nation children are more susceptible to this infectious disease than others in Canada.

1.3.1.2 Epidemiology of asthma and bronchiolitis among Aboriginal population in Canada

In the past, chronic respiratory conditions such as asthma and COPD have been reported to be uncommon among Aboriginal people in Canada. In the early 1970s, the

rates of asthma hospitalization in the Aboriginal communities were similar to those in the non-Aboriginal population. However, this pattern changed in the late 1980s. A study conducted in Saskatchewan, Canada, showed that asthma hospitalization rates among Registered Indian children under 4 years dramatically increased from 12.7 per 1000 in 1979 to 21.7 per 1000 in 1989 (Senthilselvan, Habbick, 1995), and this rise has been at a faster rate in the Aboriginal community compared to the rest of the population. At present, asthma appears to be one of the most common chronic diseases affecting First Nations (First Nation Regional Longitudinal Survey, 2002/2003).

Several population based surveys indicate that Aboriginal Canadians and American Indians, including children, bear a higher burden of chronic airway diseases such as asthma and COPD as well as bronchiolitis (in infants) than the general populations of Canada and the USA.

D.D. Sin et al. (2002) studied a population-based cohort of people residing in Alberta, Canada, which included 2.8 million; of these more than 100,000 were Aboriginal. They observed that Aboriginal residents were 1.6 times more likely to have office visits and 2.1 times more likely to use emergency services for asthma and COPD relative to non-Aboriginals. Aboriginal people had these higher rates of physician visits within all age groups. In the next study of this cohort over the 10 years, authors (Sin et al., 2004) obtained evidence of increased risk for emergency visits for asthma among Aboriginal children.

Canadian population-based study in Saskatchewan (Senthilselvan et al., 2003) reported that Registered Indians had greater prevalence of bronchitis compared to other populations of the Province in all age groups. Physician-diagnosed bronchitis prevalence

rate in Registered Indians schoolchildren (15.6%) was two to three times higher than those observed among non-Aboriginal rural and urban populations.

A survey conducted among American Indian and Alaska Native children in Washington State showed that Indian infants had hospitalization rates for asthma (528 per 100,000) and bronchiolitis (2954 per 100,000) two to three times higher than those of non-Aboriginals (232 per 100,000; and 1190 per 100,000, respectively) (Liu e.a., 2000).

S.A. Lowther e.a. (2000), employing U.S. Indian Health Service data, investigated bronchiolitis rates in hospitals. They found that hospitalization rates for bronchiolitis among American Indian and Alaska Native infants to be nearly double those for all U.S. children younger than 1 year (61.8 vs 34.2 per 1000). Hospitalization rate associated with asthma was also considerably higher among American Indian infants than national estimates (19.7 per 1000 vs 10.0 per 1000) (Lowther e.a., 2000).

According to the Centers for Disease Control and Prevention (USA), in 2003 bronchiolitis-associated outpatient visit and hospitalization rates for American Indian and Alaska Native children aged 0 to 5 years were more than twice the rates among US children overall.

Thus, these reports highlight a disproportionate burden of asthma and bronchiolitis in Aboriginal/Indian children relative to the general population.

1.3.1.3 Epidemiology of rheumatoid arthritis among Aboriginal population in Canada

Rheumatoid arthritis (RA) is a prevalent chronic condition in Canada. Arthritis and its associated conditions are a major cause of disability, morbidity and increased health care utilization (Badley, Wang, 1998). Native American populations have been reported to have the highest known prevalence rates for RA worldwide. Among some, the prevalence is five times higher than that of surrounding Caucasian population (Peschken, Esdaile, 1999).

According to the Canadian Community Health Survey in 2000, the prevalence of RA of Canadian Aboriginal people was 27% (almost twice as greater) compared with 16% in the non-Aboriginal population. Arthritis was the most prevalent chronic condition in the Aboriginal population. The RA seen in Native Americans is generally severe, seropositive, with an early age of onset, and frequent extra-articular manifestations (Oen e.a., 1998; Peschken, Esdaile, 1999).

Thus, epidemiological findings indicate marked ethnic disparities between First Nation and Caucasian populations in the prevalence and incidence for certain infectious, allergic, autoimmune diseases characterized by inflammation and altered immune responses. The reasons for these observed differences are poorly understood. Potential mechanisms by which ethnicity might have impact on disease susceptibility remain largely unknown and unstudied. Ethnic diversity in disease susceptibility cannot be explained entirely by socioeconomic factors. Host immune responses and inflammation might also play an important role in this variability.

It well recognized that TLRs control both innate and adaptive immunity and orchestrate inflammatory responses (Iwasaki, Medzhitov, 2004). A number of studies

demonstrating potential involvement of TLRs in disease pathogenesis are reviewed below.

1.3.2 The role of TLRs in the pathogenesis of several infectious and inflammatory diseases

1.3.2.1 The role of TLRs in tuberculosis

TLRs appear likely represent major receptors for sensing antigens of *M. tuberculosis* and activating cells of innate immunity (Stenger, Modlin, 2002; Heldwein, Fenton, 2002). Several investigators examine the role of TLRs in host resistance against tuberculosis infection in vivo using TLR gene-deficient mice.

N. Reiling e.a. (2002) revealed that TLR2-, but not TLR4-defective mice were more susceptible to high dose aerosol infection with *M. tuberculosis* than wild-type controls, suggesting that TLR2 signaling might contribute to host defense. B. Abel e.a. (2002) demonstrated that TLR4 expression is required to control chronic *M. tuberculosis* infection in mice. A. Bafica e.a (2005) showed that TLR2/9 double knockout mice exhibit greatly enhanced susceptibility to aerosol infection with *M. tuberculosis* compare to single TLR2- or TLR9-deficient animals. This illustrates that optimal host resistance requires TLR collaboration. Indeed, mice deficient in MyD88, an adaptor protein for signaling by many TLRs, have very high susceptibility to mycobacterial infection (Feng e.a., 2003; Fremont e.a., 2004; Nicolle e.a., 2004), indicating that TLR-based recognition is critical for successful protection against tuberculosis.

Many groups have shown that TLR2 in association with TLR1/TLR6 and TLR4 recognize mycobacterial components (Bulut e.a., 2001; Krutzik, Modlin, 2004). T.K. Means e.a. (1999) demonstrated that viable and killed *M. tuberculosis* bacilli (virulent and attenuated) activate CHO cells and murine macrophages that express either TLR2 or TLR4. A soluble heat-stable factor has been found to induce TLR2-dependent activation, whereas a heat-sensitive cell-associated mycobacterial factor mediated activation via TLR4. D.M. Underhill e.a. (1999) found that expression of inhibitory TLR2 on mouse macrophage cell line completely abrogated TNF-alpha production elicited by whole *M. tuberculosis*. Macrophages of TLR4 deficient mice infected in vitro with *M. tuberculosis* also secreted lower levels of TNF-alpha (Abel e.a., 2002). H.D. Brightbill e.a. (1999) demonstrated that mycobacterial 19-kDa lipoprotein triggers the production of TNF-alpha and nitric oxide by human macrophages via TLR2. Other *M. tuberculosis* lipoprotein, LprA, has been found to be effective TLR2 agonist for induction of maturation and cytokine secretion by murine dendritic cells (Pecora e.a., 2006).

The mycobacterial cell wall lipoglycan, lipomannan, also interacts with TLR2 on macrophages to induce TNF-alpha and IL-12 synthesis and nitric oxide release (Vignal e.a., 2003; Quesniaux e.a., 2004; Dao e.a., 2004). Similarly, phosphoinositol-capped lipoarabinomannan (Means e.a., 1999) and soluble tuberculosis factor (Jones e.a., 2001) and phosphatidylinositol mannosides (Gilleron e.a., 2003) of *M. tuberculosis* have been shown to activate macrophages in a TLR2-dependent fashion. B. Abel e.a. (2001) revealed that mycobacterial glycolipid, phosphatidylinositol mannoside has been able to induce cellular activation via TLR4.

M. tuberculosis DNA has been found to be a potent TLR9 stimulus for IL-12 secretion by murine DCs and macrophages. In vitro responses of these cells to live mycobacteria are also partially dependent on TLR9 (Bafica e.a., 2005). Recently, I. Fricke e.a. (2006) have demonstrated that *M. tuberculosis*/BCG triggers TLR2 on human dendritic cells to induce the production of IFN-gamma, a key cytokine controlling tuberculosis infection.

Several human studies provide evidence that polymorphisms of the TLR2 could be a risk factor for tuberculosis. P.Y. Bochud.e.a. (2003) found that the TLR2 gene polymorphism caused severe impairment of the macrophage responses in vitro to *M. tuberculosis* and *M. leprae*. M. Ben-Ali e.a. (2004) demonstrated the relationship between TLR2 Arg677Trp polymorphism and increased susceptibility to tuberculosis in Tunisian patients. Oguz e.a. (2004), examining Arg753Gln polymorphism of TLR2 among Turkish population, showed that risk of developing of tuberculosis is increased 6- and 1.6-fold for carriers of the AA and GA genotypes, respectively. The association between guanine-thymine repeat polymorphism in intron II of the TLR2 gene and the occurrence of tuberculosis has been documented in Korean population. (Yim e.a., 2006).

Thus, available evidence supports the role of TLRs (predominantly TLR2 and possibly TLR4, TLR9) in sensing *M. tuberculosis* (whole bacteria and its components) and mounting anti-mycobacterial responses in vitro and in vivo. Several human TLR2 polymorphisms are associated with enhanced susceptibility or resistance to disease.

1.3.2.2 *The role of TLRs in respiratory syncytial virus bronchiolitis*

Bronchiolitis, an acute lower respiratory tract infection, is mainly caused by respiratory syncytial virus (RSV) in infants and children. TLR4 has long been known to be key for the host defenses against bacterial infections. Recently, several studies have addressed the hypothesis that TLR4 is also able to recognize RSV and participate in antiviral immune responses.

E.A. Kurt-Jones e.a. (2000) first demonstrated that RSV F glycoprotein triggers the production of pro-inflammatory cytokines by human peripheral blood monocytes in TLR4-dependent fashion. Also, RSV has been observed to replicate to a higher level and persist longer in TLR-deficient mice relative to normal control mice (Kurt-Jones e.a., 2000).

L.M. Haynes e.a. (2001) further showed that TLR4-deficient mice infected with RSV had impaired NK cell and CD14⁺ cell pulmonary trafficking and IL-12 expression, leading to delayed virus clearance and more severe RSV bronchiolitis. Additionally, it has been demonstrated that RSV potently and specifically activates NF- κ B in vivo and this activation is substantially reduced in mice lacking a functional TLR4-signaling pathway (Haeberle e.a., 2002), again suggesting that innate immune responses to RSV might be TLR4-dependent. However, a recent reinvestigation of immunity to RSV in several mouse strains lacking a functional TLR4 generated findings that clearly argue against a role for TLR4 (Ehl e.a. 2004).

B. Puthothu e.a. (2006), genotyping two common TLR4 amino acid variants (D259G, and T359I) in 131 German infants with severe RSV associated diseases and 270 controls, found an association of haplotypes with severe RSV infection. Similarly, Tal e.a. (2004) demonstrated that these TLR4 mutations are associated with an increased risk

of severe RSV bronchiolitis in Jewish infants. R. Badolato e.a. (2004) reported the TLR4 polymorphism confers susceptibility to pneumonia in Italian children.

Mandelberg e.a. (2006) showed that hyporesponsiveness of PBMC to LPS in infants with RSV bronchiolitis appears to be a risk factor for intensive care unit hospitalization. A negative correlation has been observed between an increased expression of TLR4 on blood monocytes and minimal oxygen saturation in infants in acute phase of RSV bronchiolitis (Gargo e.a., 2004)

These observations suggest that TLR4 signaling may play a role in innate immunity to RSV and suggest its relation to the severity of RSV bronchiolitis in children.

1.3.2.3 The role of TLRs in asthma

A series of experimental studies provide evidence for a potential role of TLRs in modulating allergic responses and airway inflammation in mice (Chisholm e.a., 2004; Redecke e.a., 2004; Rodriguez e.a., 2003; Velasco e.a., 2005)

Several groups have studied the effects of TLR polymorphisms on asthma and atopy in humans. W. Eder e.a. (2004), examining TLR2 polymorphisms in 609 children living in rural areas in Germany and Austria in the ALEX study, revealed that farmers' children carrying the TLR2/-16934 T allele were less likely to have a diagnosis of asthma, current asthma and hay fever symptoms and atopy. They conclude that genetic variation in TLR2 might be a major determinant of the susceptibility to asthma and atopy in children of European farmers.

On the contrary, a study of 137 asthmatic families in Japan demonstrated that polymorphisms in TLR2 gene were not associated with either asthma or total serum IgE

levels (Noguchi e.a., 2004). K. Tantisira e.a. (2004) found that TLR6 Ser249Pro polymorphism is associated with a decreased risk of asthma in African-American population; while the same trend is not significant for European Americans.

S. Hoffjan e.a. (2005) investigated the polymorphism occurs in the TLR6 gene in 68 adults and 132 children with asthma living in Germany. They observed an association of the 249Ser allele with childhood asthma. However, no association was evident for adult asthma, possibly, due to small sample size of adult asthmatics.

M. Werner e.a. (2003) genotyped for the TLR4 mutations 334 adults who were participated in a follow-up to the European Community Respiratory Health Survey in Germany. It was found that non-carriers of D299G/T399I polymorphisms in the TLR4 gene have greater likelihood of asthma if they were exposed to high levels of endotoxin in house dust, whereas among carriers higher endotoxin exposure appears to be protective for bronchial hyperresponsiveness.

M. Fageras Bottcher e.a. (2004) reported that SNP (D299G) in the TLR4 gene are associated with a 4-fold higher prevalence of asthma and a 7-fold higher prevalence of atopic asthma in 115 Swedish school-aged children examined. The relationship between TLR4 polymorphisms (D299G, T399I) and mild form of allergic asthma has been observed among 613 Turkish asthmatic children (Sackesen e.a, 2005).

I.A. Yang e.a. (2004), examining 336 Caucasian families in UK, demonstrated that asthmatic siblings carrying the D299G TLR4 allele have increased severity of atopy, however, having TLR4 polymorphisms has not been associated with the risk of developing of asthma. In ALEX study (Eder e.a., 2004), genetic variation in TLR4/+4434 was shown to be inversely associated with specific IgE to common allergens in German

children heavily exposed to endotoxin; no clear association was seen between TLR4 SNPs and either asthma or hay fever. B.A. Raby et al. (2002), assessing five common polymorphisms in the TLR4 gene in two family-based cohorts in North America: heterogeneous U.S. cohort (589 families) and a more heterogeneous population from Quebec, Canada (167 families), found no evidence to support a significant association with asthma- and atopy-related phenotypes.

J. Cheng et al. (2004) revealed that the 6444C/T polymorphism in the TLR3 gene is likely to be associated with allergic rhinitis-related phenotypes including clinical severity, serum levels of total and allergen-specific IgE, eosinophil counts in nasal secretions among Japanese population. In contrast, another small case-control study in Japan failed to find any relationship between TLR3 gene variants and asthma susceptibility or total serum IgE levels (Noguchi et al., 2004).

A study of R. Lazarus et al. (2003) demonstrated that a polymorphism -1237T>C in the TLR9 gene is associated with increased risk for asthma in European but not African Americans. Singla et al. (2004) found the relationship between genetic variation in TLR9 and atopy: total serum IgE levels, skin-test positivity in 200 Dutch families examined.

In contrast, B. Berghofer et al. (2005) observed no association between five common TLR9 SNPs tested and markers of atopy: levels of total and specific IgE and history of atopy in 527 healthy German blood donors. Similarly, TLR9 polymorphisms are not likely to be associated with atopy-related phenotypes and asthma in Japanese population (Noguchi et al., 2004)

R. Lazarus et al. (2004) observed an association between two TLR10 SNPs, synonymous 1031G>A and non-synonymous 2322A>G, and asthma in a case-control

study of European Americans. This association was replicated in an independent cohort of asthmatic families, suggesting that TLR10 genetic variation might contribute the development of asthma.

Thus, several genetic studies have documented positive associations of SNPs in TLR2, TLR3, TLR4, TLR6, TLR9, TLR10 with asthma and atopy. However, in some other studies, no differences in the risk for asthma/atopy between carriers of TLR polymorphisms and non-carriers were evident. These inconsistent results might be due to the following reasons. First, environmental differences across the populations studied could modify genetic associations. Since the majority of genetic studies to date did not measure environmental factors (for example, endotoxin levels), they were unable to explore gene-environment interactions.

Second, the strength of association for different genes may vary across ethnic and racial populations. It has been shown that the genes encoding TLRs are highly variable in human populations. TLR4 Asp299Gly polymorphism has been observed in European American, African American and Hispanic American populations (Raby e.a., 2002), however, it has not been detected in Japanese individuals (Okayama e.a., 2002). Noguchi e.a. (2004) reported that most of the TLR2 and TLR3 allele frequencies for African Americans were significantly different from those in the Japanese. K. Tantisira e.a. (2006), sequencing TLR6 in DNA samples from African Americans, European Americans, and Hispanic Americans, noted significant differences in SNP frequencies among the three populations. TLR2 Arg677Trp polymorphism has been reported among Koreans (Kang e.a., 2002) and Tunisians (Ben-Ali e.a., 2002) does not occur among Germans (Schroder e.a., 2003). Therefore, the association between TLR polymorphism

and asthma/atopy might be ethnic-group specific. This notion is generally in agreement with some studies discussed above (Lazarus e.a., 2003; Tantisira e.a., 2004).

A key point relevant to virtually all this literatures is that functional implications of TLR gene variants (i.e. changes in intermediate phenotypes linking a particular TLR SNP and altered cytokine and chemokine production) to the development of asthma/atopy are yet undefined. The associations reported attempt to link genetic differences with clinical end-points independent of the many factors in between.

1.3.2.4 The role of TLRs in rheumatoid arthritis

A number of investigations obtained evidence for possible involvement of TLRs in the pathogenesis of RA. R. Seibl e.a. (2003), using in situ hybridization and immunohistochemical analysis, revealed intense TLR2 expression in synovial tissues from RA patients predominantly at sites of attachment and invasion into cartilage and bone. Similarly, T.R. Radstake e.a (2004) demonstrated elevated expression of both TLR2 and TLR4 in RA synovial tissue compared to those seen in patients with osteoarthritis and healthy controls. Also, TLR3 (Brentano e.a., 2005; Roelofs e.a., 2005) and TLR7 (Roelofs e.a., 2005) were expressed at higher levels in synovium of RA versus osteoarthritis patients. Several studies have shown that TLR agonists have the capacity to up-regulate the expression of TLRs and cytokines/chemokines in synovial fibroblasts and monocytes/DCs obtained from RA patients in vitro.

R. Seibl e.a. (2003) observed an increased expression of TLR2 mRNA in RA synovial fibroblasts after stimulation with synthetic bacterial lipopeptide, a TLR2 ligand. Study by D. Kyburz e.a (2003) demonstrated that PGN is able to activate synovial

fibroblasts of RA patients to enhance the expression of mRNA for TLR2, integrins, matrix metalloproteinases, and synthesis of proinflammatory cytokines, IL-6 and IL-8. In contrast, CpG ODNs had no stimulatory effect: TLR9 mRNA expression remained at baseline levels.

TLR2 and TLR4 agonists have been found to increase the production of TNF-alpha, IL-6, IL-8 in synovial membrane cell cultures from RA tissue (Sacre e.a., 2007).

Y.O. Jung e.a. (2007) reported that PGN induces IL-15 synthesis by fibroblast-like synoviocytes of RA patients. On the contrary, LPS did not increase the level of IL-15 although it augmented the stimulatory effect of PGN on IL-15 production.

M. Pierer e.a. (2004) demonstrated a strong up-regulation of gene expression and protein synthesis of various CC and CXC chemokines such as MCP-1, MCP-2, RANTES, GCP-2, IL-8, GRO-2 by RA synovial fibroblasts in response to TLR2 ligands.

F. Brentano e.a. (2005) found that TLR3 agonist has been an effective stimulator of cytokine/chemokine production. Stimulation of cultured RA synovial fibroblasts with poly(I:C) resulted in the secretion of high levels of IFN-beta, CXCL10, CCL5 and IL-6. These cytokines were induced to a lesser extent by LPS, whereas Pam3CSK4, a TLR2 ligand, induced only CCL5 (Brentano e.a., 2005).

M. Frasnelli and A. So (2005) found elevated expression of TLR2 and TLR4 on monocytes derived from RA patients compare to controls. Monocyte-derived DCs from individuals with RA exhibited increased production of pro-inflammatory, IL-6, TNF-alpha, and anti-inflammatory IL-10 cytokines in response to lipoteichoic acid or LPS, TLR2 and TLR4 ligands, respectively. In contrast, cytokine responses to TLR3/poly(I:C) and TLR7/8/R848 agonists were equivalent among RA patients and healthy controls.

Together, these data suggest that TLR-dependent mechanisms might contribute to the activation of synovial cells (and monocytes), leading to RA induction and/or maintenance

A series of studies have investigated the possibility whether the TLR polymorphisms contribute to disease susceptibility in patients RA. E.Y. Lee e.a. (2006) demonstrated that the genotype containing shorter GT repeats in intron II of the TLR2 gene may confer susceptibility to RA in Korean population. E. Sanchez e.a. (2003) examined 224 Spanish patients with RA and a control group of 199 healthy individuals. They demonstrated that polymorphisms of TLR2 (Arg677Trp and Arg753Gln) and TLR4 (Asp299Gly and Thr399Ile) genes do not influence the susceptibility or severity of RA. In contrast, T.R. Radstake e.a (2004) observed an association between RA disease susceptibility and the common TLR4 gene variant, Asp299Gly, in a cohort of 282 White individuals with RA and 314 healthy subjects in the Netherlands.

Thus, available data regarding the effect of TLR polymorphisms on the risk of the development of RA point to a possible ethnic-group specific association between TLR gene variants and clinical phenotype. This possibility obviously requires future studies.

To our knowledge, possible ethnic differences in TLR functional responsiveness between distinct populations have not been studied so far.

In summary, extensive epidemiologic studies clearly indicate that susceptibility to certain diseases is dramatically influenced by ethnicity. The prevalence of tuberculosis, bronchiolitis asthma, rheumatoid arthritis and other chronic inflammatory diseases is markedly different among First Nation and Caucasian populations in Canada. Toll-like receptors contribute to development of immune protection and several infectious or

inflammatory conditions. The prevalence and nature of possible ethnic differences in the function of TLRs remain unknown. We developed the hypothesis that functional responsiveness to TLR stimulation by threshold concentrations of ligands differs in First Nation vs. Caucasian children.

1.4 Hypotheses tested in the study

TLRs are major receptors of innate immunity and play critical role in regulating the nature of the adaptive immune response that subsequently develops. Alterations in TLR responsiveness in distinct individuals as a result of differences in environmental exposures (exposures to ETS) and/or ethnicity (First Nations vs. Caucasians) may influence their likelihood of exhibiting asthmatic clinical phenotype.

In the present study, we developed hypotheses to test as follows

- (i) Functional responsiveness to TLR stimulation differs in asthmatic vs non-atopic children;
- (ii) ETS exposures affect TLR responsiveness in asthmatic children;
- (iii) Functional responsiveness to TLR stimulation differs in First Nation vs. Caucasian children.

In order to increase sensitivity we tested the hypothesis of a novel approach of using “threshold” concentrations of TLR ligands (more closely approximate natural environmental levels) than widely utilized “maximal” stimulation conditions.

2 MATERIALS AND METHODS

2.1 Study participants

This study was approved by the University of Manitoba Faculty Committee on Use of Human Subjects in Research. In collaboration with Dr. Allan Becker and Dr. Anita Kozyrskyj, written informed parental consent was obtained for 723 children (of which 272 were randomly recruited for detailed immunological analysis) within a general population survey case-control cohort. This 1995 Manitoba Birth Cohort (SAGE, Study of Allergy Genes and Environment) was created from the provincial health care registry. It includes 16,320 live births in Manitoba that year. Of these, 2340 children moved out of the province or died over the next seven years and were excluded from the cohort at the time of its construction. A one-page survey was mailed out to the households of all children to inquire about their health and home environment exposure and for permission to be contacted for future study. From the returned surveys, children were stratified according to the diagnosis of asthma ($n = 392$), to the diagnosis of allergic rhinitis or food allergy ($n = 192$) or neither ($n = 3002$). All children in the asthma and allergy strata were enrolled in the case-control study, as were a random sample ($n = 200$) from the strata of children with neither condition. Consented children and parents came to the Paediatric Allergy clinic in Winnipeg or mobile Paediatric Allergy clinic in their community (over a 2 year period) for the detailed assessment used to characterize the subjects studied in this publication.

The candidate's component of SAGE study was conducted with 272 randomly recruited children at 8-9 years of age.

Two hundred twenty nine participants were Caucasian children. Among them 85 were non-atopic healthy subjects, 29 were asymptomatic allergics, 68 had allergic asthma and 47 were non-allergic asthmatics.

Forty-three participants were First Nation children All of them were non-atopic healthy individuals.

The diagnosis of allergic asthma was established on the basis of a clinical history of asthma symptoms as well as airway hyperresponsiveness to methacholine (PC20 < 2 mg/ml)

Atopy was defined as one or more positive epicutaneous test reactions (wheal diameter > 3 mm that of a negative control) to a panel of 14 common environmental allergens tested (grass, weed, ragweed, tree pollens; Alternaria, Cladosporium, Penicillium; D. pteronyssinus, D. farinae; cat and dog extracts, cockroach and feather) and to peanut allergens.

Allergic asthmatic children had a history of asthma diagnosed by a specialist physician (Dr. A. Becker or Dr. J. Liem) and exhibited one or more positive skin prick tests. Non-allergic asthmatics were children with established diagnosis of asthma and negative skin prick test. Asymptomatic allergics were identified based upon positive skin prick test and absence of history of allergic diseases. Non-atopic healthy children had no history of asthma or other allergic disorders and demonstrated negative skin prick test to the panel of allergens tested.

None of the study participants had received allergen immunotherapy, had taken anti-inflammatory medication such as inhaled or oral corticosteroids within the previous two months, or anti-histamines, beta2-agonists within three days prior to blood collection.

All of the subjects were free from symptomatic respiratory infections in the two months preceding the recruitment for the study.

2.2 Isolation of Human Peripheral Blood Mononuclear Cells (PBMC)

Peripheral whole blood (20 ml) was taken by venipuncture into tube containing 1 ml of 2.7% EDTA, mixed immediately, and diluted in saline (by adding 10 ml saline to 20 ml blood) then mixed. Diluted blood (10 ml) was layered onto a 3.5 ml Ficoll gradient (Histopaque-1077; Sigma, St. Louis, MO) and centrifuged at 460 x g for 30 min (Eppendorf Centrifuge 5804) to isolate mononuclear cells from polymorphonuclear cells, red blood cells and platelets. PBMCs, the “buffy coat” of cells, were carefully collected from the interface between plasma and Ficoll and washed twice in saline.

Isolated PBMCs were resuspended in 2 ml of complete media (RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 10 mM L-glutamine, 2x10⁻⁵ M 2-mercaptoethanol (ME), and antibiotic-antimycotic (Life Technologies, Burlington, Canada)), counted by hemocytometer and used immediately for culture. Cell viability was determined by trypan blue exclusion. >99% viability is obtained in all samples collected.

2.3 PBMC culture

Cells were cultured in duplicate at 350,000 cells/well in 200 µl using 96-well U-bottom plates (Nunc, Roskilde, Denmark) in complete media (in RPMI 1640 supplemented with 10% heat-inactivated FCS, 10 mM L-glutamine, 2x10⁻⁵ M 2-ME, and antibiotic-antimycotic) at 37°C with 5% CO₂.

For each subject, cell cultures were established

- 1) in the absence of stimuli (medium control);
- 2) with lipopolysaccharide from *Escherichia coli* serotype 00011:B4 from Sigma (St. Louis, MO);
- 3) with peptidoglycan from *Staphylococcus aureus* (Fluka Biochemica, Switzerland);
- 4) with 3M-011 compound (3M Pharmaceuticals, St. Paul, MN);
- 5) with poly (I:C) (Amersham, Biosciences, NJ);
- 6) with CpG ODN 2216 (InvivoGen, San Diego, CA)

Culture supernatants were harvested and stored at -20°C until quantification of cytokine and chemokine levels.

Optimal culture conditions (time of cell harvest; maximal and threshold TLR ligand concentrations used) were determined in preliminary experiments (see Results: Optimization of experimental system).

In blocking experiments, PBMC were pre-incubated with 10 µg/ml of anti-human TLR4 mAb (clone HTA 125; eBioscience, San Diego, CA) for 30 min before addition of stimulants at maximal concentrations. As isotype-matched negative control normal mouse IgG2a (10 µg/ml, eBioscience, San Diego, CA) was included in parallel cultures.

2.4 ELISA: analysis of human cytokine and chemokine levels

Human cytokine and chemokine levels in culture supernatants were quantified using specific sandwich ELISAs for IL-6, IL-1beta, TNF-alpha, CCL2 (pro-inflammatory cytokines and chemokine); IL-10 (anti-inflammatory cytokine); IFN-gamma, CXCL9, CXCL10 (Th1-associated cytokine and chemokines); CCL17, CCL22 (Th2-associated chemokines).

Recombinant human cytokines/chemokines and matched pair anti-cytokine/chemokine and biotinylated anti-cytokine/chemokine antibodies were obtained from BioLegend (San Diego, CA), Antibody Solutions (Mountain View, CA), Peprotech Canada Inc. (Ottawa, ON), BD PharMingen (San Diego, CA), and R&D Systems (Minneapolis, MN) (Table 5).

