NEONATAL INNATE IMMUNITY

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ABSTRACT

The neonatal period represents a critical time period in