Table 5. Suppliers of ELISA reagents used in the study

| Cytokine/Chemokine | Capture Ab | Detection Ab | Standards |
|--------------------|--------------------|--------------------|---------------|
| IL-6 | BioLegend | BioLegend | BioLegend |
| IL-1 beta | Antibody Solutions | Antibody Solutions | Peprotech |
| TNF-alpha | BioLegend | BioLegend | Peprotech |
| IL-10 | BioLegend | BioLegend | BD PharMingen |
| IFN-gamma | BD PharMingen | BD PharMingen | BD PharMingen |
| CCL2 | BD PharMingen | BD PharMingen | BD PharMingen |
| CXCL9 | R&D Systems | R&D Systems | Peprotech |
| CXCL10 | BD PharMingen | BD PharMingen | Peprotech |
| CCL17 | R&D Systems | R&D Systems | Peprotech |
| CCL22 | R&D Systems | R&D Systems | R&D Systems |

To measure human cytokine and chemokine levels we employed the following ELISA protocol.

1. Coating. The ELISA plate was coated with specific capture antibody (50 ul/well) in coating buffer at an optimal concentration pre-determined in preliminary experiments (see Results: Optimization of cytokine ELISAs), then wrapped to prevent evaporation, and incubated overnight at 4°C to allow complete binding.

2. Blocking. The capture antibody solution was removed, and blocking was performed by adding blocking buffer (75 ul/well) followed by incubating for 3 hours at 37°C. Wash four times using automatic plate washer.

3. The standard curve was created on each assay plate using recombinant human cytokine/ chemokine standard at known concentration in dilution buffer. The standard was titrated in eight two-fold dilutions. The dilution range was determined by expected levels of cytokine/chemokine in experimental samples.

4. Each sample was titrated in dilution buffer in four two-fold dilutions to accurately quantify the concentration of cytokine/chemokine. The starting dilution was determined by anticipated levels of cytokine/chemokine in samples tested (1:5 -1:10 for most cytokines/chemokines tested; 1:80 for CCL2). Afterwards, assay plates were wrapped and incubated overnight at 4°C. Wash four times using automatic plate washer.

5. Detection was carried out by adding biotinylated detection antibody (50 ul/well) in dilution buffer at an optimal concentration pre-determined in preliminary experiments (see Results: Optimization of cytokine ELISAs), followed by overnight incubating at 4°C. Wash four times using automatic plate washer.

6. Streptavidin-alkaline phosphatase (Jackson ImmunoResearch, Mississauga, ON) in dilution buffer was added at 1:3000 (50 ul/well), and plates were incubated for 45 minutes at 37°C. Following washing four times, p-nitrophenyl phosphate solution (Sigma, Oakville, ON) prepared within 20 min of use by dissolving one tablet of PNPP in 5 ml of substrate buffer was added (50 ul/well).

7. The assay plate was read at 405-690 nm employing microtiter plate reader (Molecular Devices, Sunnyvale, CA).

8. A standard curve was constructed from the data produced by serial dilutions of the standard, plotting the cytokine/chemokine concentration on the x axis (log scale) and the absorbance on the y axis (linear scale) to generate a sigmoidal curve.

Representative concentration of cytokine/chemokine protein in each sample is calculated by averaging concentrations from serial dilutions, typically 3-4, that fall on the linear part of the standard curve and are within acceptable range (variation less than 10%).

2.5 Transfection and reporter assays

Human embryonic kidney (HEK) 293 cells (293/TLR clones) stably expressing a given human TLR gene were utilized in transfection experiments which were carried out in order to assess the specificity and purity of TLR ligands used. These cells obtained from InvivoGen (San Diego, CA) were as follows:

1. HEK 293 cells stably transfected with the plasmid (pDUO-hTLR4-MD2-CD14) that constitutively expresses human TLR4A and MD2/CD14
2. HEK 293 cells stably transfected with the plasmid (pUNO-hTLR3) that constitutively expresses human TLR3;
3. HEK 293 cells stably transfected with the plasmid (pUNO-hTLR8) that constitutively expresses human TLR8;
4. HEK 293 cells stably transfected with the plasmid (pUNO-hTLR9) that constitutively expresses human TLR9

Each human 293/TLR clone was grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FCS, 1% L-glutamine, blasticidin (100 ug/ml) and hydrogold (100 ug/ml) (InvivoGen, San Diego, CA) in tissue culture flasks (T-75) maintained at 37°C in 5% CO₂. The confluent cells were detached by 0.05% trypsin and passed 2-3 times a week. Cells with low passage number were used for experiments.

The day before transfection, cells were seeded in 24-well tissue culture plates. Before seeding, the plates were incubated with poly-L-lysine solution (0.5 ml/well) for 30 min at 37°C to enhance cell attachment, then washed twice with PBS. Cells were seeded at a density of 3×10^5 cells per well and incubated overnight at 37°C in 5% CO₂.

After 24 hours, cells were washed and transfected with reporter plasmid by adding 0.21 µg/well of pNiFty-SEAP reporter plasmid (InvivoGen, San Diego, CA) mixed with 1.25 ml/well of FuGENE6 transfection reagent (Roche Diagnostics Corp., Indianapolis, IN). Cells were then incubated overnight at 37°C in 5% CO₂.

The following day, stimulation of transfected 293 HEK/hTLR cells with tested samples (potential TLR ligands at “maximal” doses we used in PBMC cultures) was performed. Each cell clone was cultured for an additional 24 hours

- 1) in the absence of stimuli (medium control);
- 2) with lipopolysahharide from *Escherichia coli* serotype 00011:B4 (Sigma, St. Lois, MO) at 5 ng/ml;
- 3) with peptidoglycan from *Staphylococcus aureus* (Fluka, Biochemica, Switzerland) at 4 µg/ml
- 4) with 3M-011 compound (3M Pharmaceuticals, St. Paul, MN) at 5 µg/ml
- 5) with poly(I:C) (Amersham, Biosciences, NJ) at 125 µg/ml
- 6) with CpG ODN 2216 (InvivoGen, San Diego, CA) at 3 µg/ml

Culture supernatants were then harvested and SEAP reporter gene activity was measured using a commercial SEAP Reporter Assay kit (InvivoGen, San Diego, CA) according to manufacturer’s protocol.

2.6 Limulus Amoebocyte Lysate assay

Peptidoglycan, 3M-011 compound, poly(I:C), CpG ODN 2216 preparations used in the study were tested for endotoxin contamination employing the Limulus Amoebocyte Lysate assay (Associates of Cape Cod Inc., Falmouth, MA) according to the manufacturer's protocol. Detection limit is 1 pg endotoxin/mg of preparation.

2.7 Statistical analysis

All statistical analyses were carried out using Graph Pad Prism 3 software. (version 3.02 for Windows, GraphPad Software, San Diego CA). Since the data were normally distributed in almost all cases, the data were expressed as mean \pm SEM. Statistical significance of differences in cytokine levels between the groups was determined using an unpaired two-tailed t test. Values of $p < 0.05$ was considered as statistically significant.

3 RESULTS

3.1 Assessment of the specificity and purity of TLR ligands used

The immune system has evolved to detect very small amounts of microbial products to sense microbes. It is well known that bacterial cell wall components such as LPS (TLR4 ligand) and lipoproteins (TLR2 ligands) are extremely potent immuno-activators, and accordingly, TLRs can be activated by very low levels of these agonists. Lipopolysaccharide at a concentration of 0.1 ng/ml has been shown to be sufficient to maximally induce TNF- α release from murine macrophages (Tsan, Gao, 2004). Some variants of lipoproteins exhibited half-maximal stimulation of macrophages at levels as low as 3 pM in vitro (Muhlradt e.a., 1998; Hirschfeld e.a., 2000). Therefore, the presence of these highly active contaminants in widely used commercial preparations of TLR ligands might be a factor confounding analysis of cellular immune responses.

We will provide an illustrative example that exists in the literature to show how contaminants can lead to misleading conclusions and to highlight the importance of employing highly purified preparations (e.g. TLR ligands) free of contamination in one's experiments.

Extensive research over the last decade has focused on the identification of Toll-like receptors and TLR ligands. In 1998, using commercial preparation of LPS, two groups first reported that human TLR2 conferred responsiveness to LPS and could be a direct mediator of LPS signaling (Kirschning e.a., 1998; Yang e.a., 1998), thus, initially TLR2 (in addition to TLR4) was implicated in recognizing of LPS.

However, a year later several studies examining LPS responses in TLR2-deficient mice demonstrated that TLR2 is not required for LPS signaling when TLR4 is present (Heine e.a., 1999; Takeuchi e.a., 1999; Underhill e.a., 1999). M. Hirschfeld e.a. (2000) then found that re-purification of commercially available LPS preparations eliminated their ability to signal via human and murine TLR2, suggesting that contaminants present in those preparations responsible for TLR2-mediated signalling. A study by H.K. Lee e.a. (2003) identified these contaminants as lipoproteins, well-established TLR2 agonists (Aliprantis e.a., 1999) possessing extremely potent bioactivity (Muhlradt e.a., 1998; Hirschfeld e.a., 2000).

Thus, failure to recognize the presence of lipoproteins in the commercial LPS preparation(s), resulted in to the erroneous attribution of lipoprotein-sensing TLR2 as the LPS signal transducer (Hirschfeld e.a., 2000; Kirschning e.a., 1998; Yang e.a., 1998).

It is therefore very important to exclude completely the confounding effects of highly bioactive contaminants in commercial TLR agonist preparations and ensure that these preparations act specifically through TLRs to induce immune activation.

To assess the specificity and purity of TLR ligand preparations employed in our study we conducted 1) transfection and reporter assays; 2) experiments using blocking monoclonal antibodies in primary PBMC cultures; 3) Limulus Amebocyte Lysate assay to test for endotoxin contamination.

3.1.1 Transfection experiments to test specificity and purity of TLR ligands used

Transfection studies were carried out in collaboration with Alex Silaghi and Dr. Steven Jones (Immunology Unit, Special Pathogens and Zoonotics, National Microbiology Laboratory, Winnipeg, Manitoba, Canada).

In these experiments, we utilized human HEK293 cells stably expressing a given human TLR: TLR4-MD2-CD14, TLR8, TLR3, or TLR9. Each 293/TLR clone was transfected with pNiFty reporter plasmid followed by the addition of TLR ligands including LPS, PGN, 3M-011 compound, poly(I:C), CpG ODN 2216. After 24 hours of exposure, secreted embryonic alkaline phosphatase (SEAP) activity that reflected the levels of NF-kB activation was measured in culture supernatants.

As shown in Figure 1, stimulation of TLR4-MD2-CD14-expressing HEK 293 cells with LPS elicited strong NF-kB activation with 7.8-fold increase compared to that seen in unstimulated control cells. In contrast, TLR4-MD2-CD14 transfectants did not respond to any of the other TLR ligands tested. These data indicate that LPS utilized in this study acts directly via TLR4-MD2-CD14 complex as truly TLR4 ligand. Additional TLR agonists employed in our experiments, PGN, 3M-011 compound, poly(I:C), CpG ODN 2216, are not contaminated with LPS. (While poly(I:C) triggered SEAP release, this activation of TLR4-MD2-CD14 expressing HEK 293 cells was probably non-specific; very high concentration of poly(I:C) (125 ug/ml) used may account for these results).

HEK 293 cells expressing TLR8 exhibited a 18.5-fold increase in SEAP activity in response to 3M-011 (Figure 2). No NF-kB activation was observed in these cells after exposure to the other TLR ligands examined. This shows that 3M-001 compound is a

potent TLR8 stimulant capable of activating NF- κ B via TLR8 while LPS, PGN, poly(I:C), CpG ODN 2216 are devoid of TLR8-stimulating activity.

As evident from Figure 3, poly(I:C) induced marked NF- κ B-dependent SEAP production with a 51.9-fold increase over medium control in transfectants expressing TLR3, demonstrating that poly(I:C) directly activates TLR3. None of the other TLR agonists tested (LPS, PGN, 3M-011 compound, CpG ODN) have the ability to mediate TLR3 responses.

TLR9-expressing HEK 293 cells responded only to CpG ODN 2216 stimulation. SEAP activity was 5.2-fold higher relative to that seen in unstimulated control cells (Figure 4). These data document that CpG ODN 2611 serves as a ligand of TLR9. Exposures to LPS, PGN, 3M-011 compound, or poly(I:C) do not lead to NF- κ B activation in TLR9 transfectants, indicating the absence of contaminating TLR9 agonists in these preparations.

Collectively, the data of transfection experiments show that commercial preparations of TLR ligands utilized in our study specifically activate corresponding human TLRs expressed in transfected HEK 293 cells and are not contaminated with additional TLR agonists.

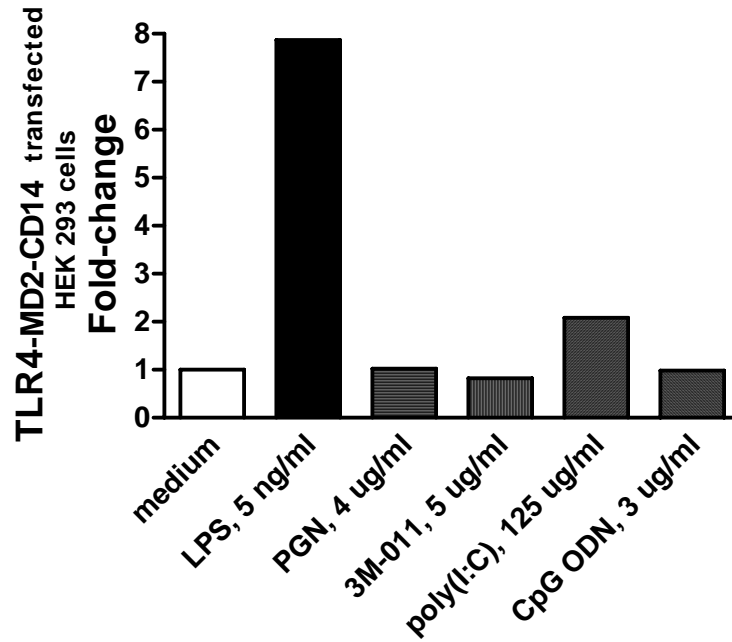


Figure 1. Human TLR4-MD2-CD14 transfectants respond to LPS, but are not activated by PGN, 3M-011 compound, poly(I:C) or CpG ODN 2216

HEK293 cell clones stably expressing TLR4, MD2 and CD14 were transfected overnight with pNiFty-SEAP reporter plasmid. Cells were then stimulated with indicated concentrations of either LPS, PGN, 3M-011 compound, poly(I:C), CpG ODN 2216 or medium alone (control) for 24 hours. SEAP activity was measured in culture supernatants using a SEAP reporter assay kit. Data are expressed as fold-change relative to control, which is arbitrarily set as 1.0, and are representative of two separate experiments

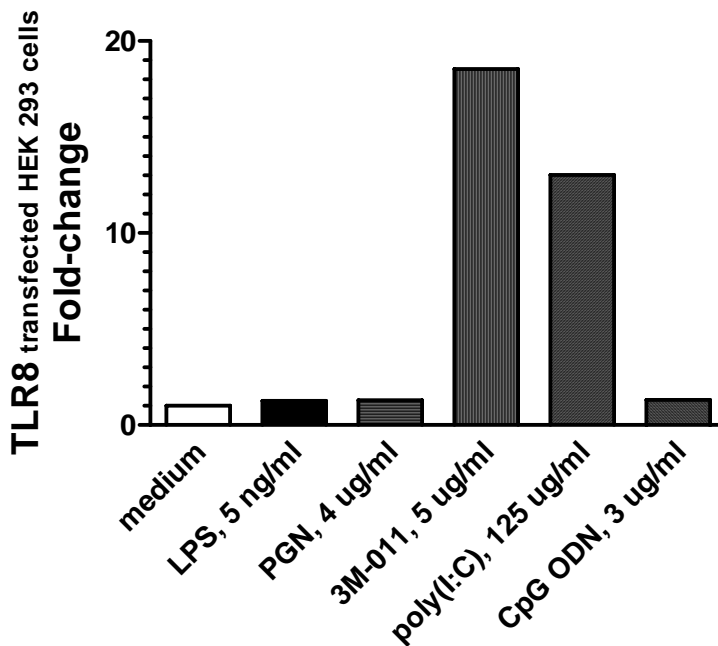


Figure 2. 3M-011 compound is a potent activator of TLR8-expressing HEK 293 cells whereas these transfectants are unresponsive to LPS, PGN, poly(I:C), or CpG ODN 2216 exposures

HEK293 cell clones stably expressing TLR4, MD2 and CD14 were transfected overnight with pNiFty-SEAP reporter plasmid. Cells were then stimulated with indicated concentrations of either LPS, PGN, 3M-011 compound, poly(I:C), CpG ODN 2216 or medium alone (control) for 24 hours. SEAP activity was measured in culture supernatants using a SEAP reporter assay kit. Data are expressed as fold-change relative to control, which is arbitrarily set as 1.0, and are representative of two separate experiments

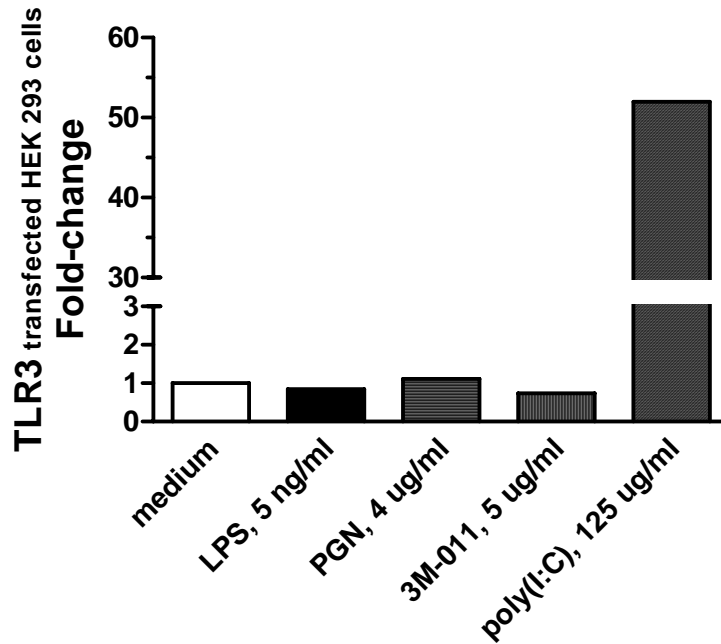


Figure 3. TLR3-expressing HEK 293 cells are activated by poly(I:C), but do not respond to LPS, PGN, 3M-011 compound

HEK293 cell clones stably expressing TLR4, MD2 and CD14 were transfected overnight with pNiFty-SEAP reporter plasmid. Cells were then stimulated with indicated concentrations of either LPS, PGN, 3M-011 compound, poly(I:C) or medium alone (control) for 24 hours. SEAP activity was measured in culture supernatants using a SEAP reporter assay kit. Data are expressed as fold-change relative to control, which is arbitrarily set as 1.0.

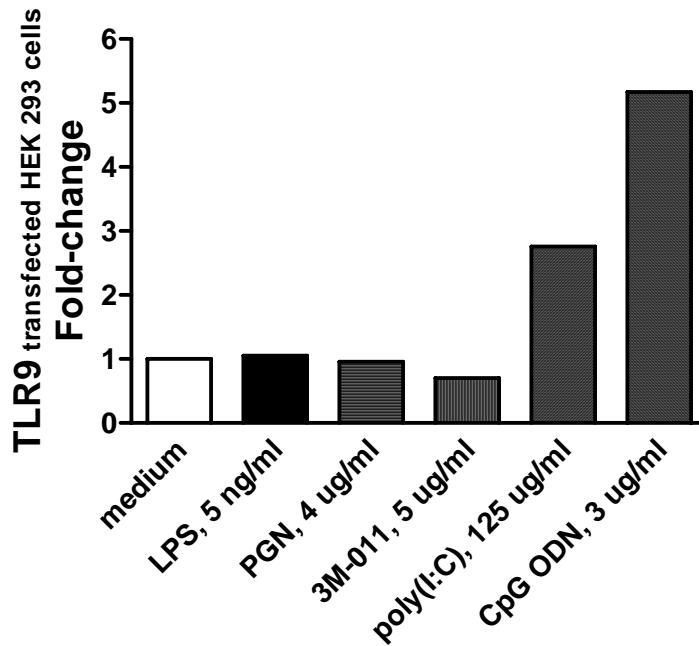


Figure 4. Human TLR4 mediates NF- κ B activation in HEK 293 cells upon stimulation with CpG ODN 2216, but is not activated by LPS, PGN, 3M-011 compound, or poly(I:C)

HEK293 cell clones stably expressing TLR4, MD2 and CD14 were transfected overnight with pNiFty-SEAP reporter plasmid. Cells were then stimulated with indicated concentrations of either LPS, PGN, 3M-011 compound, poly(I:C), CpG ODN 2216 or medium alone (control) for 24 hours. SEAP activity was measured in culture supernatants using a SEAP reporter assay kit. Data are expressed as fold-change relative to control, which is arbitrarily set as 1.0.

3.1.2 Experiments using blocking anti-TLR4 antibody in primary PBMC cultures

To confirm that in primary human PBMC cultures 1) cytokine responses to LPS are mediated specifically via TLR4 and 2) cytokine responses to PGN are not due to contamination with LPS (a TLR4 ligand), we carried out blocking experiments using anti-TLR4 mAb.

PBMC from six subjects were cultured with anti-TLR4 mAb, or an isotype-matched negative control antibody, for 30 min and then stimulated with LPS or PGN for 24hours.

As evident from Figure 5, anti-TLR4 mAb dramatically inhibited LPS-induced production of IL-1beta, TNF-alpha, IL-10 by 82%, 88%, 85%, respectively. This indicates that LPS-elicited cytokine responses are dependent on TLR4 mediated recognition..

In marked contrast, blocking of TLR4 did not affect PGN-stimulated synthesis of IL-1beta, TNF-alpha, IL-10 (Figure 6), showing that immune activation by PGN is not attributable to LPS contamination. Thus, blocking experiments demonstrated the specific ability of the LPS preparation used in this study to activate human PBMC through TLR4 as well as the lack of contaminating LPS in the PGN preparation employed in our experiments.

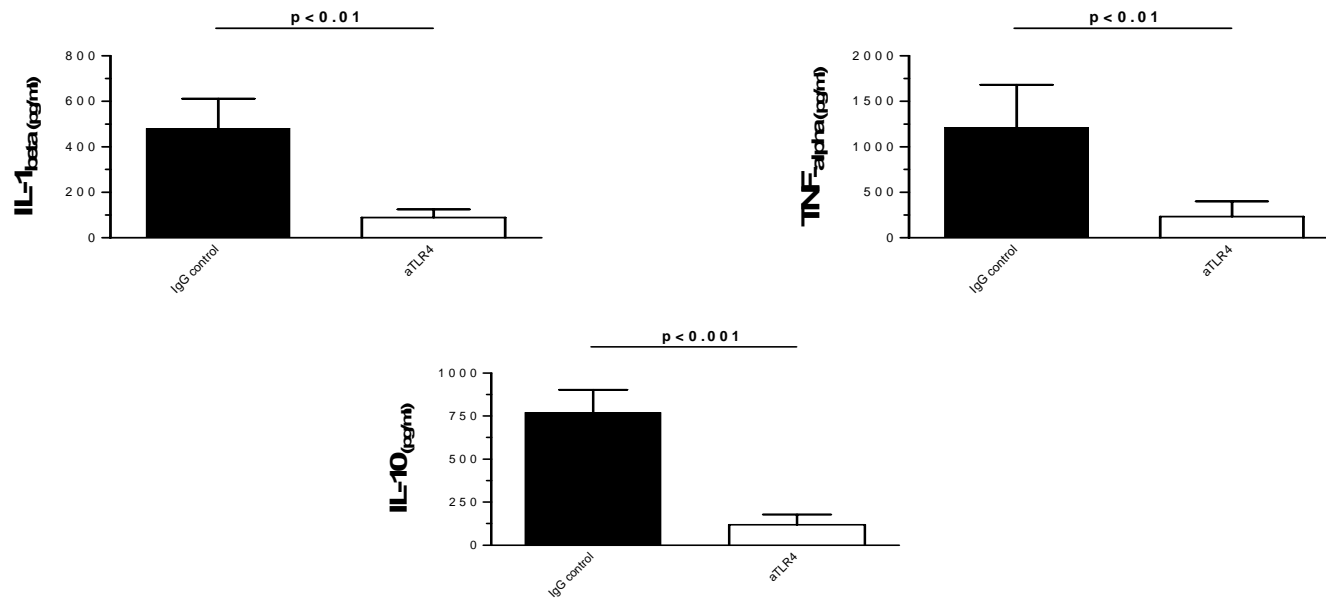


Figure 5. LPS-stimulated pro-inflammatory and anti-inflammatory cytokine responses are TLR4-dependent and are markedly inhibited by anti-TLR4 antibodies

PBMC from 6 subjects were pre-incubated with anti-human TLR4 mAb (10 μ g/ml) or mouse IgG2a isotype-matched negative control for 30 min and then stimulated with LPS at 5 ng/ml for 24h. Levels of IL-1 β , TNF- α , and IL-10 in culture supernatants were measured by specific ELISA.

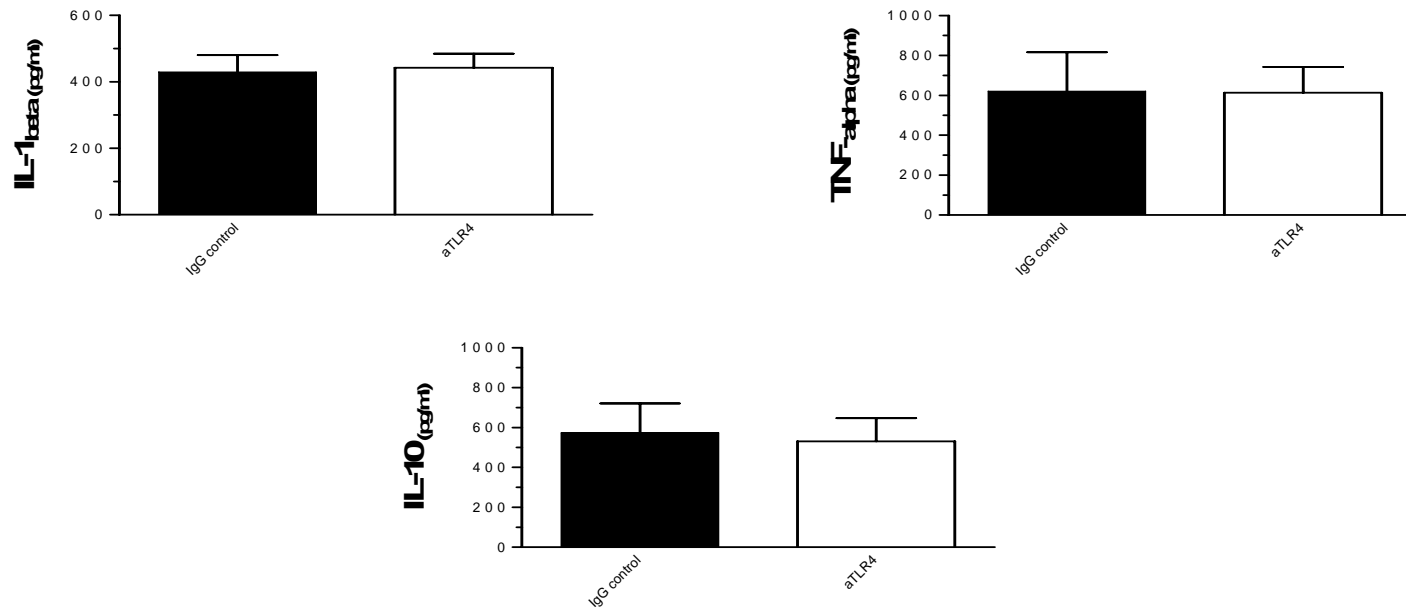


Figure 6. PGN-induced production of pro-inflammatory and anti-inflammatory cytokines is not affected by blocking of TLR4. PBMC from 6 subjects were pre-incubated with anti-human TLR4 mAb (10 ug/ml) or mouse IgG2a isotype-matched negative control for 30 min and then stimulated with PGN at 1 ug/ml for 24h. Levels of IL-1beta, TNF-alpha, and IL-10 in culture supernatants were quantified by specific ELISA.

3.1.3 Limulus Amoebocyte Lysate (LAL) assay to test for endotoxin contamination

LPS/endotoxin is known to be an extremely active immune-stimulant and consequently endotoxin contamination can play a confounding role in the analysis of cytokine and chemokine responses. Therefore, we utilized the Limulus Amoebocyte Lysate assay to test TLR (2, 3, 8, 9) ligand preparations used in the study for endotoxin contamination. These experiments were conducted in collaboration with Tina Thottingal (Dr. HayGlass's laboratory, Department of Immunology, University of Manitoba).

The results of LAL assay indicate that endotoxin levels are undetectable (<1 pg per 1 mg) in preparations of peptidoglycan, 3M-011 compound, poly(I:C), CpG ODN 2216.

Taken together, the data of these experiments confirm the specificity and purity of the TLR ligands used in the study.

3.2 Optimization of cytokine ELISAs

The main goal of this study is to identify potential differences in functional responsiveness to TLR stimulation between clinically and ethnically distinct pediatric populations.

At present, it is well established that TLR recognition of molecular patterns associated with microbes (TLR ligands) leads to the activation of signaling pathways that generate robust and rapid pro-inflammatory and anti-inflammatory cytokine and chemokine responses (Iwasaki, Medzhitov, 2004). The type of cytokine/chemokine profile has been shown to be a key (polarizing) signal responsible for the regulation of the nature of subsequent adaptive immune responses (Kapsenberg, 2003).

Hence, quantitative analysis of a broad profile of cytokines and chemokines produced by immune cells in response to TLR stimulation is a reliable method to evaluate TLR functional responsiveness. In the present study, we utilize highly specific and sensitive ELISAs capable of accurately quantifying cytokines and chemokines that could be potentially produced by human PBMCs upon stimulation with TLR ligands used. These included ELISAs for pro-inflammatory cytokines (IL-6, IL-1beta, TNF-alpha) and chemokine (CCL2), anti-inflammatory cytokine (IL-10), Th1-associated cytokine (IFN-gamma) and chemokines (CXCL9, CXCL10), and Th2-associated chemokines (CCL17, CCL22).

The candidate carried out optimization experiments to develop specific and sensitive sandwich ELISAs for the measurement of IL-6, IL-1beta, TNF-alpha, and IL-10. Additional cytokine and chemokine ELISAs we employed in the study were

previously optimized by Bill Stefura, Renee Douville, Tina Thottingal (Dr. HayGlass's laboratory, Department of Immunology, University of Manitoba).

The optimization of cytokine ELISA was conducted as follows:

We used paired purified anti-human cytokine mAb as capture antibody and biotinylated anti-human cytokine polyclonal Ab as detection antibody with human recombinant cytokine as the reference standard.

In order to determine optimal antibody concentrations which provide maximal sensitivity coupled with low background we tested a range of concentrations of both capture and detection antibodies. The ELISA is performed in accordance with the same basic protocol.

The assay was set up using three decreasing amounts of capture antibody, including one recommended by the manufacturer and two additional two-fold lower concentrations. Recombinant human cytokine was added to each lane (row A) at known concentration and titrated in seven two-fold dilutions. The control lane (row H) did not receive recombinant human cytokine and represented background. The detection antibody was then added to the plate in four decreasing amounts (two fold dilutions) ranging from that recommended by the manufacturer. The optimal concentration of capture antibody and detection antibody demonstrate low background and a large range of absorbance values for the increasing amounts of standard providing a linear standard curve. Optimal antibody concentrations, standard curves, and assay sensitivity of cytokine and chemokine ELISAs employed in the study are shown in Table 6.

Table 6. ELISA: antibody optimal concentrations, standard curve and assay sensitivity

| Cytokine /Chemokine | Optimal concentration | | Standard curve | *Assay sensitivity |
|---|-----------------------------|------------------------------|-----------------|-----------------------|
| | Capture Ab | Detection Ab | | |
| IL-6 | 0.4 ug/ml (BioLegend) | 0.125 ug/ml (BioLegend) | 2000-15.6 pg/ml | 15.6 pg/ml |
| IL-1 beta | 0.5 ug/ml (Ab Solutions) | 0.15 ug/ml (Ab Solutions) | 500-3.9 pg/ml | 3.9 pg/ml |
| TNF-alpha | 1.0 ug/ml (BioLegend) | 0.5 ug/ml (BioLegend) | 2000-15.6 pg/ml | 15.6 pg/ml |
| IL-10 | 1.25 ug/ml (BioLegend) | 0.2 ug/ml (BioLegend) | 500-3.9 pg/ml | 3.9 pg/ml |
| IFN-gamma | 1 ug/ml (BD Pharmingen) | 0.5 ug/ml (BD Pharmingen) | 40-0.31 U/ml | 0.31 U/ml |
| CCL2 | 1 µg/ml (BD Pharmingen) | 250 ng/ml (BD Pharmingen) | 6500-50.8 pg/ml | 50.8 pg/ml |
| CXCL9 | 0.35 ng/ml(R&D) | 15 ng/ml (R&D) | 4000-31.2 pg/ml | 31.2 pg/ml |
| CXCL10 | 400 ng/ml (BD) | 50 ng/ml (BD) | 1500-11.7 pg/ml | 11.7 pg/ml |
| CCL17 | 250 ng/ml (R&D) | 50 ng/ml (R&D) | 600-4.7 pg/ml | 4.7 pg/ml |
| CCL22 | 0.5 µg/ml (R&D) | 20 ng/ml (R&D) | 1100-8.6 pg/ml | 8.6 pg/ml |
| *Assay sensitivity is defined as the ability of the capture and detection antibody pair at optimized concentrations to detect the presence of a known amount of recombinant human cytokine/chemokine with absorbance values over background | | | | |

3.3 Optimization of experimental system. Optimization studies: identification of experimental conditions associated with “threshold” and “maximal” levels of Toll-like receptor stimulation

The vast majority of published studies employed LPS stimulation in vitro to mimic environmental exposures at very high concentrations. Our analysis of 25 randomly selected research reports contained within PubMed in which LPS was used as an in vitro stimulus of PBMC activation revealed that >90% of those employed LPS at >100 ng/ml (Table 7).

Table 7. Analysis of 25 randomly selected research reports contained within PubMed on cytokine responses by human PBMC to LPS stimulation in vitro

| LPS / ml | 10 ug | 1 ug | 100 ng | 50 ng | 10 ng |
|------------------|-------|------|--------|-------|-------|
| Number of papers | 5 | 8 | 10 | 1 | 1 |

In contrast, environmental field studies indicate that airborne endotoxin levels to which children are commonly exposed are very low, in the range of 0.008 -0.18 ng/m³ (Bouillard e.a., 2005; Platts-Mills e.a., 2005; Rabinovitch e.a., 2005; Sohy e.a., 2006). These are equivalent to approximately 0.18 to 1.04 ng per day, at least 1,000-10,000-fold lower than LPS levels typically used in vitro. Immune responses to LPS and other TLR ligands that would occur at levels more closely related to environmental concentrations have not been examined.

We hypothesized that use of low concentrations of LPS and other TLR ligands that more closely reflect levels of environmental exposures rather than widely utilized “maximal” concentrations would increase (i) the diversity evident in genetically different populations and (ii) sensitivity to detect possible differences in TLR responsiveness between clinically distinct populations.

Initially, we investigated cytokine and chemokine responses to TLR stimulation using ligands at concentrations ranging from the low levels observed in most environments (for LPS at 0.005 ng/ml to 0.5 ng/ml) to the high levels commonly utilized in vitro (for LPS at 50 ng/ml to 500 ng/ml).

Numerous studies have established that TLR detection of molecular patterns present on microbes (TLR ligands) causes activation of signaling pathways that lead to diverse pro- and anti-inflammatory cytokine and chemokine responses (Iwasaki, Medzhitov, 2004; Uematsu, Akira, 2006). Since the information on these immune mediators is still incomplete, we examined a wide profile of cytokines and chemokines that could be potentially evoked upon stimulation with diverse repertoire of TLR ligands.

The specific objective of these optimization studies was

- 1). to identify “threshold” and “maximal” stimulation concentrations of TLR ligands;
- 2). to determine optimal experimental conditions for quantitative analysis of a broad profile of cytokine and chemokine responses to TLR stimulation

PBMC isolated from fresh peripheral blood derived from children were cultured at 350,000 cells/well and stimulated with diverse TLR ligands over a wide range of concentrations. Dose-response titration experiments were conducted with a panel of TLR

agonists: LPS, a TLR4 agonist; PGN, a TLR2 agonist; 3M-011 compound, a TLR8 agonist; poly(I:C), a TLR3 agonist; CpG ODN 2216, a TLR9 agonist.

Levels of cytokines and chemokines in culture supernatants were quantified using ELISA. Herein, we studied a wide profile of cytokines and chemokines including (1) pro-inflammatory cytokines (IL-6, IL-1beta, TNF-alpha) and chemokine (CCL2); (2) anti-inflammatory cytokine (IL-10); (3) Th1-associated cytokine (IFN-gamma) and chemokines (CXCL9, CXCL10); (4) Th2-associated chemokines (CCL17, CCL22).

Optimal time point(s) of cytokine and chemokine production by PBMC upon stimulation with TLR ligands were first determined in kinetic experiments.

3.3.1 Time course of TLR ligand driven cytokine and chemokine production

In clinical studies, experimental conditions utilizing 24-hour period of PBMC culture have been extensively used to investigate cytokine and chemokine responses to TLR stimulation (Brunialti e.a., 2006; Sardenberg e.a., 2004; von Haehling e.a., 2003; Zeidel e.a., 2001). At this time point, robust production of most cytokines and chemokines examined was observed in many reports. However, possible shift in kinetics after stimulation with TLR ligands for longer periods has not been well-defined.

To identify optimal time point(s) of cytokine and chemokine responses to TLR stimulation, we performed time-course studies. PBMC obtained from four children were incubated with TLR ligands at wide range of concentrations in parallel cultures for 24h and 48h. Time-course analysis demonstrated that cytokine and chemokine production was virtually the same following 24h and 48 h (data not shown), indicating that there was

no change in kinetics or the interpretation of these data over this time period. Thus, studies revealed optimal cytokine responses at 24 h of culture. This time point, 24h, was selected as a working time point for the following experiments.

3.3.2 Dose-response titration experiments: identification of “threshold” and “maximal” concentrations of LPS, a TLR4 ligand

To assess the capacity of LPS to elicit cytokine and chemokine responses and verify “threshold” and “maximal” levels of stimulation, we used LPS over 1,000,000 fold range of concentrations from 500 ng/ml (a concentration approximating the widely cited in the literature 100 ng/ml- 10 ug/ml levels (Table 7) down to 0.005 ng/ml.

As demonstrated in Figures 7 and 8, LPS at high concentrations induced intense production of pro-inflammatory, IL-6, IL-1beta, TNF-alpha, and anti-inflammatory IL-10 cytokines. Increasing concentrations of LPS led to continuously augmented levels of cytokines up to the plateau response at 5 ng/ml, after which no further increases were evident. At the lower levels of stimulation, consistently detectable increases in cytokine release were observed. Specifically, stimulation with LPS at the concentration as low as 0.05 ng/ml resulted in ~10-fold increase in magnitude in IL-6 responses as compared with medium control levels.

The production of IL-1beta, TNF-alpha, and IL-10 was approximately 20-fold, ~10-fold, ~40-fold higher, respectively, than baseline levels in response to stimulation with 0.5 ng/ml of LPS.

Maximal production of cytokines, of two orders of magnitude greater relative to the medium control, was observed after stimulation with 5.0 ng/ml of LPS.

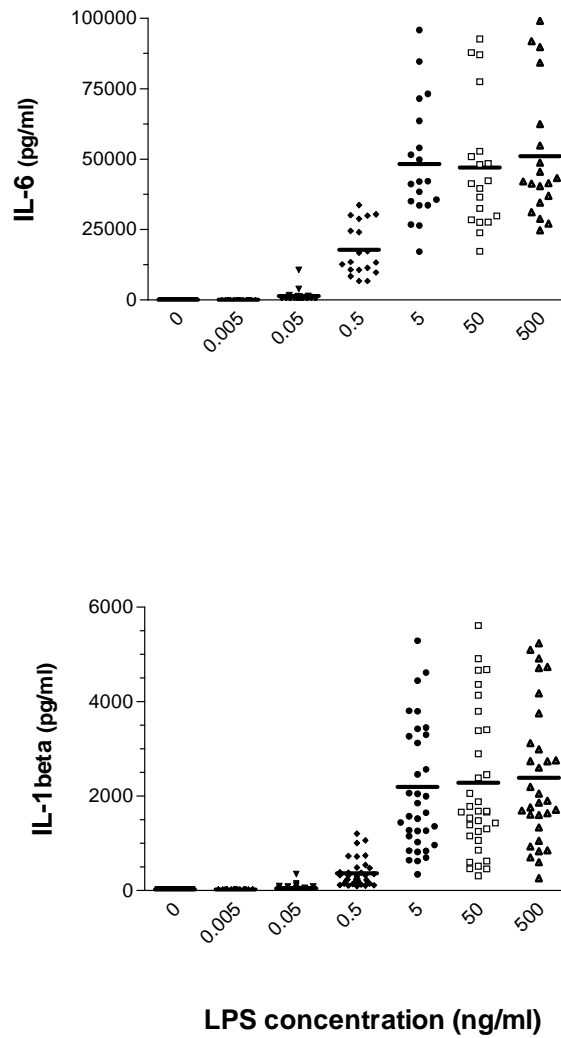


Figure 7. Dose-dependent cytokine responses elicited by LPS. IL-6 and IL-1beta production induced by different concentrations of LPS were measured by ELISA in supernatants of PBMC cultured for 24 hours. Black bars represent mean levels, with each of 15 individual children shown (•).

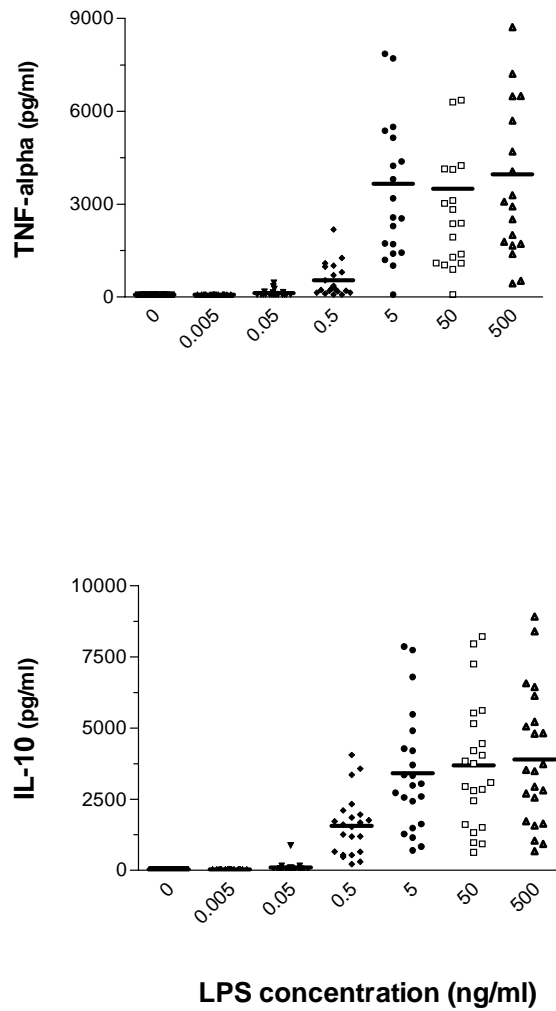


Figure 8. Dose-dependent cytokine production induced by LPS. TNF-alpha and IL-10 responses elicited by different levels of LPS were quantified by ELISA in supernatants of PBMC cultured for 24 hours. Black bars represent mean levels, with each of 15 individual children shown (•).

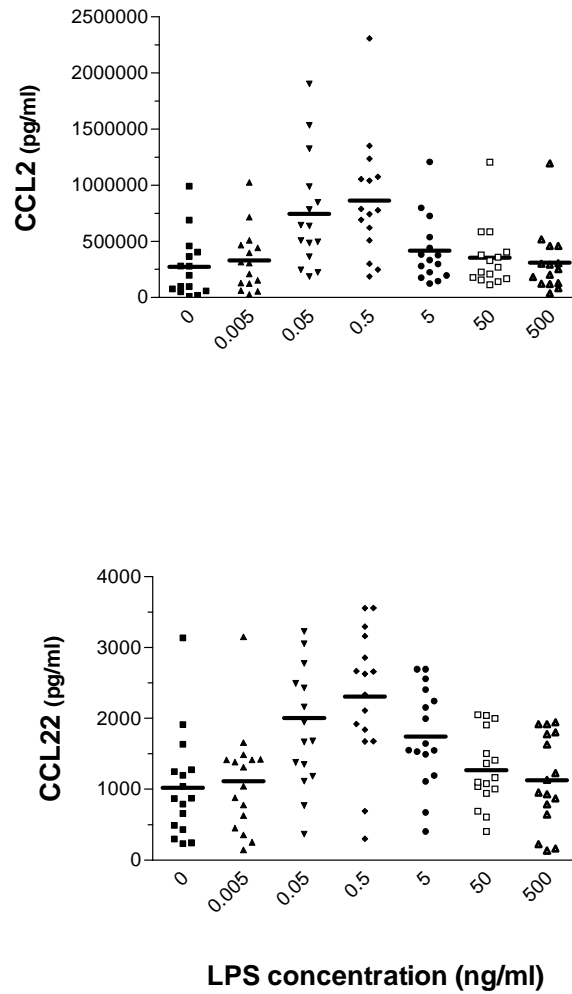


Figure 9. Optimization of chemokine responses induced by LPS. CCL2 and CCL22 secretion induced by different concentrations of LPS were measured using ELISA in supernatants of PBMC cultured for 24 hours. Black bars represent mean levels, with each of 15 individual children shown (•).

LPS also elicited robust production of CCL2 and CCL22 chemokines (Figure 9). In contrast to cytokine dose-responses, release of both chemokines also initially increased, reaching a peak level at 0.5 ng/ml, but then, at the greater levels of LPS stimulation that are typically used (50-500 ng/ml), chemokine production is progressively and substantially decreased by 64% for CCL2 and 52% for CCL22.

As evident from dose-response curves, low levels of LPS were capable of inducing chemokine release with ~2-3-fold increases at 0.05 ng/ml and maximal responses at 0.5 ng/ml.

We also investigated if stimulation with LPS elicits the production of IFN- γ , CXCL9, CXCL10, CCL17 and found that these cytokine/chemokines were not detectable (limits of detection are 0.62 U/ml, 155 pg/ml, 60 pg/ml, 24 pg/ml, respectively) in culture supernatants following stimulation with LPS over a broad range of concentrations tested (0.005-500 ng/ml) for 24 hours and 48 hours (data not shown). These data are consistent with the results of previous reports demonstrating that IFN- γ (Jansky e.a., 2003), CXCL9 (Proost e.a., 2004), CXCL10 (Proost e.a., 2003) were undetectable in LPS-stimulated human PBMC cultures.

Taking into consideration the data of published studies that extremely low levels of LPS better mimic environmental exposures in humans and on the basis of our findings we identified the “threshold” (physiologically relevant) and “maximal” (widely cited) concentrations of LPS (see Table 8) that were used to test our hypotheses.

Table 8. “Threshold” and “maximal” concentrations of TLR ligands: LPS, PGN, 3M-011 compound, poly(I:C), CpG ODN

2216

| TLR ligand | Concentration | IL-6 | IL-1beta | TNF-alpha | IL-10 | CCL2 | CCL22 | CXCL10 |
|-------------------------|---------------|-------|----------|-----------|-------|-------|-------|--------|
| LPS (ng/ml) | threshold | 0.05 | 0.5 | 0.5 | 0.5 | 0.05 | 0.05 | ND |
| | maximal | 0.5 | 5.0 | 5.0 | 5.0 | 0.5 | 0.5 | ND |
| PGN (ug/ml) | threshold | 0.025 | 0.1 | 0.1 | 0.1 | 0.025 | 0.025 | ND |
| | maximal | 0.1 | 1.0 | 1.0 | 1.0 | 0.1 | 0.1 | ND |
| 3M-011 (ug/ml) | threshold | 0.5 | 0.5 | 0.5 | 0.5 | 0.05 | ND | ND |
| | maximal | 5.0 | 5.0 | 5.0 | 5.0 | 0.5 | ND | ND |
| Poly(I:C) (ug/ml) | threshold | ND | ND | ND | ND | 5.0 | ND | ND |
| | maximal | ND | ND | ND | ND | 125.0 | ND | ND |
| CpG ODN 2216 (ug/ml) | threshold | ND | ND | ND | ND | ND | ND | 1.0 |
| | maximal | ND | ND | ND | ND | ND | ND | 3.0 |
| ND – not detected | | | | | | | | |

3.3.3 Dose-response titration experiments: identification of “threshold” and “maximal” concentrations of PGN, a TLR2 ligand

In human studies, PGN has been commonly utilized at very high concentrations (10 ug/ml and higher) in analysis of immune capacity in vitro (Netea e.a., 2005; Sha e.a., 2004; Uehori e.a., 2005; Remer e.a., 2006).

Here, PGN was first examined over a 100,000 fold range of concentrations from 0.001 ug/ml to 10 ug/ml. As seen in Figures 10 and 11, PGN is a potent inducer of pro-inflammatory, IL-6, IL-1beta, TNF-alpha and anti-inflammatory, IL-10, cytokine responses.

Cytokine production rose with increasing concentrations of PGN and reached a plateau at 1.0 ug/ml. The consistently detectable releases of IL-6, with a ~25-fold increase compared to medium control level, were detected in response to 0.025 ug/ml. The magnitude of IL-1beta, TNF-alpha, IL-10 production was approximately 7-fold, ~8-fold, and ~37-fold, respectively, greater than controls in response to 0.1 ug/ml of PGN. Maximal levels of cytokines were detected after stimulation with 1.0 ug/ml of PGN.

As evident from Figure 12, PGN also induced strong chemokine, CCL2 and CCL22, responses. The production of both chemokines progressively augmented with rising levels of stimulation, peaking at 0.1 mg/ml, but then gradually declined at the higher concentrations of PGN by 55% for CCL2 and 66% for CCL22.

These data show that relatively low concentrations of PGN are able to elicit chemokine responses and that supra-optimal concentrations (often widely used in the literature) elicit poorer responses than do levels of TLR ligands more commonly found in

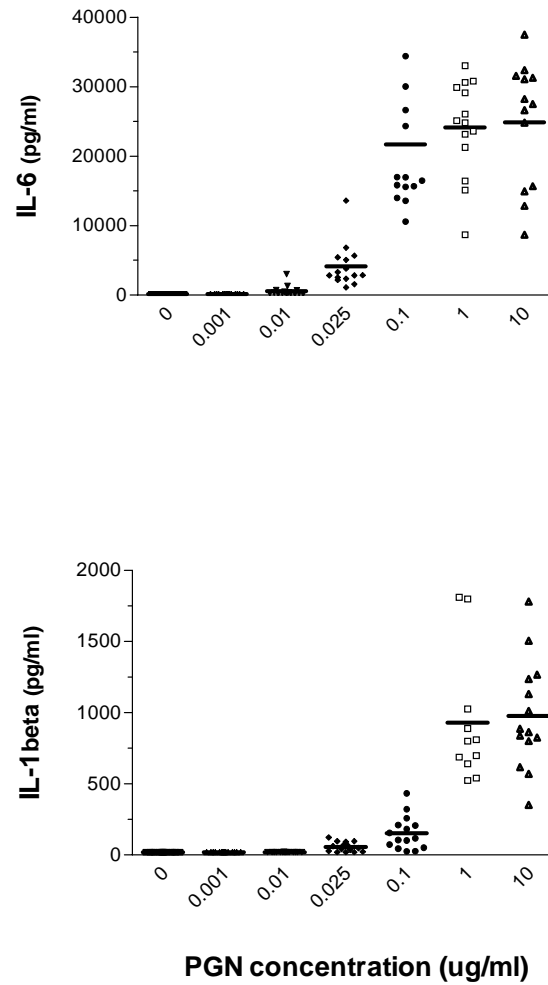


Figure 10. Dose-dependent cytokine responses elicited by PGN. IL-6 and IL-1beta production induced by different concentrations of PGN were measured by ELISA in supernatants of PBMC cultured for 24 hours. Black bars represent mean levels, with each of 15 individual children shown (•).

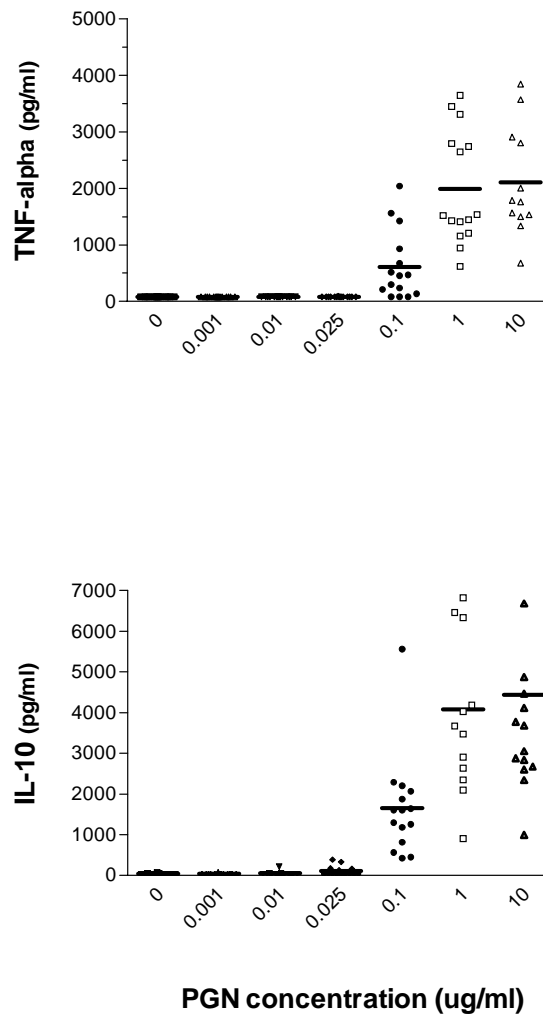


Figure 11. Dose-dependent cytokine production induced by PGN. TNF-alpha and IL-10 responses induced by different concentrations of PGN were quantified by ELISA in supernatants of PBMC cultured for 24 hours. Black bars represent mean levels, with each of 15 individual children shown (•).

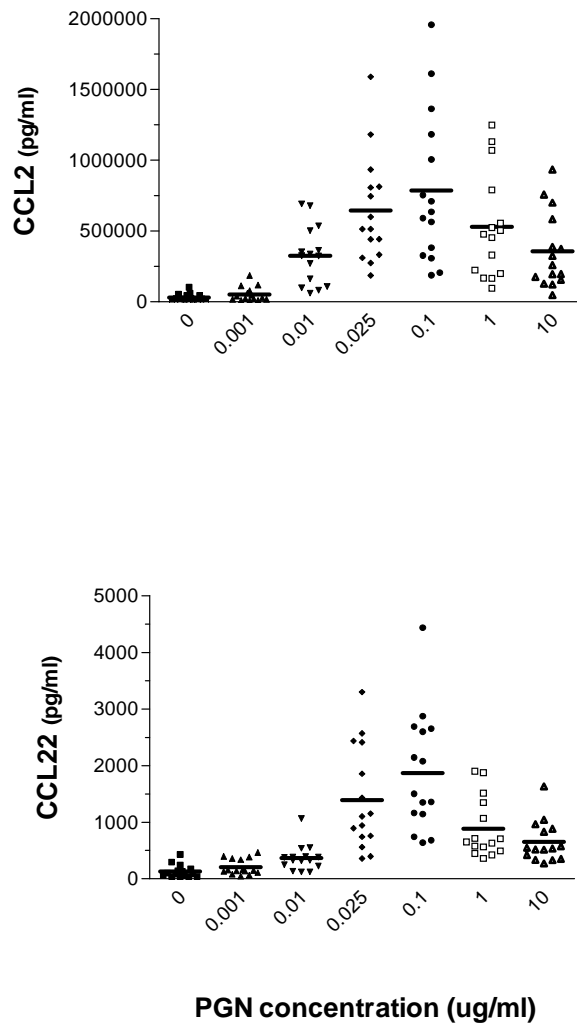


Figure 12. Optimization of cytokine responses elicited by LPS. CCL2 and CCL22 production induced by different concentrations of PGN were measured by ELISA in supernatants of PBMC cultured for 24 hours. Black bars represent mean levels, with each of 15 individual children shown (•).

natural environments. Thus, markedly detectable increases are evident at 0.025 ug/ml; maximal levels are induced by 0.1 ug/ml of PGN.

We also examined the capacity of PGN to induce the production of IFN-gamma, CXCL9, CXCL10, CCL17, and found that these cytokine/chemokines were undetectable (detection limits are 0.62 U/ml, 155 pg/ml, 60 pg/ml, 24 pg/ml, respectively) in culture supernatants upon stimulation with PGN over the 100,000 fold range of concentrations used (0.001-10 ug/ml) for 24 hours and 48 hours (data not shown). This is in line with previous studies by others showing that stimulation of human PBMC with PGN, even at supra-maximal concentrations (10-100 ug/ml), did not result in the production of measurable levels of CXCL9 (Proost e.a., 2004) and CXCL10 (Re, Strominger, 2004).

Based upon the findings of our dose-response titration experiments we determined the “threshold” and “maximal” concentrations of PGN (see Table 8) that were utilized to test our hypotheses.

3.3.4 Dose-response titration experiments: identification of “threshold” and “maximal” concentrations of 3M-011 compound, a TLR8 ligand

Recently, the groups from 3M Pharmaceuticals (Ghosh e.a., 2006; Gordon e.a., 2005) have demonstrated that three different 3M compounds (3M-001, a TLR7 agonist; 3M-002, a TLR8 agonist; and 3M-003, a TLR7/8 agonist) examined were able to induce a diverse panel of cytokine responses by donors' PBMC. Importantly, authors revealed the key difference in cytokine profiles evoked by distinct 3M compounds: TLR7 agonist preferentially elicited IFN-alpha secretion whereas TLR8, TLR7/8 agonists were more effective at inducing pro-inflammatory cytokines such as IL-1 beta, TNF-alpha, IL-6.

The ability of 3M-011 compound (another small molecule imidazoquinoline-like TLR8 agonist) to induce cytokine responses in humans has not yet been investigated.

To determine whether 3M-011 compound is capable of eliciting cytokine and chemokine responses and identify “threshold” and “maximal” stimulation conditions, we examined 3M-011 compound over a 10,000 fold range of concentrations from 0.005 ug/ml to 50 ug/ml.

As demonstrated in Figures 13 and 14, 3M-011 compound was a potent stimulus of pro-inflammatory, IL-6, IL-1beta, TNF-alpha, and anti-inflammatory IL-10 cytokine production. Marked increases in cytokine secretion were evident in response to 0.5 ug/ml of 3M-011 compound, with ~100-fold in IL-6 release, and ~50-fold in IL-1beta, TNF-alpha, IL-10 release relative to medium controls. Cytokine production reached maximal levels at 5.0 ug/ml, and then plateauing (for IL-1beta, TNF-alpha) or decreasing (for IL-6, IL-10) at the greater concentration.

As seen in Figure 15, stimulation with 3M-011 compound resulted in intense CCL2 chemokine release. CCL2 levels were initially elevated with increasing concentrations of a TLR8 ligand; consistently detectable responses with ~12-fold increases were at 0.05 ug/ml of 3M-011 compound, reaching a peak at 0.5 ug/ml, and diminishing at higher levels of ligand stimulation.

3M-011 compound did not induce detectable levels of other chemokines tested including CXCL9, CCL17, CCL22 (limits of detection are 155 pg/ml, 24 pg/ml, 43 pg/ml respectively) after stimulation over a wide range of concentrations for 24 hours and 48 hours (data not shown).

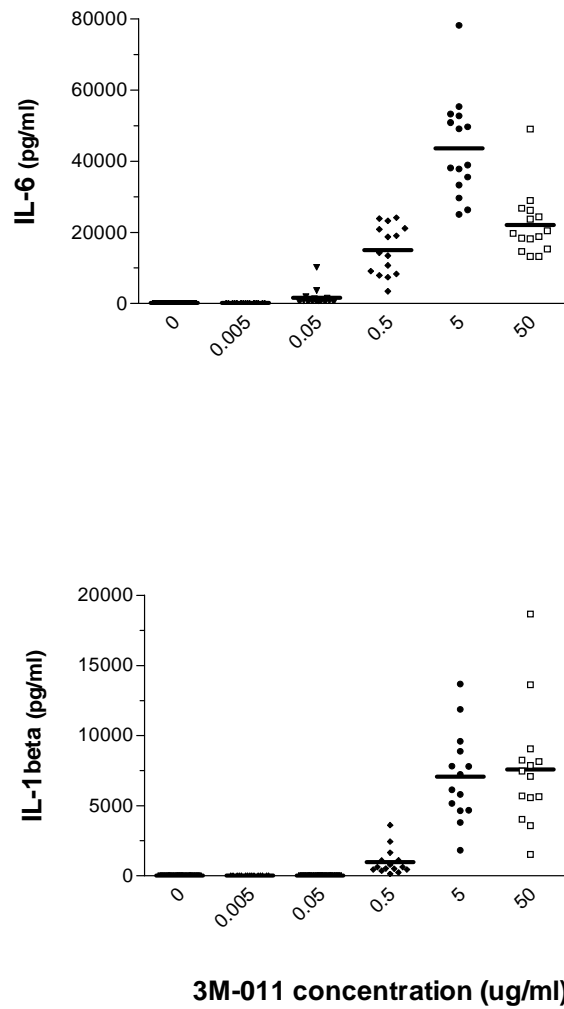


Figure 13. Dose-dependent cytokine responses induced by 3M-011 compound. IL-6 and IL-1beta production elicited by different concentrations of 3M-011 compound were measured by ELISA in supernatants of PBMC cultured for 24 hours. Black bars represent mean levels, with each of 15 individual children shown (•).

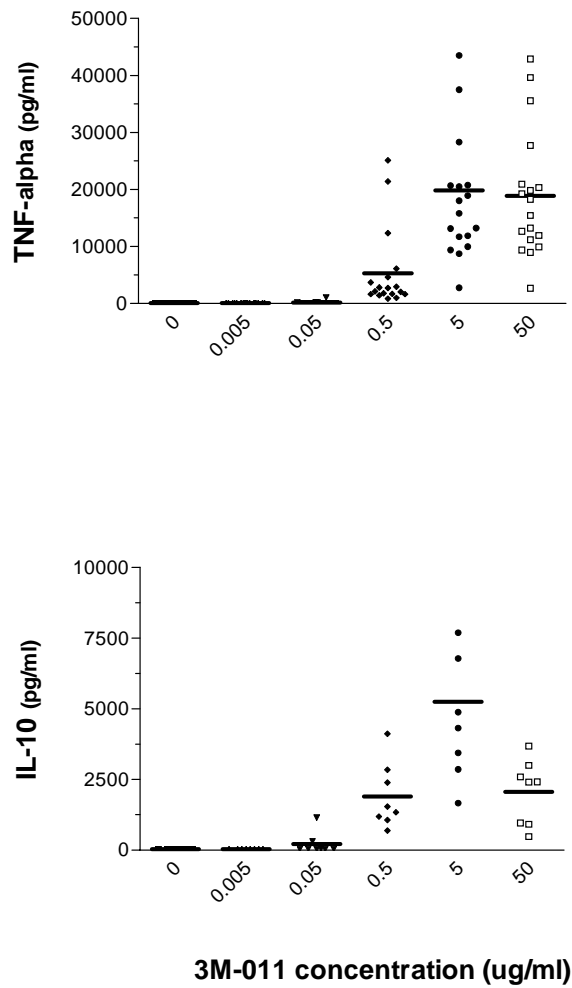


Figure 14. Dose-dependent cytokine responses elicited by 3M-011 compound. TNF-alpha and IL-10 production induced by different concentrations of 3M-011 compound were quantified using ELISA in supernatants of PBMC cultured for 24 hours. Black bars represent mean levels, with each of 15 individual children shown (•).

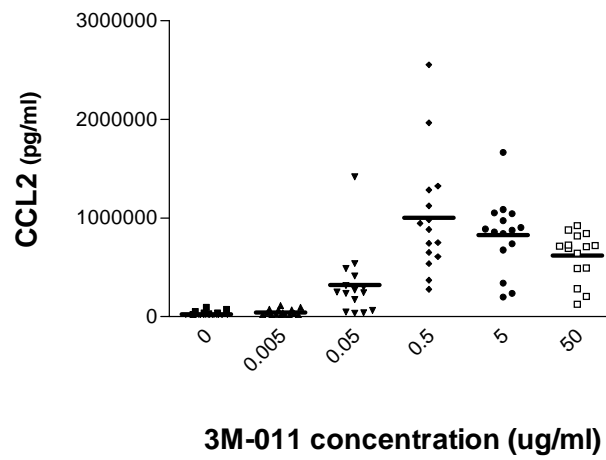


Figure 15. Optimization of chemokine responses elicited by 3M-011 compound. CCL2 concentrations induced by different levels of LPS were measured by ELISA in supernatants of PBMC cultured for 24 hours. Black bars represent mean levels, with each of 15 individual children shown (•).

The cytokine response pattern elicited by 3M-011 compound in our study is comparable to those observed with 3M-002 (TLR8 agonist) and 3M-003 (TLR7/8 agonist) compounds by other investigators (Ghosh e.a., 2006; Gorden e.a., 2005).

Thus, the results of dose-response titration experiments allow us to identify the “threshold” and “maximal” stimulation concentrations of 3M-011 compound (see table 8) that were employed in the study to test our main hypotheses.

3.3.5 Dose-response titration experiments: identification of “threshold” and “maximal” concentrations of poly(I:C), aTLR3 ligand

Poly(I:C) is widely employed at concentration of 50 ug/ml in human studies in vitro (Kadowaki e.a., 2001; Re, Strominger, 2004; Remer e.a., 2006). Here, we examine poly(I:C) over a two log range of concentrations from 5 ug/ml to 125 ug/ml to stimulate cells.

As evident from Figure 16, TLR3 agonist had the ability to induce high levels of CCL2 chemokine. The production of CCL2 in response to 5 ug/ml was approximately 7-fold higher than medium control level, and slightly rose with increasing levels of the ligand up to 125 ug/ml. Levels of other cytokines and chemokines (IL-6, IL-1beta, TNF-alpha, IL-10, IFN-gamma; CXCL9, CCL17, CCL22) studied were undetectable (limits of detection are 156 pg/ml, 20 pg/ml, 80 pg/ml, 40 pg/ml, 0.62 U/ml, 155 pg/ml, 24 pg/ml, 43 pg/ml, respectively) in culture supernatants after stimulation with poly(I:C) for 24hours and 48 hours (data not shown)

These findings are consistent with data of recent study by Ghosh e.a. (2007) showing that stimulation of human PBMC with poly(I:C) led to CCL2 secretion but did not induce any significant pro- and anti-inflammatory cytokine responses.

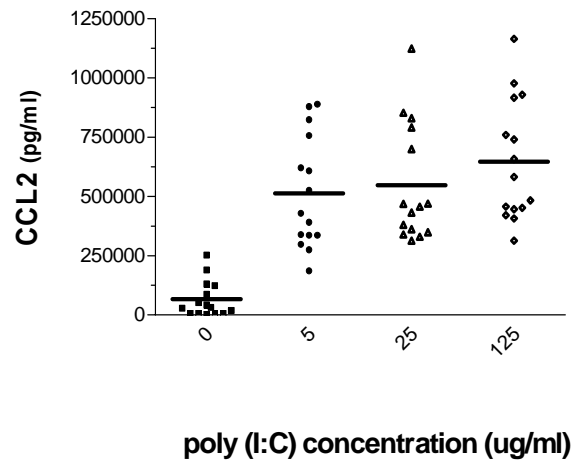


Figure 16. Optimization of chemokine responses induced by poly(I:C). CCL2 production elicited by different concentrations of LPS were measured by ELISA in supernatants of PBMC cultured for 24 hours. Black bars represent mean levels, with each of 15 individual children shown (•).

On the basis of dose-response titration experiments we choose to utilize poly(I:C) at 5 ug/ml (as “threshold” concentration) and at 125 ug/ml (as “maximal” concentration) (see Table 8) in our following studies. However, given the findings from the transfection experiments (see Results: Assessment of the specificity and purity of TLR ligands used) demonstrating that the poly(I:C) preparation used could induce the activation (SEAP) of transfectants when used at supra-optimal concentrations, we regard these data with caution.

3.3.6 Dose-response titration experiments: identification of “threshold” and “maximal” concentrations of CpG ODN 2216, a TLR9 ligand

To assess if CpG ODN 2216 has a potency to elicit cytokine and chemokine responses, PBMCs were incubated with this TLR9 agonist at concentrations of 1 ug/ml and 3 ug/ml.

As seen in Figure 17, CpG ODN 2216 induced robust CXCL10 responses, with ~8-fold increases at 1 ug/ml, and ~40 increases in magnitude at 3 ug/ml. Thus, consistently detectable “threshold” responses were observed at 1.0 ug/ml; “maximal” levels were elicited by 3.0 ug/ml of CpG ODN 2216 (see Table 8).

Stimulation with CpG ODN 2216 at both concentrations for 24hours and 48 hours does not lead to induction of detectable levels of other cytokines and chemokines (IL-6, IL-1beta, TNF-alpha, IL-10, IFN-gamma, CXCL9, CCL17, CCL22) (limits of detection are 156 pg/ml, 20 pg/ml, 80 pg/ml, 40 pg/ml, 0.62 U/ml, 155 pg/ml, 24 pg/ml, 43 pg/ml, respectively) (data not shown). These results are generally in line with recently published

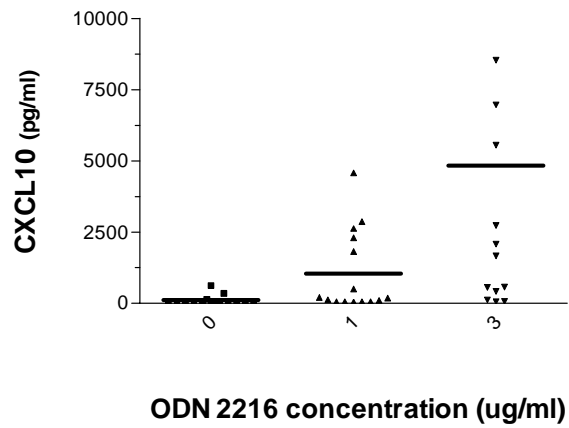


Figure 17. Dose-dependent chemokine responses elicited by CpG ODN 2216. CXCL10 production induced by different concentrations of CpG ODN 2216 was measured by ELISA in supernatants of PBMC cultured for 24 hours. Black bars represent mean levels, with each of 15 individual children shown (•).

data demonstrating that neither pro-inflammatory cytokines nor IL-10 was measurable in CpG ODN 2216-stimulated human cell cultures (Kirkmann e.a., 2003).

In summary, optimization data show that LPS, PGN, 3M-011 compound are potent stimuli of cytokine and chemokine production by PBMC derived from children. Poly(I:C) induced production of CCL2; CpG ODN 2216 elicited CXCL10 responses. “Threshold” and “maximal” stimulation concentrations of TLR ligands: LPS, PGN, 3M-011 compound, poly(I:C), and CpG ODN 2216 were identified (Table 8). These optimal experimental conditions were subsequently used to test our main hypotheses.

3.4 Cytokine responses elicited using threshold rather than maximal level of TLR stimulation differentiate allergic asthmatic versus non-atopic children

Environmental reports indicate that humans are typically exposed to extremely low levels of LPS in most natural environments (Platts-Mills e.a., 2005; Rabinovitch e.a., 2005; Sohy e.a., 2006). In marked contrast, the great majority (~90%) of clinical studies utilize LPS at very high levels (100 ng/ml and higher) for analysis of immune responses in vitro (see Table 7). In general, efforts to identify differences in TLR responsiveness between populations of distinct clinical phenotype, the great majority of which were carried out using high concentrations of TLR ligands in culture, have had limited success (Borish e.a., 1996; Hew e.a., 2006; John e.a., 1996; Mitsuta e.a., 2003; Saeki e.a., 2004).

We speculated that using low physiological concentrations of LPS and other TLR ligands that may better approximate environmental levels rather than typically utilized “maximal stimulation” concentrations could provide highly enhanced sensitivity to detect possible differences in TLR responsiveness between clinically distinct human populations.

In the present study, we tested the hypothesis that functional responsiveness to TLR stimulation by threshold concentrations of ligands differs in allergic asthmatic and non-atopic children, by evaluating responsiveness with widely used “maximal” and lower level “threshold” TLR ligand concentrations.

We randomly recruited 145 participants for this component of the study. All subjects were children 8-9 years old, Caucasians, born to non-smoking parents. Forty-six

individuals were allergic asthmatics, 26 had non-allergic asthma, 21 children were asymptomatic allergics and 52 were non-atopic controls.

The diagnosis of allergic asthma was established by physician (Dr. A. Becker or Dr. J. Liem) based on a clinical history of asthma symptoms as well as airway hyperresponsiveness to methacholine. Atopy was defined as a positive skin prick test reactions to at least one of 14 common environmental allergens tested.

Allergic asthmatics had a history of asthma and demonstrated positive skin prick test. Non-allergic asthmatics were children with established diagnosis of asthma and negative skin prick test. Asymptomatic allergics were identified based upon positive skin prick test and absence of history of allergic diseases. Non-atopic healthy children had no history of asthma or other allergic disorders and exhibited negative skin prick test.

None of the study participants had received anti-inflammatory medication such as inhaled or oral corticosteroids within the previous two months, or anti-histamines, beta2-agonists within three days prior to blood collection. All of the subjects were free from symptomatic respiratory infections in the two months preceding the recruitment for the study.

In the experiments presented, for each subject, PBMC were cultured at 350,000 cells/well and stimulated with “threshold” and “maximal” concentrations of TLR ligands: LPS, a TLR4 ligand, and PGN, a TLR2 ligand, for 24 hours. We examined the levels of pro-inflammatory (IL-1beta, IL-6, TNF-alpha) and anti-inflammatory (IL-10) cytokines in culture supernatants using ELISA.

As evident from Figure 18, non-atopic control children exhibit markedly increased IL-1beta production compared to asthmatics, both allergic and non-allergic,

upon stimulation with threshold concentration of LPS ($p < 0.05$ and $p < 0.01$, respectively). In contrast, under conditions of maximal responses, we did not observe any difference between the groups ($p > 0.05$).

IL-6 synthesis tended to be elevated in non-atopic subjects compared to asthmatics in response to threshold levels of LPS ($0.1 > p > 0.05$), and is of equivalent intensity among the groups to maximal stimulation with LPS ($p > 0.05$) (Figure 19).

TNF- α responses (Figure 20) to LPS at threshold concentration are higher among non-atopic individuals than allergic and non-allergic asthmatic children ($p < 0.05$). Again, no differences between the groups were evident under conditions of maximal stimulation ($p > 0.05$).

Conversely, IL-10 synthesis in response to threshold stimulation with LPS is elevated among allergic asthmatics relative to non-atopic controls ($p < 0.01$) (Figure 21). There were no significant differences between the groups under conditions of maximal responses ($p > 0.05$).

A similar picture emerged when we examined cytokine production in response to PGN. As demonstrated in Figure 22, use of the threshold concentration of PGN induces markedly greater IL-1 β responses in non-atopic individuals compared to allergic and non-allergic asthmatics ($p < 0.01$). On the contrary, under conditions of maximal stimulation, no differences were seen between the groups ($p > 0.05$).

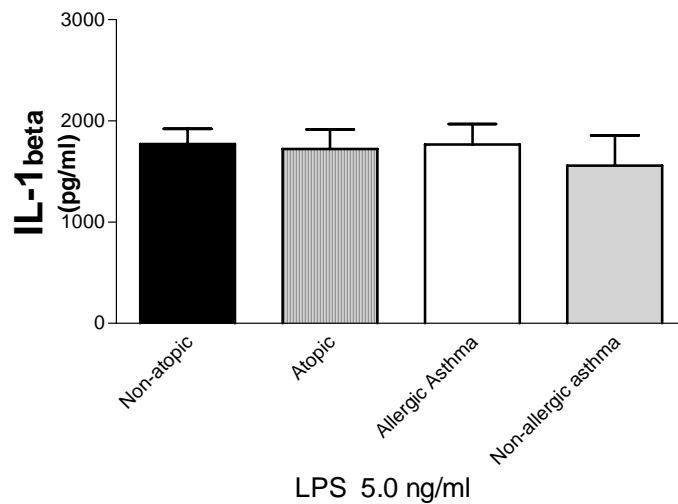
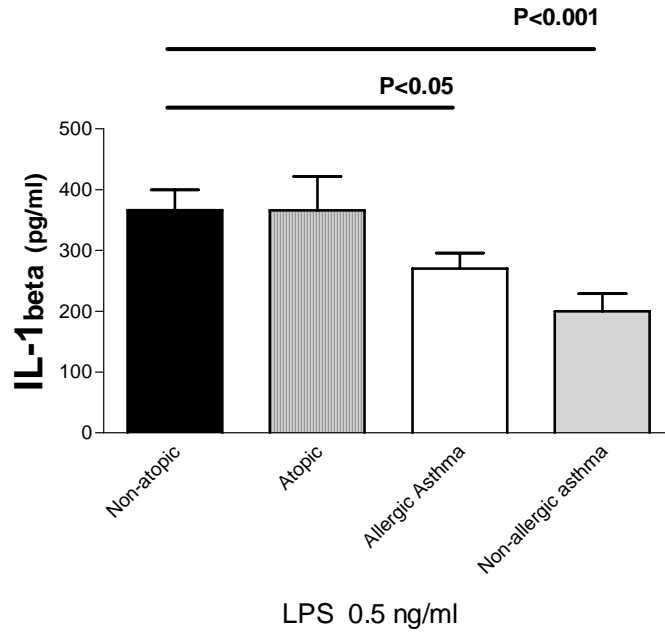


Figure 18. IL-1beta synthesis in response to threshold stimulation with LPS (0.5 ng/ml) is elevated among non-atopic control children relative to asthmatics. PBMC isolated from fresh peripheral blood of non-atopic control children (n=52), asymptomatic allergics (atopics) (n=21), allergic asthmatics (n=46), and non-allergic asthmatics (n=26) were stimulated with LPS at threshold (0.5 ng/ml) and maximal (5.0 ng/ml) concentrations for 24h. Levels of IL-1beta in culture supernatants were quantified by ELISA. Data are expressed as mean cytokine production \pm SEM.

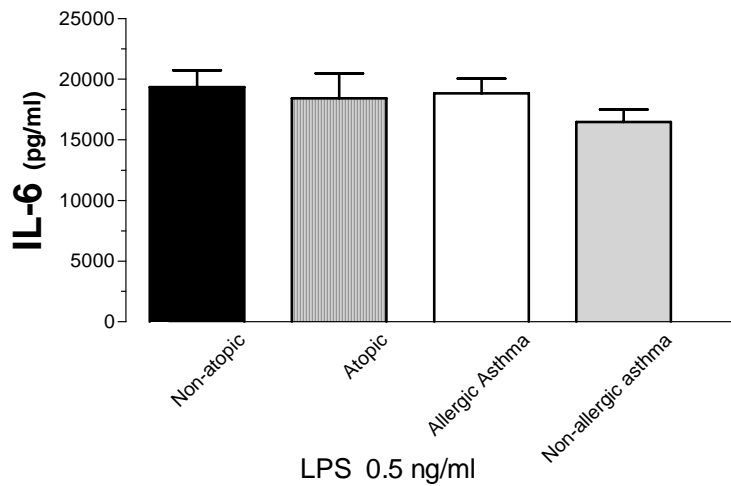
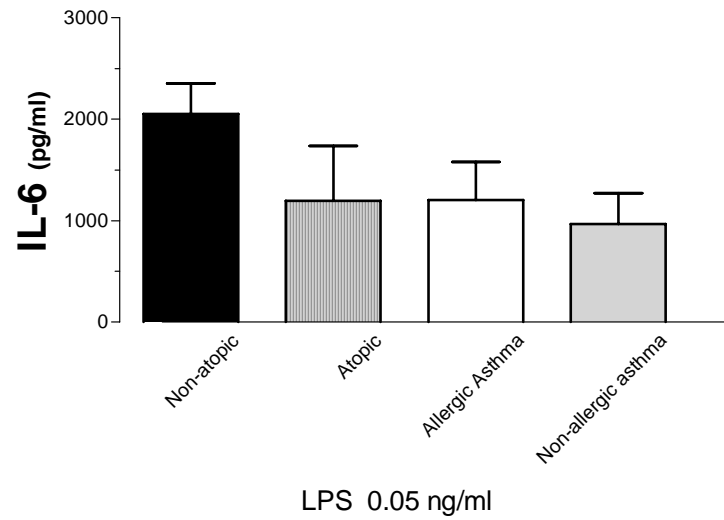


Figure 19. IL-6 production does not differ detectably among non-atopic, asymptomatic allergic (atopic) and asthmatic children in response to both threshold (0.05 ng/ml) and maximal (0.5 ng/ml) levels of LPS. Cultures were established as described for Figure 18, culture supernatants were assayed for IL-6 protein level by ELISA. Data are expressed as mean cytokine production \pm SEM.

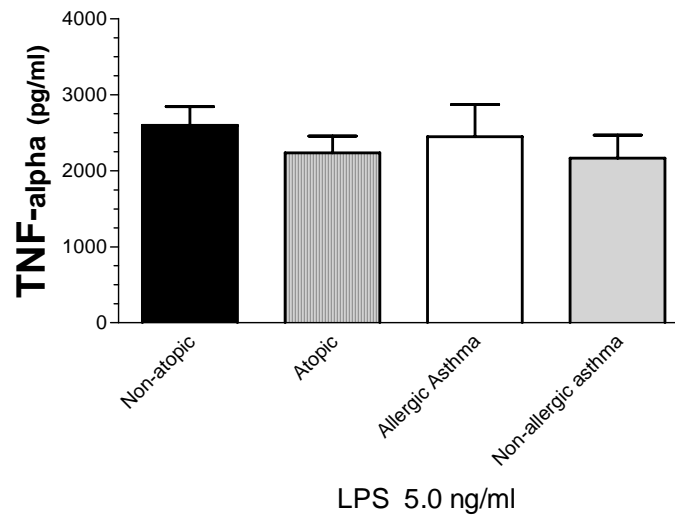
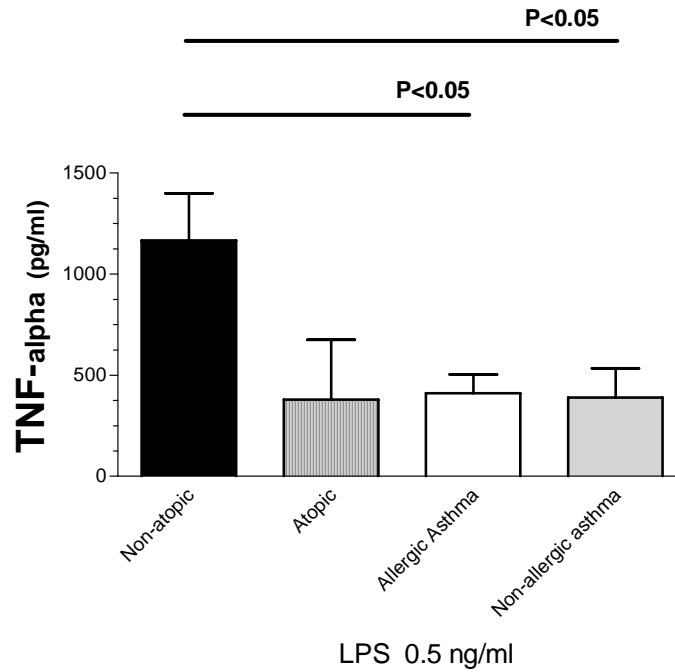


Figure 20. Non-atopic control children produced higher levels of TNF-alpha relative to controls to threshold stimulation with LPS (0.5 ng/ml). Cultures were established as described for Figure 18, levels of TNF-alpha in culture supernatant were measured using ELISA. Data are expressed as mean cytokine production \pm SEM.

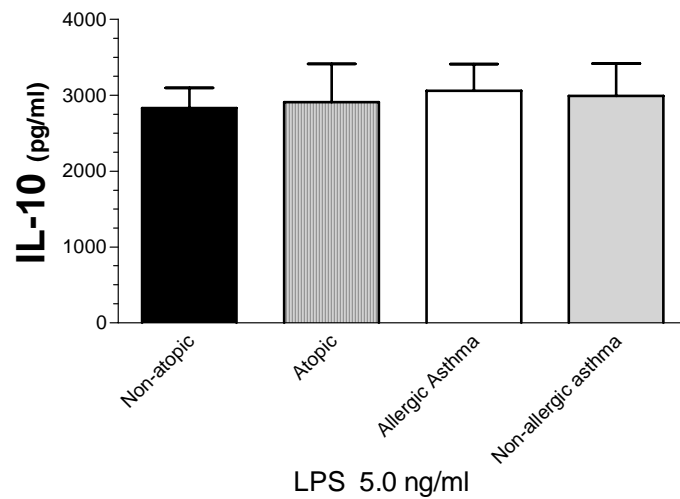
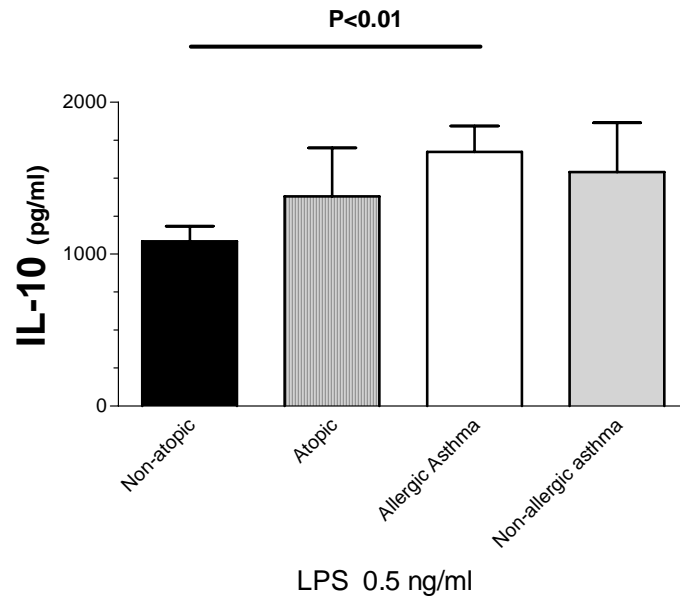


Figure 21. Asthmatic, both allergic and non-allergic, children exhibit greatly increased IL-10 production compared to controls upon stimulation with threshold dose of LPS (0.5 ng/ml). Cultures were established as described for Figure 18, culture supernatants were assayed for IL-10 protein level using ELISA. Data are expressed as mean cytokine production \pm SEM.

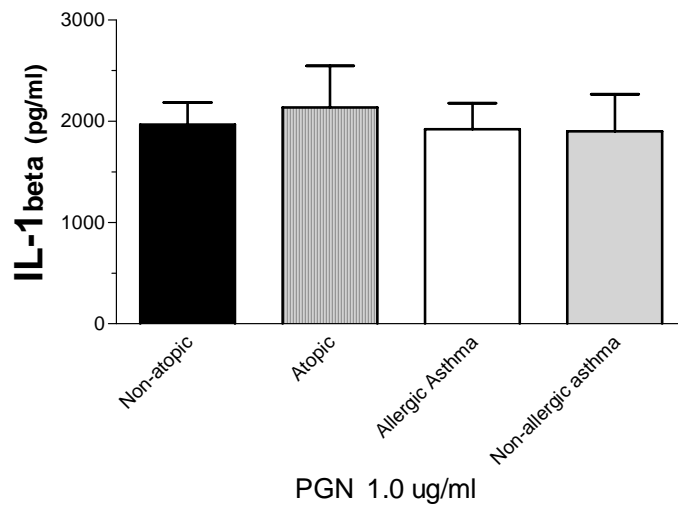
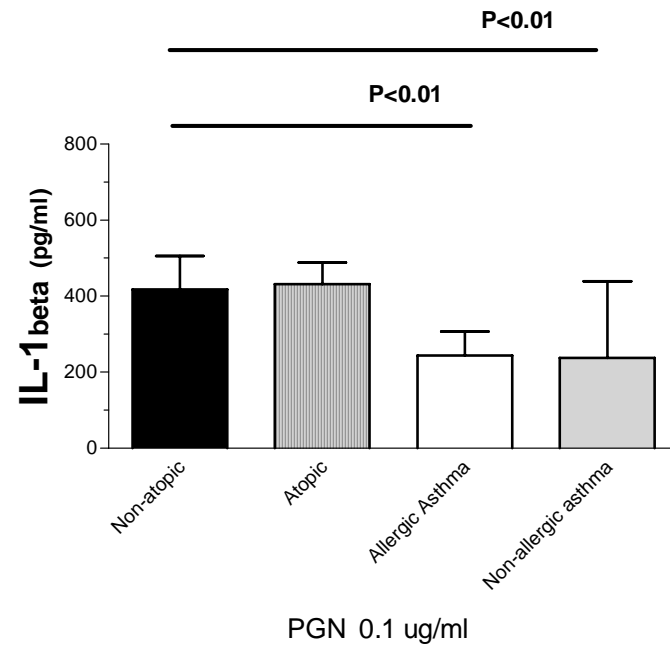


Figure 22. IL-1beta responses to threshold stimulation with PGN (0.1 ug/ml) is significantly higher among non-atopic control children compared to asthmatics. PBMC isolated from fresh peripheral blood of non-atopic control children (n=52), asymptomatic allergics (atopics) (n=21), allergic asthmatics (n=46), and non-allergic asthmatics (n=26) were stimulated with PGN at threshold (0.1 ug/ml) and maximal (1.0 ug/ml) concentrations for 24h. Levels of IL-1beta in culture supernatants were measured using ELISA. Data are expressed as mean cytokine production \pm SEM.

IL-6 release do not differ detectably among the groups in response to either threshold or maximal levels of PGN ($p>0.05$) (Figure 23)

Non-atopic control children also produce significantly higher levels of TNF-alpha in comparison to both groups of asthmatics upon stimulation with threshold dose of PGN ($p<0.05$) (Figure 24) No differences between the groups were observed under conditions of maximal responses ($p>0.05$).

IL-10 production (Figure 25) to threshold concentration of PGN is increased among asthmatics, both allergic ($p<0.0001$) and non-allergic ($p<0.01$), relative to controls. In contrast, under conditions of maximal stimulation, all groups examined exhibit virtually equivalent IL-10 synthesis ($p>0.05$).

Taken together, these data demonstrate that use of threshold levels of TLR stimulation reveal striking differences in cytokine responses between asthmatic and control children. Specifically, non-atopic children exhibit more robust pro-inflammatory (IL-1beta, TNF-alpha) cytokine responses; levels of IL-10 are elevated in asthmatics.

In marked contrast, commonly used maximal concentrations of LPS, PGN elicit strong, indistinguishable patterns of cytokine production.

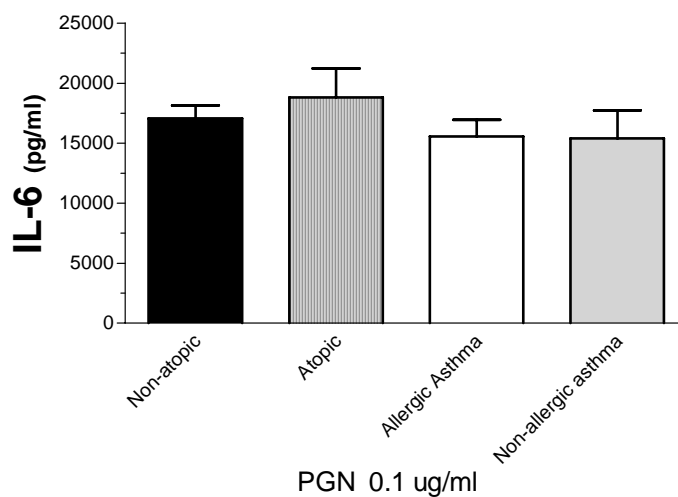
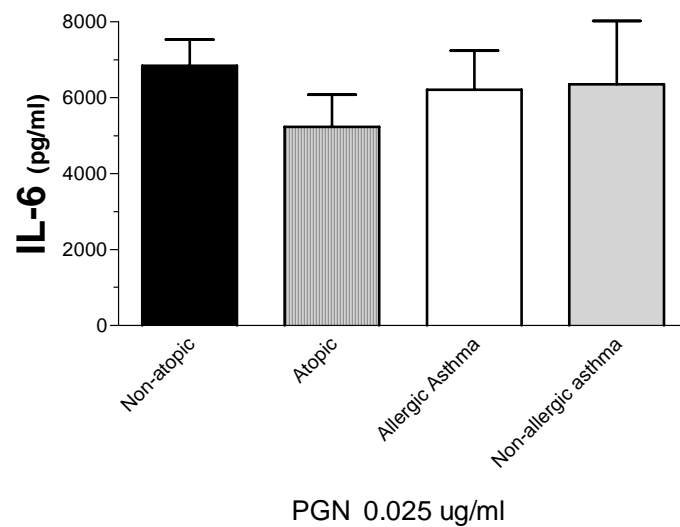


Figure 23. IL-6 levels are of equivalent intensity among non-atopic, asymptomatic allergic (atopic) and asthmatic groups both to threshold (0.025 ug/ml) and maximal (0.1 ug/ml) stimulation with PGN. Cultures were established as described for Figure 22, IL-6 release were quantified in culture supernatants by ELISA. Data are expressed as mean cytokine production \pm SEM.

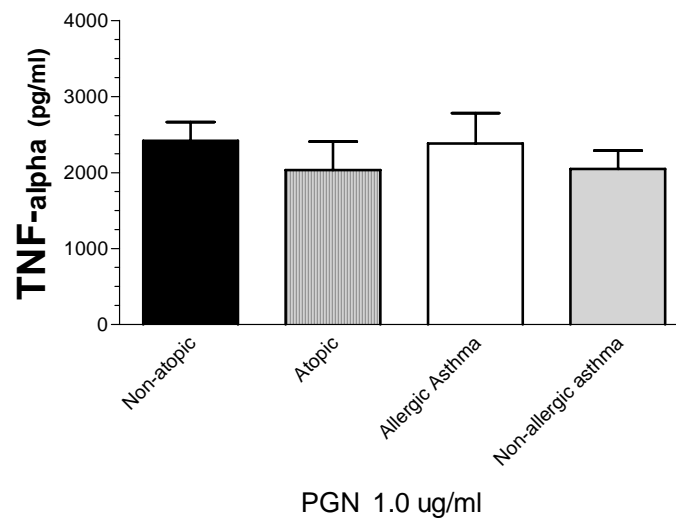
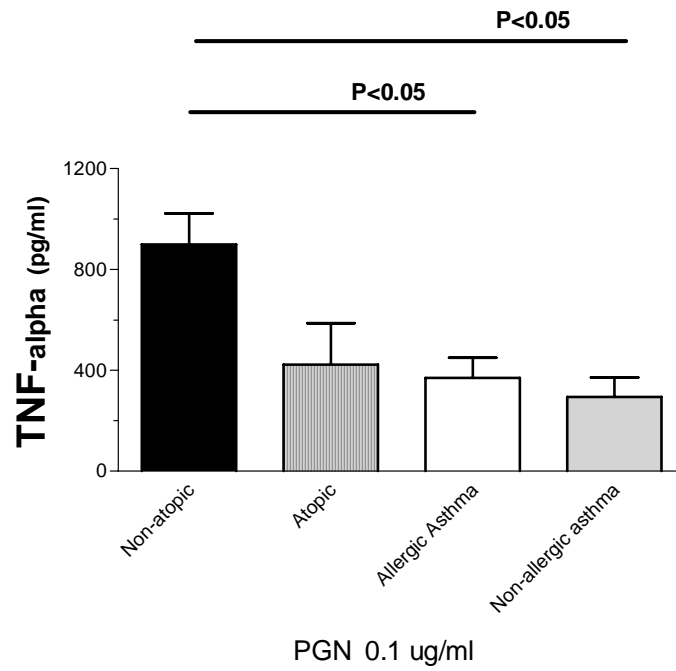


Figure 24. Non-atopic children exhibit markedly increased IL-10 production relative to asthmatics in response to threshold concentration of PGN (0.1 ug/ml). Cultures were established as described for Figure 22, culture supernatants were assayed for TNF-alpha protein level by ELISA. Data expressed as mean cytokine production \pm SEM.

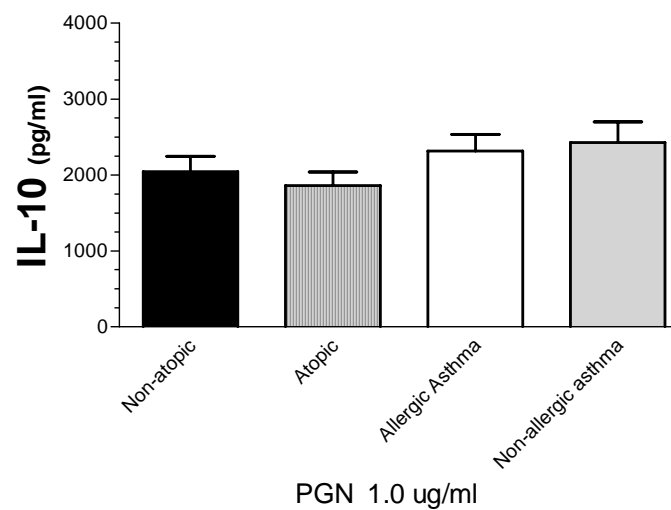
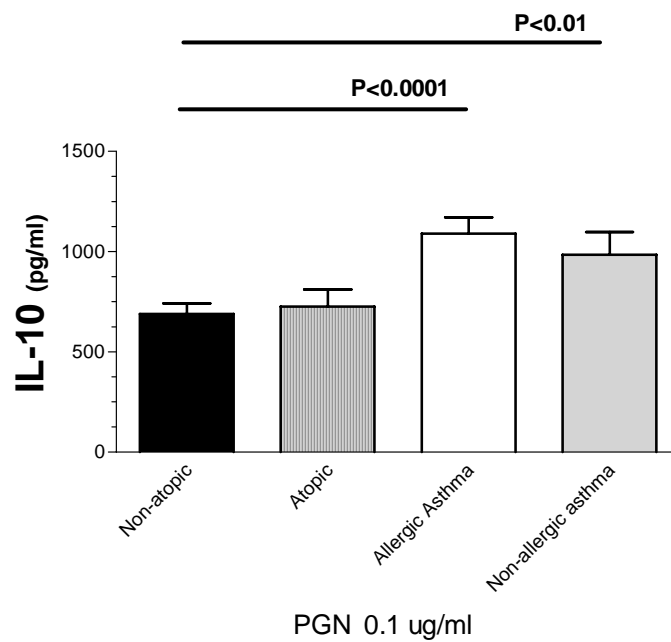


Figure 25. 10 responses to threshold stimulation with PGN (0.1 ug/ml) are markedly higher among asthmatic children compared to non-atopic controls. Cultures were established as described for Figure 22, IL-10 protein levels were quantified in culture supernatants using ELISA. Data are expressed as mean cytokine production \pm SEM.

3.5 Environmental tobacco smoke exposures modify Toll-like receptor responsiveness in asthmatic children

Epidemiological studies provide sufficient evidence for a causal relationship between ETS exposures and increased prevalence and severity of asthma in children (Pattenden e.a., 2006; Strachan, Cook, 1998). Yet, the potential mechanism of this phenomenon remains largely unknown. Differences in environmental exposures might impact the function of TLRs in different individuals, and these alterations in TLR responsiveness, we speculated, may influence the likelihood of exhibiting asthmatic clinical phenotype.

Here, we tested the hypothesis that ETS exposures affect TLR responsiveness in asthmatic children.

A total of 229 participants were randomly recruited for this study. All subjects were children at 8-9 years of age, Caucasians, born to smoking and non-smoking parents. Eighty-five individuals were non-atopic controls, 29 were asymptomatic allergics, 68 had allergic asthma and 47 were non-allergic asthmatics.

Each clinical group of children was stratified on the basis of smoking status of their parents and household members (“ETS-exposed” vs. “non-exposed to ETS”). Study population groups are presented in Table 9.

Exposure to ETS was assessed using questionnaire completed by the parents or/and experienced field staff. Information was collected on the past and current household smoking histories of each participant's mother, father, other adult household members, and regular household visitors.

Children were defined as “ETS-exposed” if:

mother smoked during pregnancy and/or in the first 12 mo of child's life and/or
father smoked in the first 12 mo of child's life and/or
other smokers were living in the home during child's first year of life and/or
visitors were allowed to smoke inside the home during child's first year of life
and/or

child went to daycare with smokers in first year of life and/or

mother smoked in the last 12 mo and/or

father smoked in the last 12 mo and/or

smokers were living in the home during last 12 mo and/or

visitors were allowed to smoke inside the home during last 12 mo

We attempted to identify children who were exposed to ETS in utero or in early life and not after age 1 but within our cohort of 229 children for whom blood was cultured, there were extremely few parents who smoked during pregnancy/early life then stopped afterwards. Thus, we were unable to make more detailed comparison of the impact of the time of ETS exposure on immune responses.

PBMC obtained from 8-9 year old children were cultured in the presence of TLR ligands: LPS, PGN, 3M-011 compound or poly(I:C) for 24 hours. In the present study, we utilized "threshold" concentrations of ligands based on the results of our previous investigation that demonstrated that this novel approach provide markedly enhanced sensitivity to reveal functional alterations in TLR responsiveness that associate with clinical status.

Levels of pro-inflammatory (IL-1beta, TNF-alpha, IL-6, CCL2), anti-inflammatory (IL-10) and Th2-associated (CCL22) cytokine and chemokine production were quantified by ELISA.

Table 9. Study population groups

| | Non-atopics | Asymptomatic allergics | Allergic asthmatics | Non-allergic asthmatics |
|----------------------------|-------------|------------------------|---------------------|-------------------------|
| ETS-exposed * | 33 | 8 | 22 | 21 |
| Non-exposed to ETS | 52 | 21 | 46 | 26 |
| Physician-diagnosed asthma | - | - | + | + |
| Skin test | - | + | + | - |

* Children were defined as “ETS-exposed” if: mother smoked during pregnancy and/or in the first 12 mo of child’s life; and/or father, other household members, regular visitors smoked in the first 12 mo of child’s life; and/or child went to daycare with smokers in first year of life; and/or mother, father other household members, regular visitors smoked in the last 12 mo inside the home.

As evident from Figure 26, IL-1beta responses to LPS or PGN are markedly higher in non-atopic children of smoking parents compared to that seen in asthmatics, both allergic and non-allergic, exposed to ETS ($p < 0.001-0.05$). There are no detectable differences in IL-1beta levels among ETS-exposed and non-ETS-exposed groups ($p > 0.05$). These findings suggest that IL-1 beta production in response to LPS or PGN is not modified by exposures to ETS.

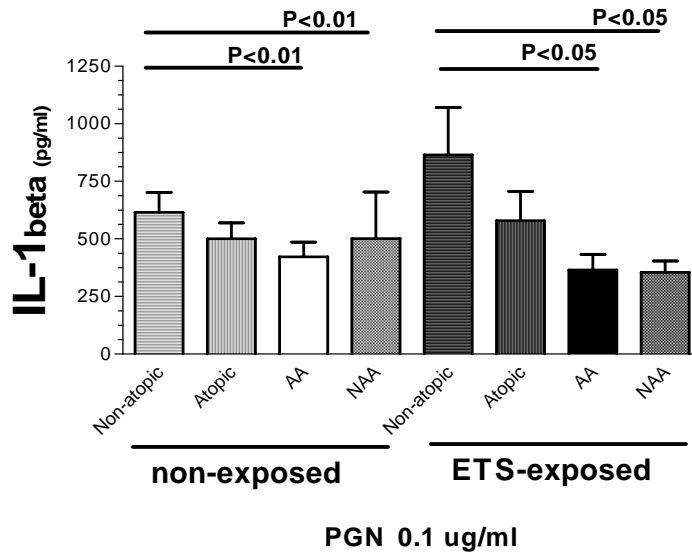
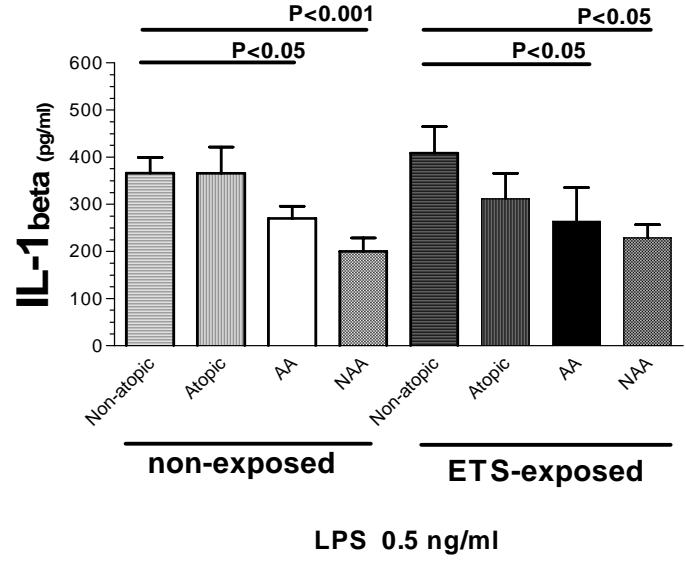


Figure 26. IL-1beta responses to LPS or PGN are not altered by exposure to ETS in asthmatic, asymptomatic allergic (atopic), and non-atopic children. Non-atopic individuals exhibit markedly increased levels of IL-1beta relative to asthmatics in response to LPS or PGN.

PBMC obtained from fresh peripheral blood were stimulated with LPS or PGN at the indicated concentrations for 24h. Levels of IL-1beta in culture supernatants were quantified by ELISA. Data are expressed as mean cytokine production \pm SEM.

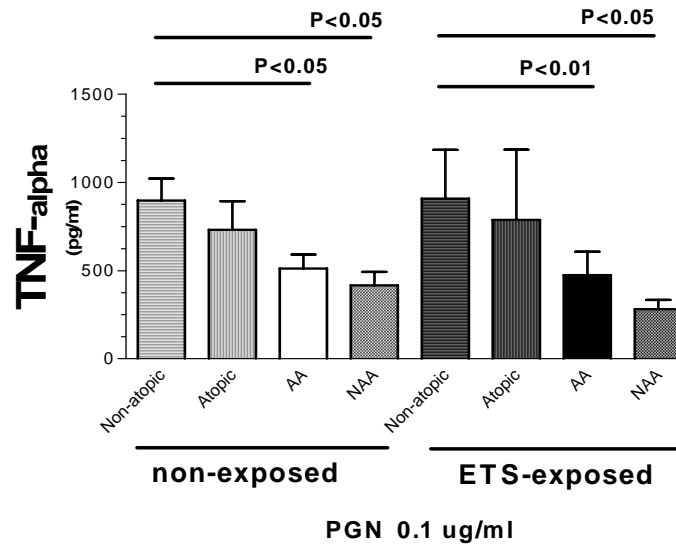
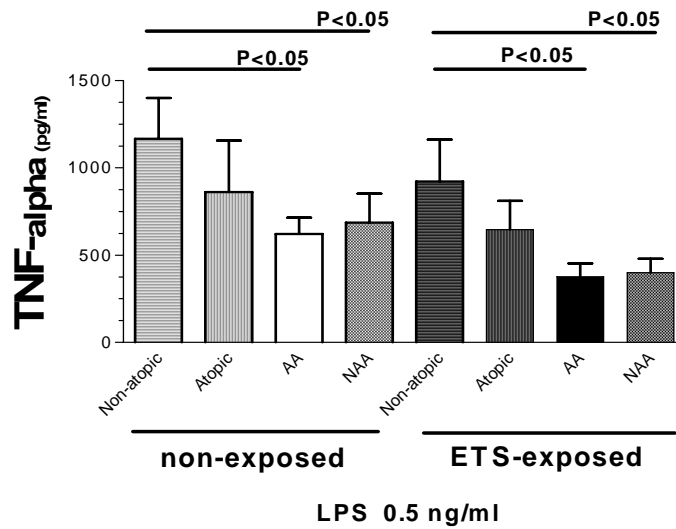


Figure 27. Exposure to ETS does not modify TNF-alpha responses to LPS or PGN in non-atopic, asymptomatic allergic (atopic) and asthmatic children. TNF-alpha production is significantly higher in non-atopic, control subjects than that in asthmatics. PBMC were cultured as described for Figure 2, levels of TNF-alpha in culture supernatants were quantified by ELISA. Data are expressed as mean cytokine production \pm SEM.

Similarly, non-atopic children whose parents smoke exhibit greatly increased LPS- or PGN-elicited TNF- α production relative to asthmatics exposed to passive smoking ($p < 0.01-0.05$) (Figure 27). No differences are evident between ETS-exposed and unexposed groups ($p > 0.05$).

IL-6 responses to LPS or PGN stimulation are of equivalent intensity among asthmatics, asymptomatic allergics and controls, as well as between exposed and non-exposed to ETS groups ($p > 0.05$) (data not shown).

These data demonstrate that passive smoking has no detectable effects on pro-inflammatory cytokine responses to LPS or PGN stimulation in asthmatic, asymptomatic allergic and non-atopic children.

As seen in Figure 28, IL-10 production in response to LPS or PGN is elevated among asthmatic children of parents who smoke compared to non-atopic individuals exposed to ETS ($p < 0.0001-0.05$). No differences are observed among exposed and non-exposed to passive smoking groups ($p > 0.05$). These results indicate that anti-inflammatory, IL-10, cytokine responses to LPS or PGN are not altered by parental smoking in non-atopic, asymptomatic allergic, and asthmatic children.

Exposure to ETS enhances LPS- or PGN-elicited CCL2 production in allergic asthmatics relative to non-ETS-exposed asthmatic and control groups ($p > 0.01-0.05$) (Figure 29). Levels of CCL2 are equivalent among groups of non-exposed to ETS children (non-atopics, asymptomatic allergics, asthmatics) ($p > 0.05$).

CCL22 responses to stimulation with LPS or PGN are markedly higher among ETS-exposed asthmatics compared to non-atopic children ($p < 0.01-0.05$) (Figure 30).

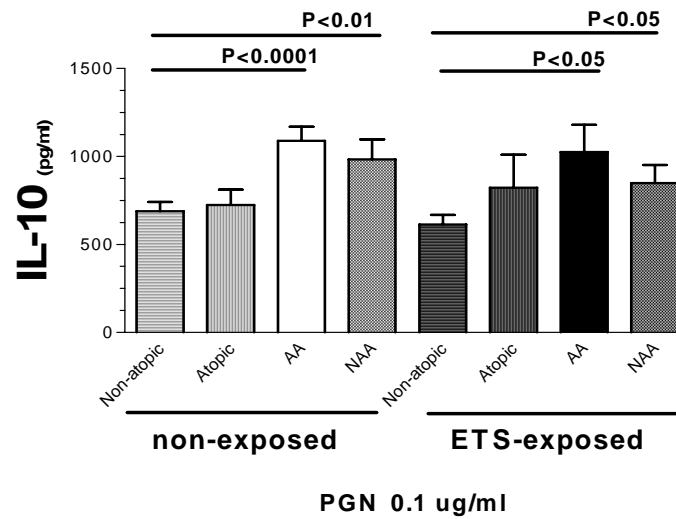
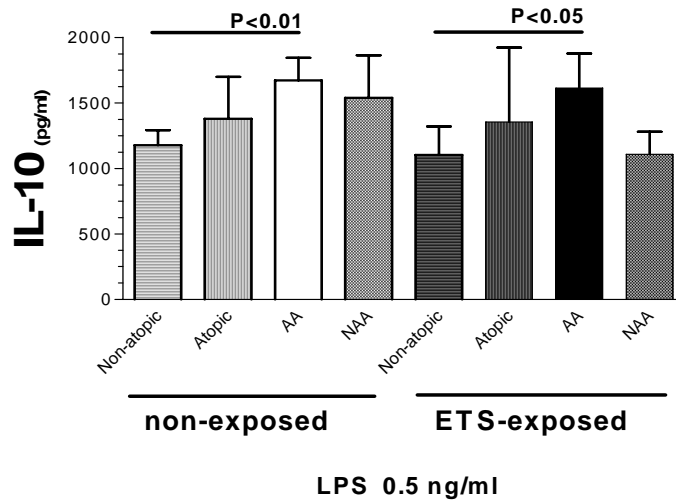


Figure 28. Parental smoking has no detectable effect on IL-10 responses to LPS or PGN in non-atopic, asymptomatic allergic (atopic), and asthmatic children. Cultures were established as described for Figure 2, levels of TNF-alpha in culture supernatants were measured using ELISA. Data are expressed as mean cytokine production \pm SEM.

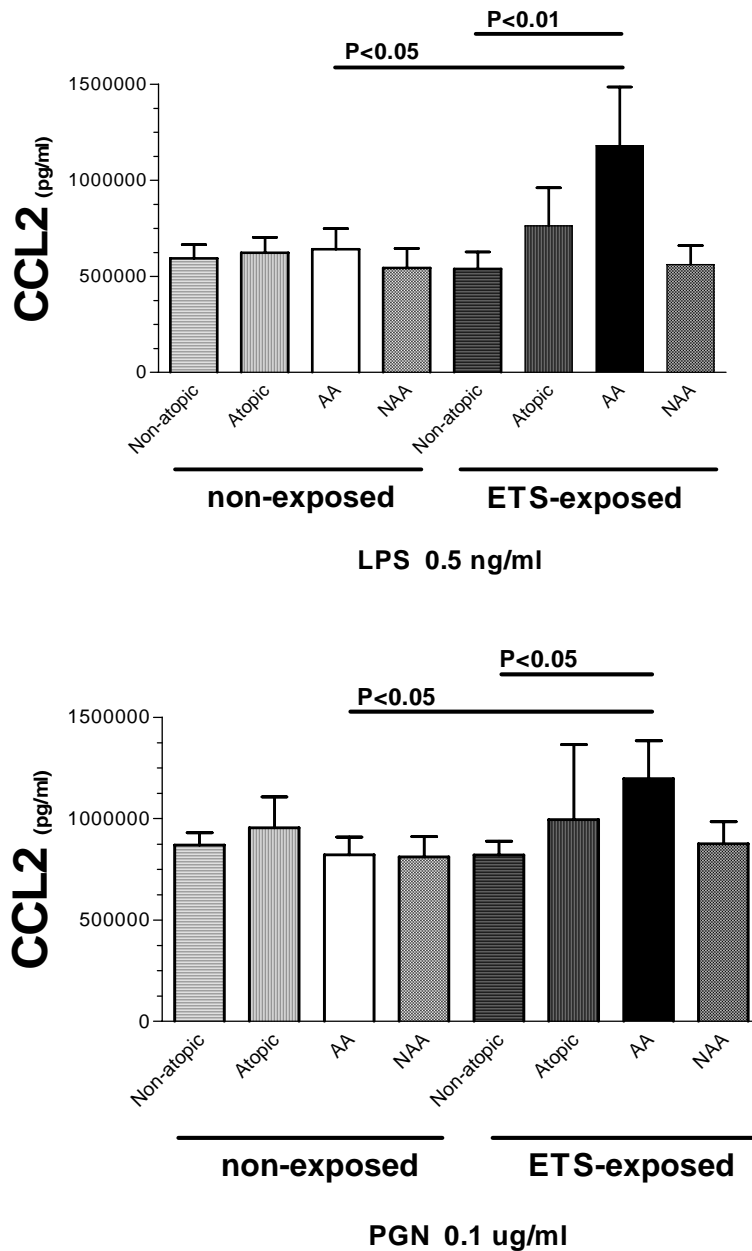


Figure 29. ETS exposures resulted in increased CCL2 responses to LPS or PGN in allergic asthmatics compared to non-ETS-exposed asthmatic and control groups. No differences are found between groups of children non-exposed to ETS. PBMC isolated from fresh peripheral blood were stimulated with LPS or PGN at the indicated concentrations for 24h. Levels of CCL2 in culture supernatants were measured using ELISA. Data are expressed as mean chemokine production \pm SEM.

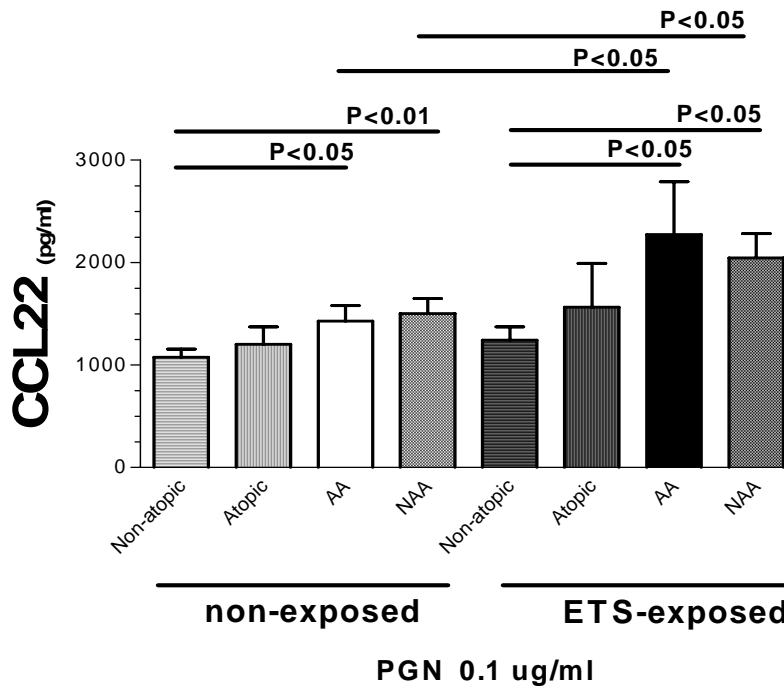
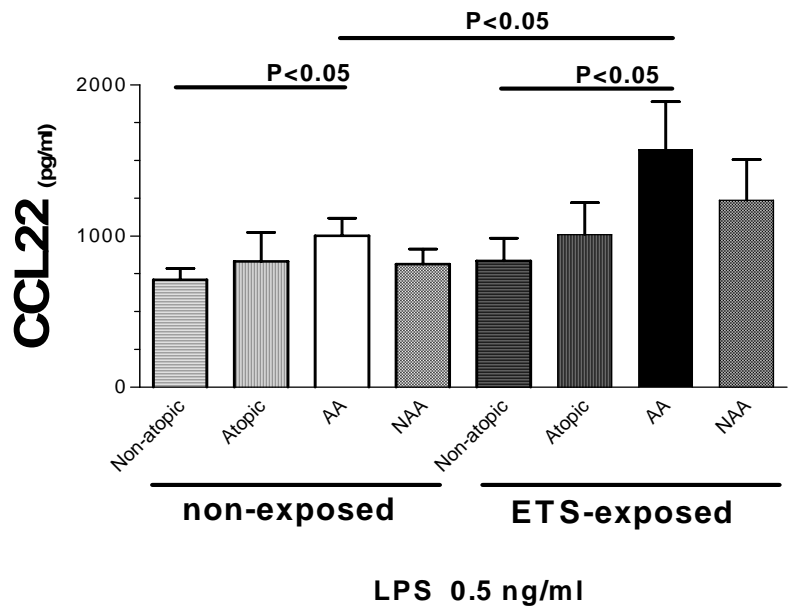


Figure 30. Exposure to ETS markedly augments CCL22 responses to LPS or PGN in asthmatic children relative to unexposed asthmatics and controls. PBMC were cultured in the presence of LPS or PGN at the indicated concentrations for 24 hours. Culture supernatants were assayed for TNF-alpha protein level by ELISA. Data are expressed as mean chemokine production \pm SEM.

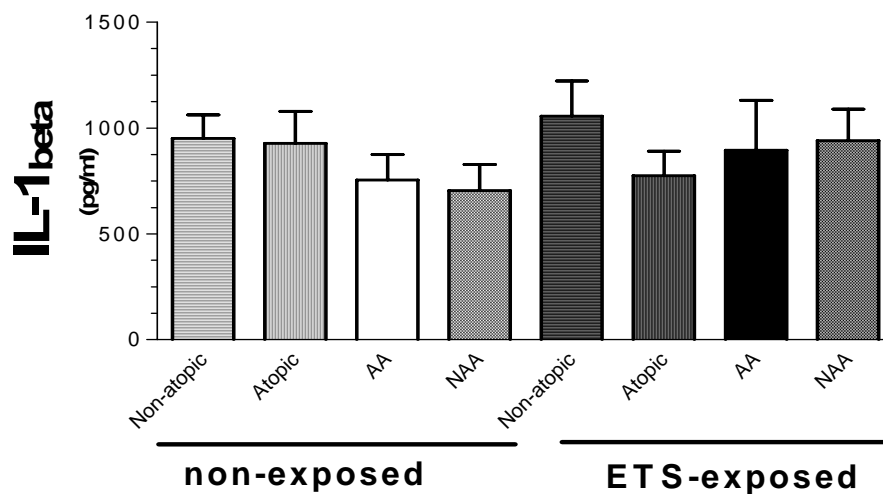
Furthermore, children with asthma whose parents smoke exhibit markedly increased levels of CCL22 relative to that seen in asthmatics unexposed to ETS ($p < 0.05$). These findings show that exposure to ETS augments CCL22 production in response to LPS or PGN stimulation in asthmatic children.

3M-011-stimulated secretion of proinflammatory cytokines, IL-1 β (Figure 31), TNF- α and IL-6 (data not shown), is equivalent among children with asthma, asymptomatic allergic and non-atopic controls as well as between ETS-exposed and non-ETS-exposed groups ($p > 0.05$).

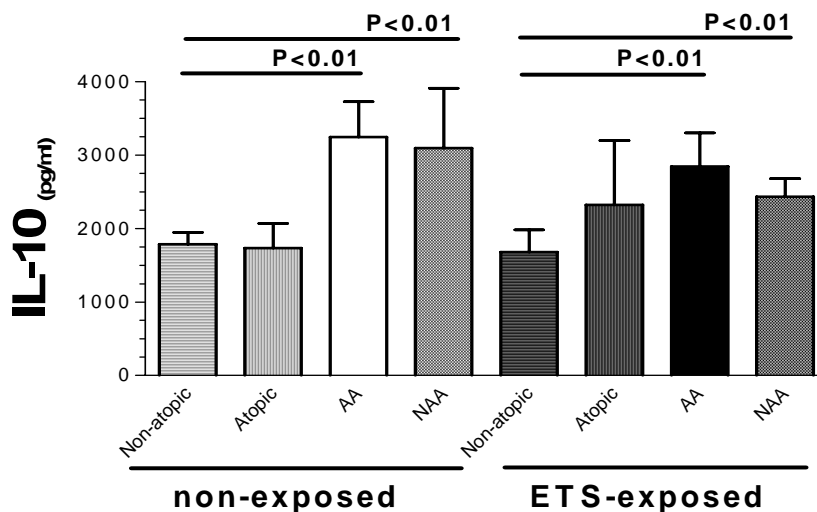
IL-10 production to stimulation with 3M-011 are increased in asthmatic children of smoking parents compared with non-atopic controls exposed to ETS ($p < 0.01$) (Figure 31). However, IL-10 responses do not differ detectably between ETS-exposed and non-ETS-exposed groups ($p > 0.05$). This suggests that 3M-011-elicited IL-10 synthesis is not modified by ETS exposures.

CCL2 responses to 3M-011 are markedly higher among children with allergic asthma exposed to ETS than those in non-ETS-exposed allergic asthmatic and non-atopic control groups ($p < 0.01-0.05$), indicating that ETS exposures increase CCL2 production in response to 3M-011 stimulation in allergic asthmatics (Figure 32).

As demonstrated in Figure 33, exposure to passive smoking results in elevated levels of CCL2 in response to poly(I:C) stimulation among allergic asthmatics compared to non-ETS-exposed children with allergic asthma and non-atopic controls ($p < 0.05$).



3M-011 0.5 ug/ml



3M-011 5.0 ug/ml

Figure 31. ETS exposures do not alter proinflammatory, IL-1beta, as well as anti-inflammatory, IL-10, cytokine responses in non-atopic, asymptomatic allergic (atopic) and asthmatic children. PBMC isolated from fresh peripheral blood were stimulated with 3M-011 at 0.5 ug/ml for 24h. Levels of cytokines in culture supernatants were quantified using ELISA. Data are expressed as mean cytokine production \pm SEM.

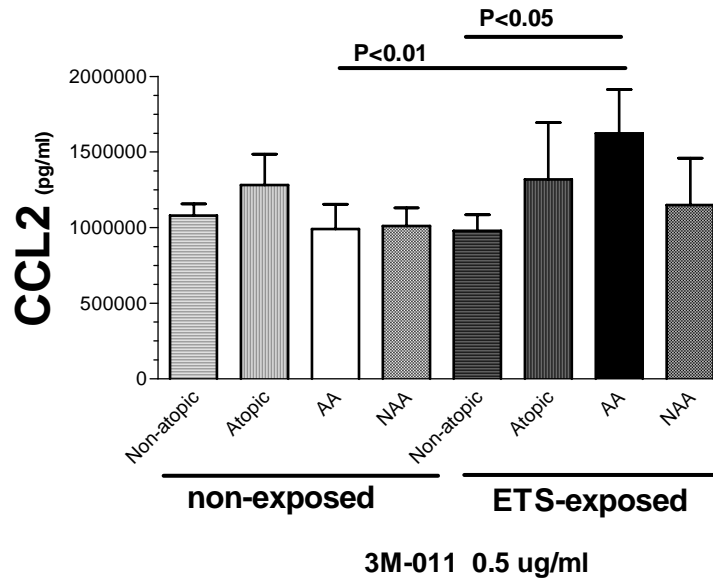


Figure 32. Exposure to passive smoking enhances CCL2 production in response to 3M-011 in children with allergic asthma relative to non-ETS-exposed allergic asthmatics and non-atopic controls. Cultures were established as described for Figure 6, levels of CCL2 in culture supernatants were measured by ELISA. Data are expressed as mean chemokine production \pm SEM.

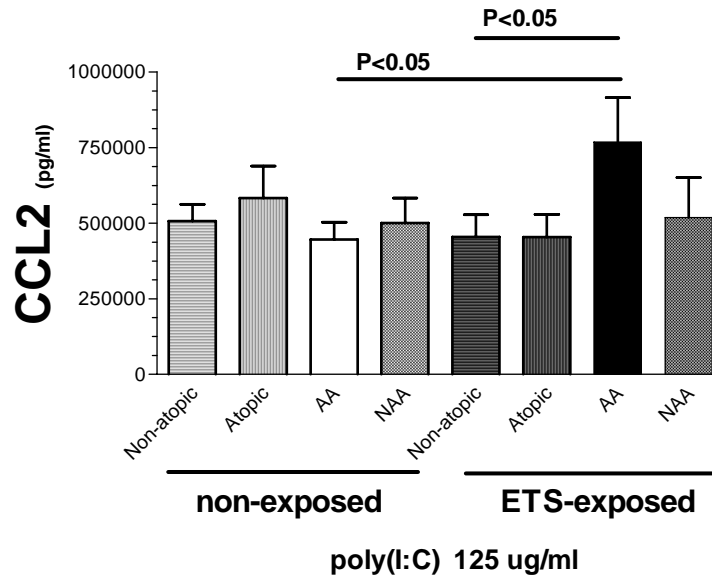


Figure 33. ETS exposures increase CCL2 responses to poly(I:C) stimulation among allergic asthmatics compared to non-ETS-exposed children with allergic asthma and non-atopic controls. PBMC isolated from fresh peripheral blood were cultured in the presence of poly(I:C) at 125 ug/ml for 24 hours. Culture supernatants were assayed for CCL2 protein level using ELISA. Data are expressed as mean chemokine production \pm SEM.

Taken together, these results show that environmental tobacco smoke exposures augment chemokine responses to Toll-like receptor stimulation in children with asthma. Specifically, asthmatic children exposed to ETS exhibit markedly increased levels of chemokines such as CCL22 (Th2-associated), CCL2 (pro-inflammatory), compared to non-ETS-exposed asthmatics. Increased production of these chemokines may contribute to asthma in part by influencing the migration of leukocytes (by CCL2) and Th2 cells (by CCL22) to the lung, thereby further promoting and amplifying inflammation.

3.6 Cytokine responses to Toll-like receptor stimulation differentiate First Nation vs Caucasian children

Epidemiological data clearly indicate marked ethnic differences in the prevalence for certain infectious, allergic and autoimmune diseases between First Nation and Caucasian populations in Canada (Blackwood e.a., 2003; Peschken, Esdaile, 1999; Senthilselvan e.a., 2003). However, mechanisms underlying ethnically distinct influences on disease susceptibility remain largely unclear. Toll-like receptors play key role in immune protection and have been implicated in the development of several infectious and inflammatory conditions. Possible impact of ethnicity on the function of TLRs is as yet undefined.

Herein, we tested the hypothesis that functional responsiveness to TLR stimulation by threshold concentrations of ligands differs in First Nation versus Caucasian children.

This study was conducted with 128 healthy, randomly recruited 8-9 year old First Nation and Caucasian children. First Nation subjects (n=43) were all residents of rural areas. Caucasian children in accordance with their domicile were grouped into Caucasian rural (n=44) and Caucasian urban (n=41) populations. All study participants had no history of allergic diseases or other serious conditions; also they were free of symptomatic respiratory infections in the two months preceding the study. None of them received anti-inflammatory or immunosuppressive drugs.

PBMC derived from children were stimulated with TLR ligands: PGN, LPS, 3M-011, or CpG ODN 2216 at “threshold” concentrations for 24 hours. We previously established highly sensitive experimental systems using “threshold” doses of TLR ligands rather than the typically used “maximal stimulation” concentrations to detect potential differences in TLR responsiveness between distinct populations. This novel approach is employed to test our hypothesis in the present study.

Levels of anti-inflammatory (IL-10), pro-inflammatory (IL-1beta, TNF-alpha, IL-6, CCL2) and Th1-associated (IFN-gamma, CXCL10) cytokines and chemokines were measured using ELISA.

As demonstrated in Figure 34, First Nation children exhibit markedly increased IL-10 synthesis upon stimulation with PGN compared to either Caucasian rural or urban populations ($p < 0.05$; $p < 0.01$, respectively). IL-10 production in response to LPS is equivalent among First Nation and Caucasian children ($p > 0.05$).

Caucasian populations, both rural and urban groups, produce higher levels of IL-6 in response to PGN relative to First Nation population ($p < 0.05$; $p < 0.01$, respectively) (Figure 35). LPS-induced IL-6 secretion does not differ detectably among the groups studied ($p > 0.05$).

IL-1beta responses to stimulation with either PGN or LPS are indistinguishable among First Nation and Caucasian children ($p > 0.05$) (Figure 36)

TNF-alpha production in response to PGN is enhanced among Caucasian children in comparison with First Nation subjects ($p < 0.05$) (Figure 37). No differences are observed in LPS-elicited TNF-alpha levels between the groups ($p > 0.05$).

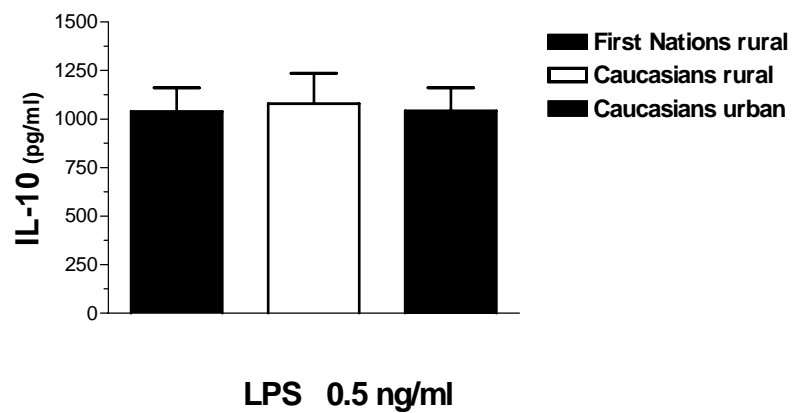
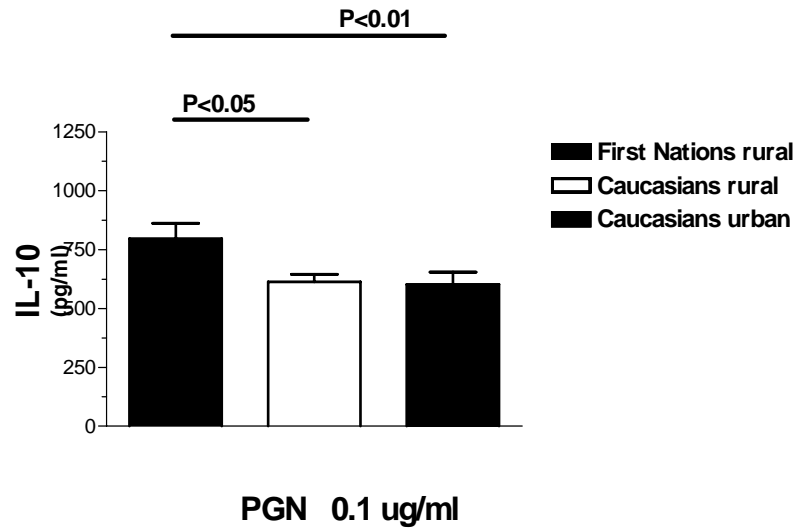


Figure 34. IL-10 responses to stimulation with PGN are elevated among First Nation children relative to Caucasians. PBMC obtained from fresh peripheral blood of healthy First Nation (n=43) and Caucasian rural (n=52) and urban (n=46) 7-8 year old children were stimulated with PGN or LPS at the indicated concentrations for 24h. Levels of IL-10 in culture supernatants were measured using ELISA. Data are expressed as mean cytokine production \pm SEM.

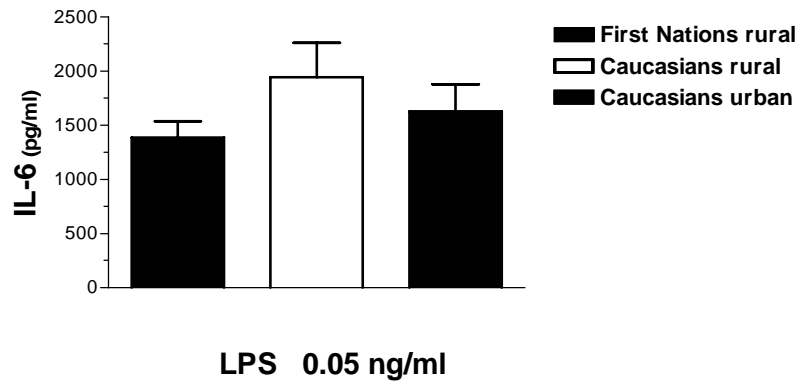
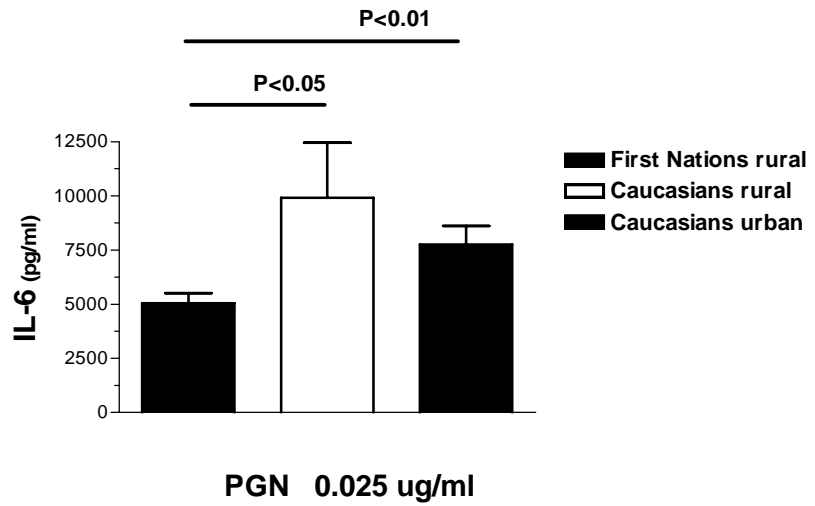


Figure 35. Caucasian children demonstrate increased synthesis of IL-6 in response to PGN compared with First Nation population. Cultures were established as described for Figure 1, culture supernatants were assayed for IL-6 protein level by ELISA. Data are expressed as mean cytokine production \pm SEM.

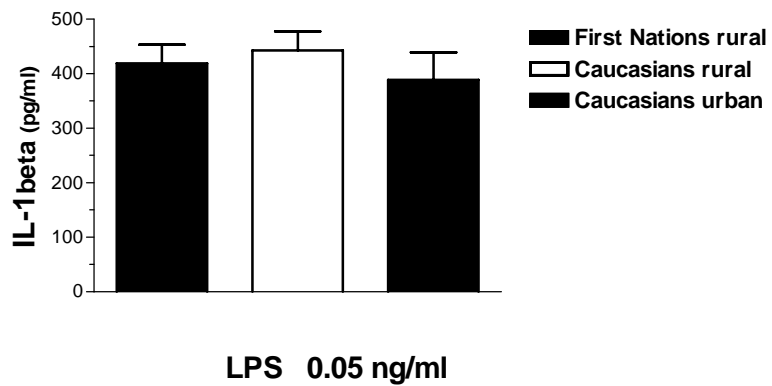
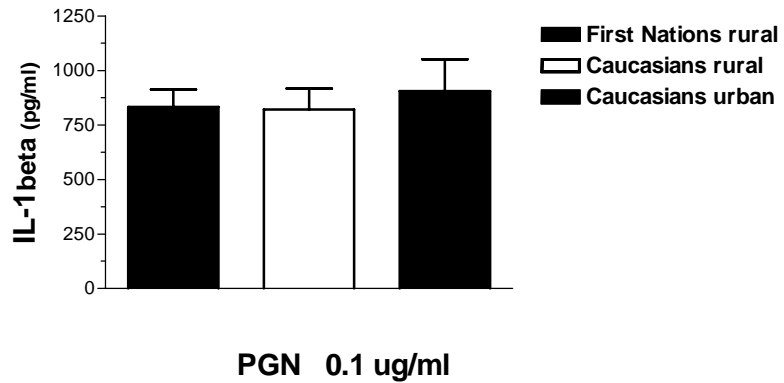


Figure 36. IL-1beta production on stimulation with either PGN or LPS does not detectably differ among First Nation and Caucasian children. PBMC were cultured as described for Figure 1, levels of IL-1beta in culture supernatants were quantified by ELISA. Data are expressed as mean cytokine production \pm SEM.

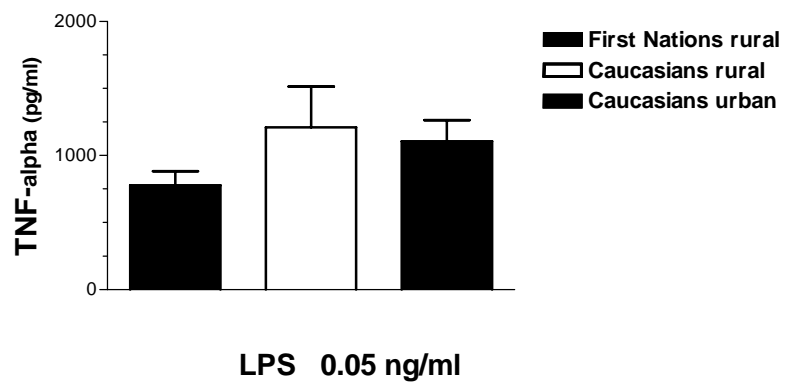
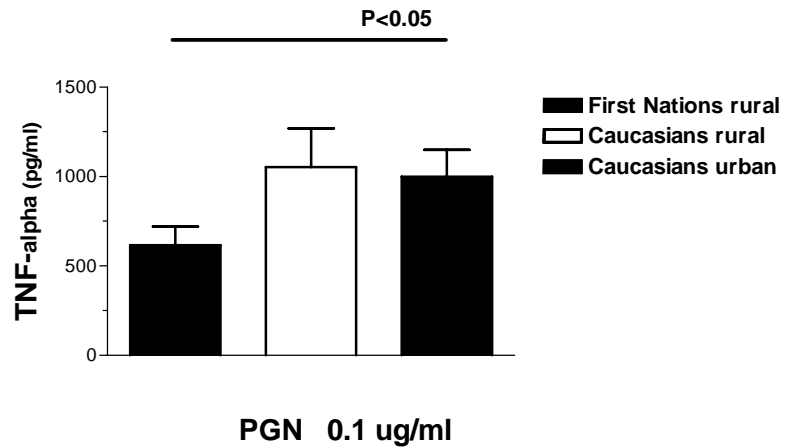


Figure 37. Caucasian populations produce higher levels of TNF- α in response to PGN in comparison with First Nation children. Cultures were established as described for Figure 1, levels of TNF- α in culture supernatants were measured using ELISA. Data are expressed as mean cytokine production \pm SEM.

Both Caucasian populations, rural and urban, exhibit greatly elevated levels of CCL2 upon stimulation with either PGN or LPS relative to First Nation children ($p < 0.001 - 0.05$) (Figure 38).

As evident from Figure 39, Caucasian children, both living in rural and urban areas, produce markedly increased IFN-gamma levels in response to 3M-011 compared with First Nations ($p < 0.05$; $p < 0.0001$, respectively). There are no significant differences in 3M-011-stimulated production of IL-10 and IL-6 (Figure 40), TNF-alpha, IL-1beta, CCL2 (data not shown) among the groups ($p > 0.05$). TLR9 stimulation with CpG ODN 2216 resulted in greatly higher CXCL10 responses among Caucasians relative to First Nation population ($p < 0.05$) (Figure 41). These data indicate that Th1-associated cytokine (IFN-gamma) and chemokine (CXCL10) responses are influenced by ethnicity.

These findings show that ethnicity influences anti-inflammatory (IL-10), and pro-inflammatory cytokine/chemokine (IL-6, TNF-alpha, CCL2) responses to TLR2 stimulation.

Taken together, our results demonstrate remarkable differences in functional TLR responsiveness between ethnically distinct populations: First Nation vs. Caucasian. Specifically, First Nation children favor anti-inflammatory IL-10 responses to TLR2 stimulation. Conversely, Caucasian population respond to TLR (2, 4, 8, 9) activation by production of more robust pro-inflammatory and Th1 biased cytokine/chemokine responses. These distinct cytokine profiles observed between the populations examined might in part explain ethnic differences in the risk of developing certain infectious and inflammatory diseases.

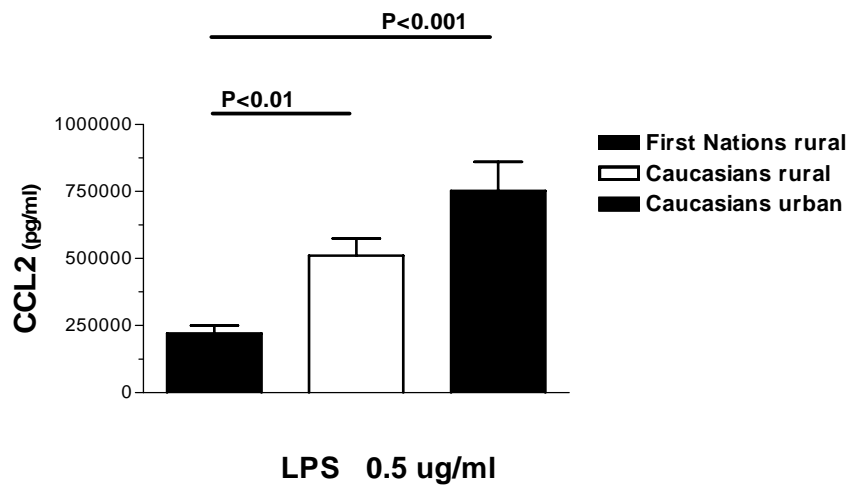
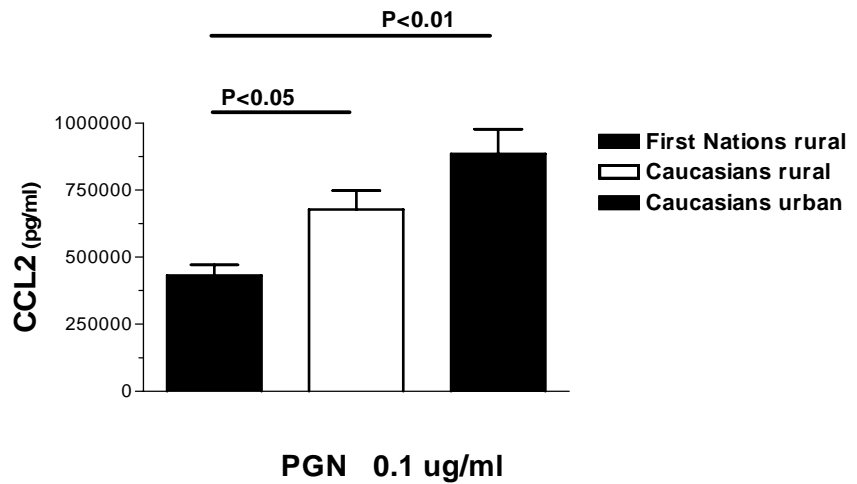


Figure 38. CCL2 responses to either PGN or LPS are elevated in Caucasians relative to First Nation children. PBMC were cultured as described for Figure 1, levels of CCL2 in culture supernatants were quantified by ELISA. Data are expressed as mean chemokine production \pm SEM.

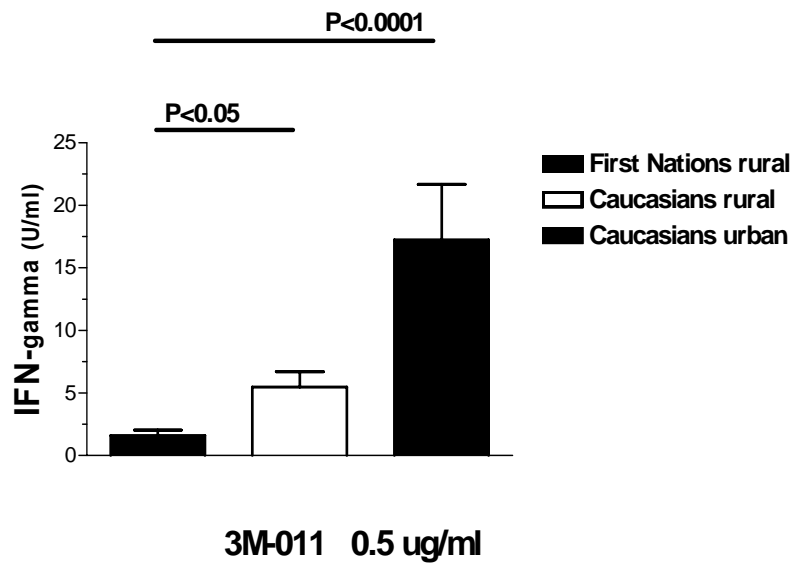


Figure 39. Caucasian populations exhibit increased IFN-gamma synthesis in response to 3M-011 compared to First Nation population. PBMC were cultured in the presence of 3M-011 at 0.5 ug/ml for 24 hours. Culture supernatants were assayed for IFN-gamma protein level using ELISA. Data are expressed as mean cytokine production \pm SEM.

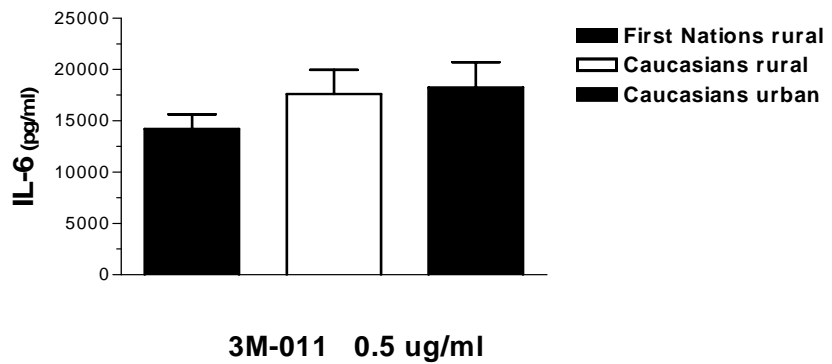
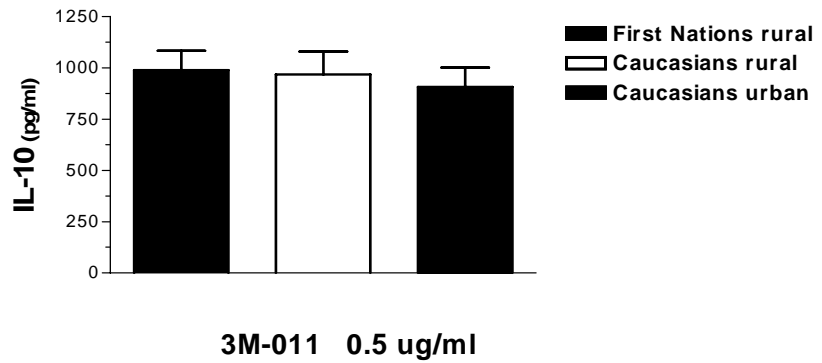


Figure 40. IL-10 and IL-6 responses to stimulation with 3M-011 are equivalent among First Nation and Caucasian populations. Cultures were established as described for Figure 7, levels of these cytokines in culture supernatants were measured using ELISA. Data are expressed as mean cytokine production \pm SEM.

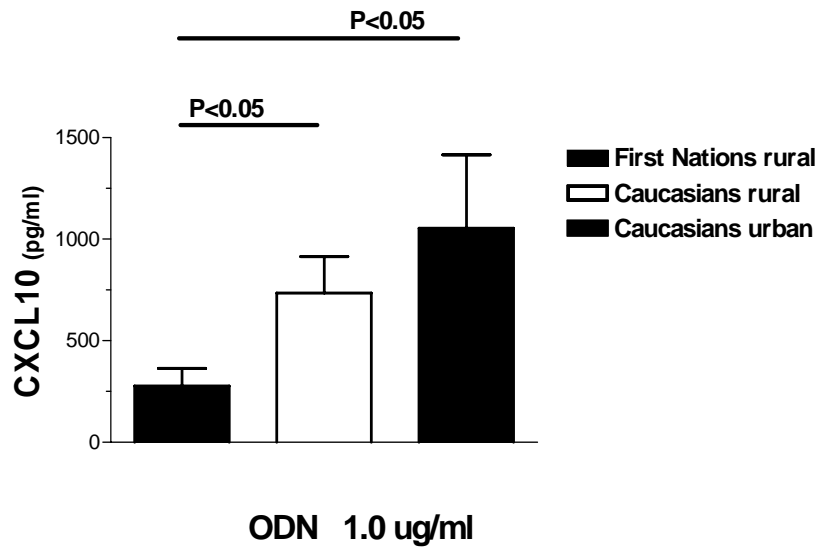


Figure 41. Caucasian children produce higher levels of CXCL10 in response to CpG ODN 2216 compare to First Nation population. PBMC were stimulated with CpG ODN 2216 at 1.0 ug/ml for 24 hours. Levels of CXCL10 in culture supernatants were quantified by ELISA. Data are expressed as mean chemokine production \pm SEM.

4 DISCUSSION

4.1 Differential Toll-like receptor responsiveness is revealed in asthmatic versus non-atopic children using threshold levels of TLR stimulation

In the present proof-of-concept study aimed at testing our hypothesis of a novel approach to enhance sensitivity, we demonstrated that use of threshold concentrations of TLR4 and TLR2 ligands stimulation revealed striking differences in cytokine responses between asthmatic and non-atopic control children. Specifically, non-atopic controls produce higher levels of pro-inflammatory cytokines IL-1beta and TNF-alpha, whereas asthmatics exhibit greatly increased anti-inflammatory IL-10 responses. In contrast, commonly used maximal concentrations of LPS and PGN elicit strong, indistinguishable patterns of cytokine production.

Tumor necrosis factor-alpha and Interleukin-1beta are key mediators of inflammation with a broad spectrum of effects in immune responses and host defense (Rosenwasser, 1998; Thomas e.a, 2002).

A number of observations suggest that these cytokines might contribute to the severity of asthma and/or are involved in the exacerbation of the disease.

I. Tillie-Leblond e.a. (1999) demonstrated that patients with “status asthmaticus” had markedly higher levels of TNF-alpha and IL-1beta in bronchial compartment than either those with mild asthma or healthy volunteers.

Elevated concentrations of these pro-inflammatory cytokines were also found in bronchoalveolar lavage fluid (Broide e.a., 1992), sputum (Konno e.a., 1996) and serum (Koizumi e.a, 1995; Pellegrino e.a, 1996; Subratty, Hooloman, 1998; Yoshida e.a, 1996)

of symptomatic (during asthma attacks) versus asymptomatic asthmatics (those in stable conditions). In addition, V. Ackerman e.a. (1994) revealed that symptomatic asthmatic subjects had increased expression of TNF-alpha and IL-1beta in bronchial biopsies compared to the asymptomatic patients and normal controls.

Similarly, allergen challenge has been shown to result in substantial increase of pro-inflammatory cytokines in patients with allergic asthma. Levels of TNF-alpha, IL-1beta in bronchoalveolar lavage fluid (Virchow e.a., 1995, Virchow e.a., 1998) and a rise in IL-1beta release by bronchial epithelial cells (Hastie e.a., 1996) as well as TNF-alpha secretion by alveolar macrophages (Gosset e.a., 1991) *ex vivo* were found to be elevated following segmental allergen provocation in allergic asthmatic subjects with dual responses. Also, challenge with allergen in allergic asthmatics with documented late-phase reaction led to marked increases in the number of TNF-alpha mRNA positive cells in bronchoalveolar lavage fluid (Ying e.a., 1991) and in levels of TNF-alpha in the sputum (Keatings e.a., 1997). These findings point to a potential involvement of pro-inflammatory cytokines, TNF-alpha and IL-1beta, in the development of late asthmatic responses.

Recently, P.H. Howarth e.a. (2005) have studied a role of TNF-alpha in severe asthma by evaluating the synthesis of this cytokine in severe asthmatic patients (steroid-treated) vs. mild asthmatic subjects (non-steroid treated) vs. healthy controls. They have observed higher levels of TNF-alpha in bronchoalveolar lavage fluid as well as increased TNF-alpha mRNA expression and greater numbers of TNF-alpha-immunoreactive cells in bronchial biopsies of subjects with severe corticosteroid dependent disease. In contrast, no differences were evident between mild asthmatics and healthy individuals. These

findings led authors to conclude that the “increase in TNF-alpha seen in severe disease is a feature of more persistent and corticosteroid refractory asthma rather than asthma per se” (Howarth e.a., 2005).

Thus, it could be suggested that severe persistent asthma, exacerbations of disease, late asthmatic responses after allergen challenge are characterized by elevated levels of pro-inflammatory cytokines, TNF-alpha and IL-1beta. The up-regulation of these cytokines is not a hallmark of stable mild asthma (Howarth e.a., 2005).

In the present study we examined asthmatic children with mild to moderate disease in stable conditions. Subjects with severe asthma were not included in the investigation because they required treatment with anti-inflammatory and immunosuppressive medications. These medications would potentially interfere with analysis of cytokine and chemokine responses to TLR stimulation. It has been shown that glucocorticoids were capable of increasing the expression of TLR2, 3 and 4 on myeloid dendritic cells (Rozkova e.a., 2006) and TLR2 on respiratory epithelial cells (Homma e.a., 2004) in humans. Also, treatment with glucocorticoids has been found to enhance the ability of macrophages to produce TNF-alpha, IL-6, IL-12 in response to LPS (Zhang e.a., 2007) as well as to suppress IFN-alpha secretion by TLR stimulated plasmacytoid dendritic cells (Boor e.a., 2006).

Most previous studies, investigating pro-inflammatory cytokine production in response to LPS stimulation in vitro, revealed equivalent synthesis of IL-1 beta and TNF-alpha among allergic asthmatics and healthy controls. It is important to note that LPS was commonly used at high concentrations (1-10 ug/ml and higher) in cell cultures. The

results we obtained at our high concentrations support this finding of no apparent difference in cytokine response to TLR ligation.

It was also reported that LPS-stimulated (at 1-10 ug/ml) monocytes derived from asthmatics have the same ability to release IL-1beta and TNF-alpha as those of controls (Gosset e.a, 1988; Enk, Mosbech, 1988; Vecchiarelli e.a, 1992). The production of IL-1beta by alveolar macrophages in response to LPS (at 1 ug/ml) was found to be similar in asthmatic and healthy subjects (Gosset e.a., 1988). Equal levels of TNF-alpha secreted by whole blood to stimulation with LPS (at 10 ng/ml) were shown among severe asthmatics, patients with mild asthma and healthy individuals (Tomita e.a., 2002). M.M. Hagendorens e.a. (2004), examining intracellular synthesis of TNF-alpha and IL-1beta by dendritic cells in whole blood cultures after exposure to LPS (at 1 ug/ml) in vitro, did not observe any differences between asthmatics and non-atopic children. Similarly, several groups have found comparable levels of TNF-alpha and IL-1beta produced by PBMC of asthmatic patients and control subjects in response to LPS at high (10 ug/ml) concentrations (Borish e.a., 1996; Mitsuta e.a., 2003; Hew e.a, 2006). Our results obtained utilizing maximal concentrations of TLR ligands are consistent with these findings. This suggests that intense pro-inflammatory cytokine responses to maximal TLR stimulation do not discriminate asthmatic and healthy control subjects.

In the present study, we, using lower levels (“threshold” concentrations) of TLR ligands that more closely approximate natural environmental exposures, have detected marked differences in pro-inflammatory cytokine (IL-1-beta, TNF-alpha) responses between asthmatic and non-atopic control children.

Interleukin-10 is a pleiotropic cytokine with anti-inflammatory and immunoregulatory properties (Barnes e.a., 2001; Moore e.a, 2001). It is known to inhibit the production of a number of proinflammatory cytokines and chemokines (Berkman e.a., 1995; Berkman e.a., 1996; de Waal Malefyt e.a, 1991; Fiorentino e.a, 1991) and enhances the expression of some anti-inflammatory mediators, including IL-1 receptor antagonist (Cassatella e.a, 1994; Jenkins e.a., 1994) and soluble TNF receptors (Joyce e.a., 1994). This argues that IL-10 plays a pivotal role in orchestrating the inflammatory reaction.

IL-10 has also been shown to interfere with antigen presentation by monocytes/macrophages and dendritic cells to T cells by reducing their expression of MHC class II (de Waal Malefyt e.a, 1991; Peguet-Navarro e.a, 1994), co-stimulatory molecules (Ding e.a, 1993; Mitra e.a, 1995), and some adhesion molecules (Chang e.a., 1994; Chatelain e.a, 1998), thus indirectly inhibiting T cell activation.

In addition, it exerts some direct effects on T lymphocytes (Asadullah e.a, 2003). Specifically, IL-10 inhibits the antigen-specific proliferation of CD4⁺T cells (de Waal Malefyt e.a., 1993), and downregulates cytokine production by both Th1 and Th2 cells (de Waal Malefyt e.a., 1993; Del Prete e.a., 1993).

IL-10 is widely considered to play an important role in the regulation of allergic inflammation (Barnes e.a., 2001; Lamblin e.a, 2000). The production of IL-10 in human asthma was examined by a large number of studies. However, available data are apparently contradictory.

Several previous studies have provided evidence that IL-10 synthesis might be deficient in allergic asthma. Early report by L. Borish e.a. (1996) demonstrated reduced levels of this cytokine in bronchoalveolar lavage fluid, and its decreased production in

response to LPS at high (10 ug/ml) concentration by PBMC in allergic asthmatics. Similarly, release of IL-10 was shown to be diminished in whole blood cultures stimulated with LPS at 1 ug/ml (Hagendorens e.a., 2004) and PHA-activated PBMCs (Kenyon e.a., 2000) derived from allergic asthmatics.

Additionally, H. Koning e.a. (1997) found a decrease in IL-10 mRNA expression by peripheral blood T lymphocytes in asthmatic children. S. Takanashi e.a (1999) documented lower levels of IL-10 protein and reduced number of IL-10-expressing cells in the sputum of a group of subjects with allergic or non-allergic asthma compared to controls.

Some authors suggest that defective IL-10 synthesis might result in the development of severe persistent asthma. K. Tomita e.a (2002) observed attenuated IL-10 release in whole blood cultures on LPS stimulation and decreased production of intracellular IL-10 in monocytes of patients with severe allergic asthma vs. mild asthmatics and healthy controls. Another recent study by K. Matsumoto e.a. (2004) has shown that individuals with severe unstable asthma had a lower frequency of IL-10-producing CD4⁺ T lymphocytes in peripheral blood relative to asthmatics with mild disease. Decreased IL-10 levels were also seen in serum of patients with severe atopic dermatitis (Niwa e.a., 2000). Thus, the impairment of IL-10 production might contribute to the severity of asthma.

These results are in contrast to our current findings that asthmatic children exhibit increased levels of IL-10 in response to “threshold” levels of TLR stimulation. Possible explanations could include differences in patient populations studied (we have predominantly examined mild asthmatics whereas other abovementioned studies

investigated mostly individuals with severe asthma), and differences in experimental systems and levels of TLR stimulation employed (low threshold concentrations of ligands we used vs. high pharmacological doses of stimuli utilized in previous reports).

Several other groups have also obtained evidence of increased IL-10 in allergic asthma. A. Magnan e.a. (1998) demonstrated elevated production of this cytokine by LPS-stimulated alveolar macrophages in allergic asthmatic adults compared to controls. Study by J.A. Long e.a (2004) showed that monocyte-derived dendritic cells from subjects with asthma also exhibited enhanced IL-10 secretion in response to LPS plus IFN-gamma.

D.S. Robinson e.a (1996), using in situ hybridization, found an increased number of IL-10 mRNA-positive cells in bronchoalveolar lavage fluid of allergic asthmatics. Also, overexpression of mRNA for IL-10 was reported in the sputum (Zeibecoglou e.a., 2000) and gut mucosa (Lamblin e.a, 2000), and an increase in IL-10-producing T cells was shown in the blood (Matsumoto e.a, 2002) of patients with allergic asthma. Furthermore, elevated levels of IL-10 protein have been found in asthmatic's serum (Wong e.a, 2001) and BAL fluid (Colavita e.a, 2000).

Some investigators examined IL-10 synthesis in allergic asthmatics after allergen challenge. They observed upregulated IL-10 transcripts in the bronchoalveolar lavage cells (Borish e.a., 1996) as well as a rise in IL-10 release from sputum cells ex vivo (Bettiol e.a., 2002) obtained during late phase asthmatic reaction. Correspondingly, peripheral blood monocytes exhibited augmented mRNA for IL-10 and IL-10 secretion to LPS stimulation in asthmatic patients with dual responses (Lim e.a., 2000). The increase

in IL-10 levels following allergen challenge likely indicates a negative-feedback response to allergic inflammation.

It is worth noticing, that several reports also demonstrated an equivalent synthesis of IL-10 among patients with allergic asthma and non-atopic control subjects. M. John e.a. (1996), examining IL-10 mRNA expression and IL-10 release by monocytes to LPS stimulation at 1.0 ug/ml, did not observe any difference between allergic asthmatics and healthy individuals.

S. Saeki e.a (2004) have shown that immature dendritic cells obtained from patients with atopic disease (asthma, rhinitis, dermatitis) have virtually the same capacity to secrete IL-10 in response to specific *D. farina* allergen as those from controls. Equal quantities of IL-10 released by PBMC in response to a panel of inhalant allergens (Bottcher e.a, 2003) or *S. aureus* (Chou e.a., 1999) were demonstrated in asthmatic and non-atopic children. Also, the concentration of this cytokine in BAL fluid (Tillie-Leblond e.a, 1999) and expression of mRNA for IL-10 in the sputum (Grissel e.a., 2005) were found to be similar in patients with stable mild allergic asthma and controls.

A recent study by M. Hew e.a. (2006) revealed equivalent production of IL-10 by PBMC in response to LPS at high concentrations (10 ug/ml) among subjects with severe asthma, asthmatics with non-severe disease and healthy volunteers (Hew e.a., 2006). These data are in line with our findings obtained using maximal concentrations of TLR ligands. This indicates that strong cytokine responses elicited by maximal TLR stimulation does not differentiate asthmatic and non-atopic populations.

Thus, available data on the expression and role of IL-10 in human asthma are highly controversial. While some reports have found reduced IL-10 production in

asthmatic patients, others have demonstrated elevated levels, or equivalent amounts of IL-10 in asthmatics compared to non-atopic control individuals. These discrepant results of previous studies might be in part associated with the variation in clinical status of study subjects (mild versus severe asthma), and with different experimental systems and conditions used (different high levels of LPS and time of culture).

Our results obtained employing threshold concentrations of TLR ligands demonstrate significantly increased production of anti-inflammatory/immunoregulatory IL-10 in asthmatic children. These data support the notion that IL-10 overexpression could represent a homeostatic mechanism to control inflammation in asthma (Heaton e.a., 2005; Robinson e.a., 1996; Bettiol e.a, 2002; Asadullah e.a., 2003). This hypothesis is strengthened by studies in animal models of asthma showing that IL-10 is capable of inhibiting allergen-induced airway inflammation and airway hyper-responsiveness (Tournoy e.a., 2000).

In summary, this strategy of using threshold levels of TLR stimulation provides markedly increased sensitivity to reveal functional alterations in TLR responsiveness that associate with clinical phenotype. Possible mechanisms responsible for this phenomenon have not been addressed in the present study and need further investigations.

It could be speculated that markedly differential responsiveness to TLR stimulation in asthmatic vs. non-atopic children might result from alterations (defects, deficiencies, imbalances) in intracellular TLR signaling pathways (NF-kappaB and/or mitogen-activated protein kinase (MAPK) pathways) in asthmatics. Maximal TLR activation with high doses of ligands could overcome these alterations in signal transduction resulting in similar cytokine production in asthmatic and non-atopic

children. In contrast, threshold levels of stimulation allow to detect these functional differences that correlate with clinical status.

Studies to test the hypothesis that activation of signaling pathways to TLR4 stimulation by threshold concentrations of ligand differ between asthmatic and non-atopic children is currently underway in our laboratory.

4.2 Environmental tobacco smoke exposures modify Toll-like receptor responsiveness in asthmatic children

Despite sufficient epidemiological evidence for the causal relationship between exposures to ETS and increased prevalence and severity of childhood asthma, the mechanisms underlying this phenomenon are still poorly understood.

In the present study, we demonstrated that ETS exposures augment chemokine responses to TLR stimulation in asthmatic children. Specifically, asthmatic children exposed to passive smoking exhibit markedly elevated levels of pro-inflammatory CCL2 and Th2-associated CCL22 chemokines relative to non-ETS exposed asthmatics and controls.

CCL2 was first characterized as a monocyte-chemoattracting protein (Matsushima, Oppenheim, 1989). The receptor for this chemokine, CCR2, is highly expressed on monocytes (Rollins, 1997). It is well established that CCL2 is a potent chemoattractant for monocytes. In addition, CCL2 has been shown to attract activated and memory T cells, NK cells and basophils (Daly, Rollins, 2003). Thus, CCL2/CCR2

system plays an important role in regulating inflammatory cell migration and recruitment (Rose e.a., 2003).

It has been shown that CCL2/CCR2 pair not only exerts its influences on innate immunity but also has effects on the differentiation of T helper cells.

Several observations have pointed to a possible association CCL2 with Th2 responses. S.W. Chensue e.a. (1995) demonstrated that antibody blockage of CCL2 reduced the size of *Schistosoma mansoni*-elicited pulmonary granulomas filled with eosinophils and type 2 cytokines in mice. In experiments in vitro, the addition of CCL2 to naïve CD4⁺ T cells stimulated with antigen was shown to drive their differentiation in a Th2 direction (Karpus e.a., 1997).

In murine models of allergic airway disease, neutralization of CCL2 led to attenuated cellularity in bronchial alveolar lavage fluid and diminished bronchial hyperreactivity (Gonzalo e.a., 1998). The same results have been observed in CCL2 $-/-$ knockout mice (Daly, Rollins, 2003). Again, the amelioration of asthma-like disease in the absence of CCL2 points to a potential role for this chemokine in promoting Th2 responses. The global defect in type 2 immunity in CCL2 $-/-$ mice was further confirmed by the relative resistance of CCL2-deficient mice to *Leishmania major* infection (Gu e.a., 2000).

On the contrary, other studies have found that CCR2, a receptor for CCL2, may be involved in Th1 development. W. Peters e.a. (2000) reported that sensitized T cells derived from CCR2 $-/-$ mice in response to in vitro re-challenge produced far less IFN- γ , but equivalent amounts of IL-5 relative to those from wild-type mice. Additionally, it was shown that CCR2-deficient mice were more susceptible to

Leishmania major infection (Daly, Rollins, 2003) and have normal (MacLean e.a., 2000) or increased (Kim e.a., 2001) cellularity and bronchial hyperreactivity in allergic airway hypersensitivity models.

Thus, studies on mouse models demonstrated that CCL2 and CCR2 exhibit disparate effects on T-helper cell polarization: CCL2 stimulates Th2 responses, whereas CCR2 activation favors Th1 development.

In contrast to mouse models, there is no evidence so far that CCL2 influences Th2 cell differentiation in humans. Instead, M.C. Lebre e.a. (2005) demonstrated that monocyte-derived mature Th1- and Th1/Th2-promoting dendritic cells, but not Th2-promoting dendritic cells, selectively produced elevated levels of CCL2.

Thus, available data indicate a complicated role of the CCL2-CCR2 axis in adaptive immunity. The role of CCL2 in asthma requires discussion.

We found similar levels of CCL2 production in response to TLR stimulation among unexposed to ETS asthmatic, asymptomatic allergic and non-atopic children. Some previous observations are not in line with these data. Early human studies demonstrated elevated levels of CCL2 in bronchoalveolar lavage fluid obtained from subjects with asthma (Alam e.a., 1996; Jahnz-Rozyk e.a., 1997). Increased expression of CCL2 has also been documented in bronchoalveolar lavage fluid cells (Miotto e.a., 2001), sputum cells (Cataldo e.a., 2004) and bronchial tissue (Sousa e.a., 1994) of non-smoking asthmatic patients.

This disagreement might be partially explained by the usage of different experimental test systems. While most aforementioned studies investigated CCL2 expression, we examined the production of CCL2 in response to TLR stimulation. It is

known that the expression, even at protein level, can not always be extrapolated to a functional phenotype.

The results of some functional studies are compatible with our current findings. S.G. Folkard e.a. (1997) observed comparable CCL2 secretion by LPS-stimulated bronchoalveolar lavage cells in asthmatic and healthy individuals. M. Reale e.a. (2001) revealed that PHA-activated PBMC derived from asymptomatic atopics have virtually the same capacity to produce CCL2 as those from non-atopic controls. Thus, our data obtained in pediatric populations complement these observations by showing that TLR stimulated CCL2 responses do not differentiate atopic as well as stable asthmatic status.

Importantly, several studies showed that CCL2 might contribute to the inflammatory process in human asthma and be involved in late asthmatic responses.

S.T. Holgate e.a. (1997) observed elevated concentrations of CCL2 in bronchoalveolar lavage fluid as well as greatly increased release of this cytokine by bronchoalveolar lavage cells ex vivo in asthmatic patients following segmental allergen challenge. However, these steady-state asthmatics exhibited an equal CCL2 levels relative to healthy controls.

I. Tillie-Leblond e.a. (2000) documented markedly higher levels of CCL2 in bronchial compartment in patients with “status asthmaticus”. In addition, animal studies showed substantially increased levels of CCL2 in the lung tissue during allergic airway inflammation in mice sensitized intraperitoneally and challenged via airway with allergen (Kozma e.a., 2003).

Moreover, accumulating evidence implicates CCL2 in the pathogenesis of chronic obstructive pulmonary disease (COPD) in smokers. S.L. Traves e.a. (2002) demonstrated

that smoking patients with this inflammatory disorder of the lung (COPD) had elevated concentrations of CCL2 in induced sputum compared to healthy smokers and non-smokers.

S. Fuke e.a. (2004) showed that having smoking-induced airflow limitations and/or emphysema (early stage COPD) are associated with an increased expression of mRNA for CCL2 in bronchial epithelial cells. Similar findings have been reported by M. Tomaki e.a. (2006).

W.I. de Boer e.a. (2000), using an in situ hybridization technique, found higher mRNA and protein expression levels for CCL2 in bronchial epithelium and alveolar macrophages in smokers with COPD compared with those seen in smoking and ex-smoking individuals without disease. The expression of CCL2 mRNA in bronchial epithelial cells correlated with both CCR2 expression in alveolar macrophages and mast cells, pointing to a possible involvement of this chemokine and its receptor in the recruiting of macrophages and mast cells into the airway epithelium in COPD.

Thus, taken together, these observations suggest that CCL2 (i) might play a contributory role in the inflammatory process in asthmatics; and (ii) participates in the airway inflammation associated with COPD in smoking patients.

In the present study, we first provide evidence that exposure to ETS augmented CCL2 responses to TLR simulation in allergic asthmatic children compared to non-ETS-exposed asthmatics and controls. It is tempting to speculate that increased production of CCL2 as a result of alteration of TLR responsiveness caused by exposures to ETS in asthmatics could potentially promote pre-existing allergic airway inflammation.

Possible mechanisms through which CCL2 might contribute to allergic inflammation are (i) by mediating the inflammatory cell recruitment to the lung compartments and (ii) by stimulating the effector cells to release inflammatory mediators into the airways.

CCL2 is known to be potent chemoattractant for monocytes and basophils (Daly, Rollins, 2003). Gonzalo e.a. (1999) demonstrated that upregulation of CCL2 in the lungs was temporally correlated with emigration of monocytes and macrophages in the airways in a murine model of allergic airway disease. Local alveolar deposition of exogenous CCL2 has been found to elicit a monocytic influx into the alveolar air spaces in mice (Maus e.a., 2001). P. Conti e.a. (1997) reported that CCL2 is capable of inducing a migration of basophils in rat skin injection sites.

Several studies showed a potent mediator-releasing activity of CCL2. E.M. Campbell e.a. (1999) demonstrated that neutralization of CCL2 during the allergic airway response in mice decreased levels of histamine and leukotriene C4 in bronchoalveolar lavage, whereas instillation of CCL2 into the airway of normal mice caused an increase in the release of these mediators into bronchoalveolar lavage fluid. In addition, CCL2 has been found to directly mediate degranulation of pulmonary mast cells in vitro.

Similarly, others demonstrated the ability of CCL2 to induce histamine secretion by basophils and mast cells in humans (Alam e.a., 1992; Bischoff e.a., 1992) and to cause mast cell aggregation in vitro (Conti e.a., 1995).

Thus, on the basis of our findings and available literature data we could speculate that passive smoking modifies TLR responsiveness in asthmatic children; this leads to enhanced production of CCL2 that might contribute to asthma pathogenesis in part by

recruiting inflammatory cells (monocytes, basophils) and activating the effector cells (mast cells, basophils) to secrete inflammatory mediators (histamine, leukotrienes) into the lung, thus further promoting and amplifying inflammation.

Next, we demonstrated increased CCL22 responses to stimulation with TLR4 or TLR2 ligands among allergic asthmatics, both exposed and unexposed to ETS, compared to non-atopic controls. Furthermore, asthmatic children of smoking parents exhibit markedly elevated levels of CCL22 relative to that seen in asthmatics unexposed to ETS. These data show that passive smoking augments CCL22 production in response to TLR4 and TLR2 stimulation in asthmatic children.

CCL22 was first described as a macrophage-derived chemokine (Godiska e.a., 1997). It specifically acts on its functional receptor, CCR4 (Imai e.a., 1997). Since CCR4 is predominantly expressed by Th2 cells, CCL22 is considered to be key mediator of Th2 lymphocyte recruitment into sites of inflammation (Bonicchi e.a., 1998; Imai e.a., 1999). Additionally, CCL22 has been demonstrated to serve as potent chemoattractant for macrophages, monocytes, monocyte-derived dendritic cells, and NK cells (Godiska e.a., 1997).

At present, available data implicate CCL22 in the pathogenesis of asthma. In fact, highly upregulated expression of CCL22 was reported in animal models of allergic airway inflammation (Gonzalo e.a., 1999; Ritter e.a., 2005). In these studies, neutralization of CCL22 by specific blocking antibodies resulted in prevention of bronchial hyperreactivity and abrogation of pulmonary inflammation. Also, C.M. Lloyd e.a. (2000) established that CCL22/CCR4 axis mediates the long-term recruitment of Th2 cells to the lung under conditions of repeated allergen stimulation in vivo. Thus, CCL2

may be pivotal chemokine for the development of Th2-dominated experimental allergic inflammation.

Some human studies have revealed an increased expression of CCL22 and its receptor CCR4 in patients with pre-established asthma. H. Hammad e.a. (2003) showed that myeloid dendritic cells derived from allergic asthmatics enhance CCL22 production after stimulation with specific allergen Der p 1 whereas the challenge of myeloid dendritic cells of healthy donors did not affect CCL22 synthesis. Another study by H. Hirata e.a (2003) reported an augmented secretion of CCL22 by naïve CD4⁺ T lymphocytes activated with anti-CD3 and anti-CD28 antibodies in asthmatic patients. Much higher levels of CCL22 have also been detected in bronchoalveolar lavage fluid of steady-state adult asthmatics (Lezcano-Meza e.a, 2003) and asthmatic children (Hartl e.a., 2005) compared to healthy individuals.

These findings are consistent with our data showing increased CCL22 responses to TLR4 and TLR2 stimulation in allergic asthmatic children relative to controls. Thus, the current study provides additional evidence/further support for the involvement of CCL22 in the development of asthma in pediatric population.

Several groups have shown that CCL22 might contribute to the severity of asthma and late-phase asthmatic responses.

Elevated CCL22 levels in exhaled breath condensate (Ko e.a., 2006; Leung e.a., 2004), bronchoalveolar lavage fluid (Lezcano-Meza e.a, 2003), plasma (Leung e.a., 2004) have been found to be strongly associated with severe asthmatic phenotype. Similarly, an increase in the number of CCR4⁺ T cells, expressing the receptor for

CCL22, has been reported to be linked to the severity of the disease. (Kurashima e.a., 2006).

Highly enhanced levels of CCL22 in bronchoalveolar lavage fluid (Bochner e.a., 2003; Liu e.a., 2004; Pilette e.a., 2004) as well as substantial upregulation of CCL22 in airway epithelial cells (Panina-Bordignon e.a., 2001) have been observed in asthmatic patients during the late reaction in response to allergen challenge. Of note, CCL22 concentration in bronchoalveolar lavage has been closely related to the levels of Th2 cytokines such as IL-5, IL-13 (Liu e.a., 2004; Pilette e.a., 2004).

In addition, allergen challenged asthmatics demonstrated markedly increased numbers of CCR4-expressing T cells in the lungs: in bronchoalveolar lavage fluid (Kallinich e.a., 2005), airway mucosa (Panina-Bordignon e.a., 2001) and bronchial tissue (Nouri-Aria e.a., 2002).

Collectively, these findings suggest that CCL22/CCR4 system plays an important role in the pathogenesis of inflammation in asthma

Although available data regarding the effect of cigarette smoking on the expression of CCL22 are scarce, one study (Ritter e.a., 2005) found an elevated expression of CCL22 mRNA in the lungs of rats with cigarette smoke-induced pulmonary inflammation. This raises the possibility that CCL22 may be involved in chronic inflammation caused by cigarette smoke exposure.

In the present study, we first obtained evidence for enhanced CCL22 responses to TLR4 and TLR2 stimulation in asthmatic children exposed to ETS relative to non-ETS-exposed asthmatics and controls.

It might be speculated that augmented CCL22 production in consequence of modification of TLR responsiveness caused by passive smoking in asthmatic children could potentially lead to an amplification of allergic airway inflammation already present in asthmatics.

Plausible mechanisms through which CCL22 contributes to the inflammatory process in asthma might be (i) by selectively recruiting Th2 lymphocytes into the lungs (Bonecchi e.a., 1998; Imai e.a., 1999; Panina-Bordignon e.a., 2001) and (ii) by maintaining local Th2-cell dominance being/representing a crucial part of the amplification loop of polarized type 2 responses (Bonecchi e.a., 1998; Lezcano-Meza e.a., 2003; Mantovani e.a., 2000).

As discussed above, CCL22 is known to be a potent chemoattractant of Th2 cells and orchestrate their selective migration into asthmatic airways (Bonecchi e.a., 1998; Imai e.a., 1999; Panina-Bordignon e.a., 2001).

Hence, increased production of CCL22 in asthmatics (markedly higher in those exposed to ETS) results in more intensive trafficking of Th2 lymphocytes into the lung compartments. Having recruited in sites of allergic inflammation, Th2 cells produced type 2 cytokines, namely IL-4 and IL-13. These cytokines may trigger release of CCL22 that, in turn, recruits Th2 lymphocytes into asthmatic airways (as amplification circuit) (Bonecchi e.a., 1998; Lezcano-Meza e.a., 2003; Liu e.a., 2004; Mantovani e.a., 2000). The perpetuation of this Th2 milieu could then contribute to the persistence of allergic airway inflammation.

Thus, based upon the data of our study and published literature we propose the following scenario.

Alterations in functional TLR responsiveness caused by exposures to ETS results in augmented CCL2 and CCL22 responses in asthmatic children.

Elevated levels of these chemokines induce the recruitment of inflammatory cells such as monocytes, basophils (by CCL2) and Th2 lymphocytes (by CCL22) into the asthmatic lung compartments.

Once recruited, these cells may then release a number of mediators of allergic inflammation. CCL2-activated basophils and monocytes secrete histamine, leukotriene C4, pro-inflammatory cytokines (IL-8, neutrophilic chemoattractant), CCL11 (eosinophilic chemoattractant). Additionally, CCL2 may directly mediate degranulation of pulmonary mast cells. Released mediators, in turn, attract eosinophils, neutrophils and additional basophils and monocytes to the asthmatic lung, leading to the accumulation of leukocytes in the tissues.

Th2 cells produce type 2 cytokines such as IL-4, IL-5, IL-13 which may maintain local Th2 dominance via amplification loop and amplify allergic inflammatory reactions through IgE synthesis, maturation and activation of mast cells, basophils and eosinophils (Larche e.a., 2003). These events may then lead to more persistent/perpetuated allergic airway inflammation.

Thus, increased production of CCL2 and CCL22 may contribute to asthma in part by influencing the migration of leukocytes and Th2 cells to the lung, thereby further promoting and amplifying inflammation.

4.3 Differential Toll-like receptor responsiveness in First Nation versus Caucasian children

Although extensive epidemiological studies clearly indicate that susceptibility to certain infectious and inflammatory diseases is markedly influenced by ethnicity (Blackwood e.a., 2003; Peschken, Esdaile, 1999; Senthilselvan e.a., 2003), the prevalence and nature of possible ethnic differences in the function of TLRs remain unknown.

In this study, we identified remarkable differences in functional TLR responsiveness between ethnically distinct populations: First Nation versus Caucasian. Specifically, First Nation children favor anti-inflammatory IL-10 responses to TLR2 stimulation. Conversely, Caucasian population respond to TLR (2, 4, 8, 9) activation by production of more robust pro-inflammatory and Th1 biased cytokine and chemokine responses. These strikingly distinct cytokine profiles observed between the populations examined might in part explain ethnic disparities in the risk of developing tuberculosis.

It has been argued that Caucasians have a high level of resistance to *M. tuberculosis* infection, because of natural selection by tuberculosis during the epidemics originating 300 years ago in Europe. On the contrary, Aboriginal American populations may be more susceptible to this disease, since extensive contact with *M. tuberculosis* was rare prior to the early 1900s (Greenwood e.a., 2000; Stead, 1997).

The evolution of immune defense genotypes and phenotypes is strongly influenced by pathogens and their impact is not equal across time and space (Hurtado e.a., 2003).

The world's first epidemic of tuberculosis occurred in Europe. In the 18th century, the rapid urbanization of the population in crowded cities (with a great population density) during Industrial Revolution produce a change in the prevalence of tuberculosis from sparse endemics to a devastating epidemic (Stead, 1997). At the peak of this epidemic (the late 1700s and early 1800s) it was estimated that virtually everyone in European urban areas became infected with *M. tuberculosis* and 20-30% of all deaths were attributable to tuberculosis (Lipsitch, Sousa, 2002)

The infection by *M. tuberculosis* was particularly lethal for children and young adults. The elimination of highly susceptible progeny before their reproductive age would continue over many generations, thus creating a powerful selective pressure for immune resistance to infection by *M. tuberculosis* (Stead, 2001). Those individuals with greater ability to generate protective immune responses against tuberculosis survived epidemics, reproduced and ultimately became the predominant phenotype of the population (Sousa e.a., 1997). Their descendants represent much of the Caucasian population of Canada at the present time.

In contrast, tuberculosis had minimal if any influence on the evolution of immune defense mechanisms in Aboriginal North American populations (Hurtado e.a., 2003). Infection caused by *M. bovis* probably was sporadic with little or no human-to-human spread among many isolated tribes before Columbian period. The first epidemics of tuberculosis in American Indians began in the early 20th century when they were crowded to reservations, with increased contact with European settlers. Tuberculosis death rates elevated rapidly and were 10 times higher than ever observed in Europe at the height of epidemic there, suggesting a great susceptibility attributable to the lack of

ancestral experience with the infection. Every such epidemic in Aboriginal populations was separate and of short duration, and did not produce population-wide natural selection for immune resistance to tuberculosis (Stead, 1997).

Thus, it could be argued that ethnic differences in susceptibility to tuberculosis between Caucasians and Aboriginal populations of Canada might reflect the difference in the history of natural selection for resistance (ie effective immune responsiveness) to tuberculosis.

It is well recognized that cytokines play key role in the protective immunity and pathogenesis of tuberculosis (Flynn, 2004; van Crevel e.a., 2002). Recent observations strongly implicated TLRs in innate resistance to infection by *M. tuberculosis* (Krutzik, Modlin, 2004; Jo e.a., 2007).

Activation of TLRs is known to induce innate immune and inflammatory responses characterized by the production of pro- and anti-inflammatory cytokines and chemokines. These mediators play a crucial role in anti-microbial innate immunity and regulation of the nature of subsequent adaptive immune responses (Iwasaki, Medzhitov, 2004; Kaisho e.a., 2006).

Of note, we demonstrated that ethnicity influenced mostly TLR2 responsiveness among populations examined. In particular, PGN, a TLR2 ligand stimulated cytokine responses (pro-inflammatory TNF-alpha, IL-6, CCL2, and anti-inflammatory IL-10) markedly differ in First Nation and Caucasian children.

In the literature, several lines of evidence showed that TLR2 plays a predominant role in *M. tuberculosis* infection.

First, TLR2 has been demonstrated to be the principal TLR for sensing mycobacteria and initiating the host immune responses. Accumulating data indicate that *M. tuberculosis* expresses a large repertoire of ligands for TLR2 (Bhatt, Salgame, 2007). These included three lipoproteins such as LpqH, 19-kDa (Rv3763) (Brightbill e.a., 1999; Noss e.a., 2001), LprG (Rv1411c) (Gehring e.a., 2004), and LprA (Rv1270) (Pecora e.a., 2006); 38 kDa glycolipoprotein (Jung e.a., 2006); lipomannan (Quesniaux e.a., 2004); phosphatidyl-myo-inositol mannosides (PIM2, PIM6) (Gilleron e.a., 2003; Jones e.a., 2001); glycopeptidolipid (Sweet, Schorey, 2006); PE_PGRS33 protein (H37Rv) (Basu e.a., 2007); non-mannose-capped lipoarabinomannan (AraLAM) (Means e.a., 1999; Kamath e.a., 2003); soluble heat-stable tuberculosis factor (Means e.a., 1999). These distinct mycobacterial products have been shown to signal through TLR2 to induce cellular activation and subsequent cytokine responses (mainly TNF-alpha production). In addition to TLR2, some components of *M. tuberculosis*, namely, 38-kDa glycolipoprotein (Jung e.a., 2006), phosphatidylinositol mannoside (Abel e.a., 2002) are able to act via TLR4. Thus, *M. tuberculosis* antigens preferentially interact with TLR2.

Secondly, TLR2 is believed to be the major TLR that contribute to host resistance to tuberculosis infection in vivo. Studies using animal models demonstrated that TLR2 signaling was critical for successful protection against respiratory tuberculosis, particularly at an early stage of infection (Tjarnlund e.a., 2006). In addition, TLR2 knockout mice were found to exhibit increased susceptibility to high-dose aerosol *M. tuberculosis* infection relative to wild-type controls (Reiling e.a., 2002). Some groups reported that TLR4 might also play the protective role in mycobacterial infection (Abel e.a., 2002), although not to the same degree as TLR2 (Tjarnlund e.a., 2006). The greatest

effect on the progression of tuberculosis was seen in mice doubly deficient in TLR2 and TLR9 (Bafica e.a., 2005). Thus, optimal host resistance to infection with *M. tuberculosis* requires functional TLR2, possibly in combination with TLR4, TLR9.

Finally, polymorphisms in human TLR2 could be a risk factor for tuberculosis. M. Ben-Ali e.a. (2004) reported that the TLR2 Arg753Gln polymorphism is associated with susceptibility to tuberculosis in Tunisian patients. Also, high risk of developing of tuberculosis has been observed in subjects with AA and GA genotypes of TLR2 Arg753Trp polymorphism among Turkish population (Ogus e.a., 2004). J.J. Yim e.a. (2006) revealed the relationship between shorter guanine-thymine repeats in intron II of the TLR2 gene and the presence of tuberculosis in Koreans.

Thus, in the present study we revealed the greatest impact of ethnicity on TLR2 responsiveness among First Nation and Caucasian populations. Toll-like receptor 2 has been established to be the most important one (among other TLRs) for recognizing *M. tuberculosis* and triggering anti-mycobacteria immune responses. It seems reasonable to speculate that ethnic disparities in susceptibility to tuberculosis (First Nation vs Caucasians) might be substantially influenced by differential responsiveness to TLR2 stimulation.

In our study of healthy 8-9 year old children, we demonstrated that pattern of TLR stimulated cytokine production in healthy Caucasian populations is biased in favor of higher levels of pro-inflammatory (TNF-alpha, IL-6, CCL2) and Th1 (IFN-gamma, CXCL10) responses compared to First Nations. This suggests that the baseline TLR-dependent response, (ie that of uninfected individuals), is also markedly different between the two ethnic groups.

There is compelling evidence for important roles of these cytokines and chemokines in protective immune responses to *M. tuberculosis* infection.

It is well established that IFN-gamma is essential for the protective immunity against tuberculosis (Flynn, 2004; Bhatt, Salgame, 2007). This Th1-type cytokine is highly effective in restricting mycobacterial growth in macrophages in vitro (Flesch, Kaufmann, 1991). Moreover, in murine models of tuberculosis, IFN-gamma, in conjunction with TNF-alpha, has been shown to induce nitric oxide synthase and reactive nitrogen intermediates within phagosome, resulting in *M. tuberculosis* killing (Chan e.a., 1992; Flynn e.a., 1993).

IFN-gamma-gene knockout mice have been found to be extremely susceptible to *M. tuberculosis*; unable to control a normally sublethal dose of mycobacterium and succumb to infection (Cooper e.a., 1993; Flynn e.a., 1993; MacMicking e.a., 1997; Pearl e.a., 2001). Also, increased susceptibility to *M. tuberculosis* was associated with impaired IFN-gamma production in T-bet-deficient (Sullivan e.a., 2005) and genetically predisposed C3HeB/FeJ mice (Chackerian e.a., 2001).

Similarly, humans with genetic deficiency of the IFN-gamma receptor and polymorphisms of the IFN-gamma promoter are prone to recurrent, serious, sometimes lethal *M. tuberculosis* infections (Holland e.a., 1998; Levin, Newport, 1999; Ottenhoff e.a., 2005; Rosenzweig, Holland, 2005).

There is ample clinical data showing depressed IFN-gamma responses in patients with tuberculosis. Several studies have demonstrated strongly reduced IFN-gamma production by *M. tuberculosis*-stimulated PBMC among individuals with active tuberculosis compared to healthy controls (Lee e.a., 2003; Sahiratmadja e.a., 2007; Song

e.a., 2000; Torres e.a., 1998; Zhang e.a., 1995). Likewise, others observed decreased capacity of PBMC derived from patients with tuberculosis to produce IFN-gamma in response to PPD (Demissie e.a., 2004; Hirsch e.a., 1999) or BCG (Roberts e.a., 2007, Salina, Morozova, 2004). In addition, IFN-gamma mRNA expression in PBMC of subjects with tuberculosis was found to be substantially diminished (Torres e.a., 1998; Zhang e.a., 1995).

The production of IFN-gamma is further depressed with the development of anergy. It has been demonstrated that PBMC obtained from tuberculosis patients with anergy do not respond (Boussiotis e.a., 2000) or released markedly lower levels (Delgado e.a., 2002) of IFN-gamma to stimulation with PPD or mycobacterial antigens relative to PPD-positive individuals with tuberculosis. Additionally, increased expression of IFN-gamma was seen in well-developed granulomas in the lungs of tuberculosis patients (Bai e.a., 2004), indicating that this cytokine may contribute to granuloma formation and mycobacterial containment. Thus, available data suggest that reduced IFN-gamma responses might result in defective host control of *M. tuberculosis* infection and subsequent development of disease.

TNF-alpha, a pro-inflammatory cytokine, has been shown to be critical in maintaining host resistance and regulating the pathology of tuberculosis (Stenger, 2005; Flynn, Chan, 2005; Bhatt, Salgame, 2007). Its production, in synergy with IFN-gamma, strengthens the mycobactericidal capacity of macrophages by induction of nitric oxide synthase and reactive nitrogen intermediates (Chan e.a., 1992; Flynn e.a., 1993).

Studies in mice models demonstrated the importance of TNF-alpha in controlling of *M. tuberculosis* infection and granuloma formation. V. Kindler e.a. (1989) showed that

mice treated with anti-TNF-alpha antibodies became more susceptible to BCG infection and displayed malformed granulomas. In addition, *M. tuberculosis* infection of mice deficient in TNF-alpha (Bean e.a., 1999; Saunders e.a., 2005) or 55 kDa TNF receptor (Flynn e.a., 1995) resulted in rapid death, with markedly higher mycobacterial burdens relative to controls. The granulomatous responses in these mice were defective and depressed. In mice with chronic infection, neutralization of TNF-alpha has been found to lead to loss of granuloma organization, aberrant pathology and subsequent death (Mohan e.a., 2001), supporting the role for TNF-alpha in maintenance of structural integrity of an established granuloma in latent tuberculosis. This study also showed that TNF-alpha contributes to the prevention of reactivation of persistent tuberculosis as indicated by the increased mycobacterial burden in the lungs of TNF-alpha-neutralized animals (Mohan e.a., 2001).

More recently, the significance of TNF-alpha has been revealed in human tuberculosis. Several groups observed that treatment with TNF blockade using neutralizing antibodies (Infliximab) in patients with inflammatory disorders, such as rheumatoid arthritis and Crohn's disease, results in reactivation of tuberculosis in some latently infected individuals (Gardam e.a., 2003; Keane e.a., 2001; Nunez Martinez e.a., 2001). There were also high rates of disseminated tuberculosis and a notable absence of granulomas in the lung tissue. These observations suggest that anti-TNF-alpha therapy causes dissolution of granulomas and this might lead to disseminated disease (Algood e.a., 2003). Therefore, TNF-alpha appears to play a role in the long-term containment of residual *M. tuberculosis* in tissues (Jakobs e.a., 2007).

Some studies obtained evidence of decreased TNF-alpha production among tuberculosis patients. T.R. Sterling e.a. (2001) showed that TNF-alpha responses after stimulation of PBMC with LPS were lower in patients with extra-pulmonary tuberculosis than those of persons with latent *M. tuberculosis* infection. These defects in LPS-induced TNF-a synthesis were detected up to several years after cure of disease, pointing to possible abnormalities in innate immunity. Similarly, patients with fatal tuberculosis have been demonstrated to exhibit reduced secretion of TNF-alpha and IL-6 by whole blood leukocytes in response to LPS (Friedland e.a., 1995). Also, diminished TNF-alpha release in PBMC cultures was found to stimulation with *M. tuberculosis* in subjects with tuberculosis (Al-Attayah e.a., 2006) and patients with clinically advanced multi-drug-resistant tuberculosis (Lee e.a., 2003).

Thus, both murine and human studies indicate that TNF-alpha has important roles in tuberculosis. It activates macrophages and orchestrates the formation and the maintenance of granulomas, contributing to the innate and adaptive immune responses and to containment of disease.

Several groups demonstrated that CCL2, a proinflammatory chemokine and its receptor (CCR2), might participate in the control of tuberculosis. W. Peters e.a. (2001) found that CCR2^{-/-} mice experimentally infected with *M. tuberculosis* have a rapid and progressive course of infection that resulted in the death of 90% of the mice by day 24 post-infection compared to no deaths in the infected wild-type controls. The absence of CCR2 causes an early and persistent defect in the recruitment of macrophages and immature dendritic cells to the site of infection: lungs and draining lymph nodes, resulting in delayed and reduced priming of naïve T cells. As a consequence, CCR2^{-/-}

mice are unable to control the growth of *M. tuberculosis*. These findings of increased susceptibility of CCR2 knockout mice to infection with *M. tuberculosis* have been confirmed by a later investigation by H.M. Scott and J.L. Flynn (2002).

It has also been shown that deficiency of CCL2 inhibits type 1 responses and granuloma formation in murine models. L. Boring e.a. (1997) demonstrated that CCL2^{-/-} mice exhibit diminished formation of granulomas after instillation of PPD coupled beads accompanied by a dramatic decrease in the levels of IFN- γ , a Th1-type cytokine. Correspondingly, B. Lu e.a. (1998) observed blunted development of secondary pulmonary granulomata in CCL2^{-/-} mice in response to *Schistosoma mansoni* eggs, indicating that CCL2 might contribute to granuloma formation.

Clinical study by J.S. Lee e.a. (2003) demonstrated that individuals with pulmonary tuberculosis in the early stages had markedly reduced CCL2 secretion by PBMCs or monocytes in response to PPD or the 30-kDa antigen of *M. tuberculosis* relative to healthy tuberculin reactors. Thus, CCL2 production in vitro might be depressed in patients with early tuberculosis. These data suggest that CCL2 may serve an early and essential role in resistance to *M. tuberculosis* infection.

CXCL10, first described as human IFN- γ -inducible 10-kDa protein (IP-10), is high affinity ligand for its receptor, CXCR3, which is preferentially expressed on activated Th1 lymphocytes (Luster e.a., 1987). This chemokine has been shown to stimulate monocyte activation, T-cell progenitor maturation, and direct migration of activated Th1 cells (Farber, 1997).

A series of observations implicated CXCL10 in tuberculosis, particularly in tuberculosis-associated granuloma formation. E. Giacomini e.a. (2006) found that

supernatants from *M. tuberculosis*-exposed dendritic cells have strong ability to activate chemotaxis in Th1 cells, indicating that CXCL10 exerts a major role in *M. tuberculosis*-induced Th1 cell chemotaxis. Experimentally, granulomas elicited in mice by PPD-coated beads has been demonstrated to have a polarized type 1 immune response with elevated levels of CXCL10 and CXCL9 expression (Chiu e.a., 2002).

In lung tissues from cynomolgus macaques infected with a low dose of virulent *M. tuberculosis*, C.L. Fuller e.a. (2003) observed abundant expression of mRNA for CXCL10 within solid and caseous granulomas. In contrast, only minimal expression of this chemokine was evident in nongranulomatous regions of tissue. Similarly, human tissue-based study by E. Ferrero e.a. (2003) demonstrated *in vivo* localized expression of CXCL10 in granulomas detected in the lungs and draining lymph nodes from a patient with active tuberculosis. X. Bai e.a. (2004), using morphometric analysis of cytokine expression in tuberculosis pulmonary lesions in humans, demonstrated increased CXCL10 levels in inflammatory and fibrotic areas of tuberculosis granulomas compared to controls. These studies highlight an important role for CXCL10 in establishing normal architecture of granulomas and its maintenance in tuberculosis.

Thus, based upon our results and data from the literature, we may speculate that Caucasians with a greater tendency toward pro-inflammatory and Th1 responses would be more resistant to tuberculosis. This “protective” cytokine profile could have evolved by natural selection in ancestral epidemics of tuberculosis. In contrast, First Nations exhibited relatively high levels of anti-inflammatory/ immunosuppressive IL-10 in response to TLR2 stimulation.

Many studies have provided evidence that IL-10 might interfere with host defense against tuberculosis. In animal models, high expression of IL-10 in transgenic mice has been found to be associated with reactivation of chronic latent pulmonary tuberculosis (Turner e.a., 2002). The *M. tuberculosis*-infected macrophages from these mice exhibited reduced anti-mycobacterial capacity (Feng e.a., 2002). In line with this, IL-10-deficient mice showed increased resistance to mycobacteria (rapid elimination, a lower bacterial burden) early after infection (Jacobs e.a., 2000; Murray, Young, 1999). B.M. Sullivan e.a. (2005) recently demonstrated that elevated IL-10 production correlates with increased susceptibility of mice lacking T-bet to *M. tuberculosis* infection. These observations suggest that endogenous IL-10 is inhibitor of early mycobacterial clearance and attenuates anti-mycobacterial immunity in mice.

Interleukin 10 levels have been demonstrated to be increased in patients with tuberculosis. A large case-control study by E. Sahiratmadja e.a. (2007) showed that subjects with newly diagnosed active pulmonary tuberculosis exhibit markedly enhanced IL-10 release in LPS-stimulated whole blood cultures compared to healthy controls. Similarly, other groups demonstrated elevated production of IL-10 by PBMC in response to *M. tuberculosis* (Lee e.a., 2003; Song e.a., 2000; Torres e.a., 1998) or purified protein derivative (PPD) (Hirsch e.a., 1999) stimulation among tuberculosis patients relative to healthy household contacts. Also, the expression of mRNA for IL-10 was found to be higher in PBMC derived from patients with tuberculosis than in healthy individuals that control a latent infection (Demissie e.a., 2004). Likewise, several studies reported elevated concentrations of IL-10 in serum (Dlugovitzky e.a., 1997; Olobo e.a., 2001;

Vankayalapati e.a., 2003; Verbon e.a., 1999) and bronchoalveolar lavage fluid (Bonecini-Almeida e.a., 2004) of subjects with active tuberculosis versus healthy volunteers.

R.C. Huard e.a. (2003), examining a cohort of tuberculosis patients, found a positive correlation between levels of IL-10 and *M. tuberculosis* CFP32 protein levels (an indicator of increasing bacterial burden) in lung sputum, suggesting that a link between *M. tuberculosis* and IL-10 may play a role in the pathogenic mechanism leading to active tuberculosis. Interestingly, X. Bai e.a. (2004) observed decreased expression of IL-10 in well-formed pulmonary granulomas of patients with tuberculosis compared to controls, leading authors to conclude that IL-10 absence may be permissive for the formation of these protective lesions.

Some authors demonstrated an involvement of IL-10 in the induction of anergy (lack of skin reactivity to PPD) in tuberculosis patients. Anergic patients, both before and after treatment, have been shown to produce IL-10 constitutively by PBMC. IL-10 levels were further augmented by stimulation with *M. tuberculosis* antigens and PPD (Boussiotis e.a., 2000), suggesting that *M. tuberculosis*-induced IL-10 production suppresses an effective immune response in anergic patients. Correspondingly, J.C. Delgado e.a. (2002) detected increased levels of IL-10 in PPD-stimulated PBMC cultures of tuberculosis patients with persistent anergy relative to PPD-reactive individuals with tuberculosis.

Together, these data suggest that IL-10 is induced by *M. tuberculosis* and might down-modulate host anti-mycobacterial immunity, thereby, allowing *M. tuberculosis* to evade protective immune mechanisms, replicate and overt disease.

Thus, on the basis of literature findings and our results, it could be speculated that individuals of First Nation population that tended to produce higher levels of anti-inflammatory/ immunosuppressive IL-10 and relatively low pro-inflammatory and Th1-associated cytokines would be less resistant to tuberculosis. This cytokine profile may be due in part to the lack or the shortest duration of selective pressure by *M. tuberculosis* on the population.

In summary, our data document marked differences in functional TLR responsiveness (cytokine profiles) between ethnically distinct populations (First Nation and Caucasian) that, we speculate, may influence the ethnic disparities in susceptibility to tuberculosis. These findings contribute to the concept of natural selection for resistance to tuberculosis: *M. tuberculosis* infection has created a selective pressure on the population that over centuries has shaped the nature of human immune (cytokine) responses.

4.4 Summary

TLRs play a key role in initiating innate immunity and in regulating the nature of the adaptive immune response that subsequently develops. Alterations in TLR responsiveness in distinct individuals as a result of differences in environmental exposures (exposures to ETS) and/or ethnicity (First Nations vs. Caucasians) may influence their likelihood of exhibiting allergic disease such as asthma.

In the present study, we tested the following hypotheses:

(i) Functional responsiveness to TLR stimulation differs in allergic asthmatic vs non-atopic children;

(ii) ETS exposures affect TLR responsiveness in asthmatic children;

(iii) Functional responsiveness to TLR stimulation differs in First Nation vs. Caucasian children

In order to enhance sensitivity our study aimed at testing the hypothesis of a novel approach of employing “threshold” concentrations of TLR ligands (more closely approximate natural environmental levels) than commonly utilized “maximal” stimulation conditions.

Our data demonstrate the following:

1). Use of threshold concentrations of both TLR4 and TLR2 ligands reveals striking differences in cytokine responses between asthmatic and non-atopic control children. In particular, non-atopic controls exhibit more robust pro-inflammatory (IL-1beta and TNF-alpha) cytokine responses, whereas asthmatics produce markedly elevated

anti-inflammatory IL-10 levels. In contrast, typically utilized maximal concentrations of LPS and PGN elicit strong, indistinguishable patterns of cytokine production.

2). Exposures to ETS augment chemokine responses to TLR stimulation in allergic asthmatic children. Specifically, asthmatic children exposed to passive smoking exhibit greatly enhanced levels of pro-inflammatory CCL2 and Th2-associated CCL22 chemokines relative to non-ETS exposed asthmatics and controls. Increased production of CCL2 and CCL22 may contribute to asthma in part by influencing the migration of leukocytes and Th2 cells to the lung, thereby further promoting and amplifying allergic inflammation.

3). Use of threshold TLR stimulation reveals remarkable differences in immunoregulatory responses between ethnically different populations. In particular, First Nation children favor anti-inflammatory/ immunosuppressive IL-10 responses to TLR2 activation. Conversely, Caucasians respond to TLR (2, 4, 8 or 9) stimulation by production of more robust pro-inflammatory and Th1 biased cytokine and chemokine responses. These strikingly distinct cytokine profiles observed between the populations examined might partly explain ethnic disparities in susceptibility to tuberculosis.

Thus, this novel strategy of using threshold levels of TLR stimulation provides markedly enhanced sensitivity to reveal functional alterations and diversity in TLR responsiveness that associate with clinical status and ethnicity.

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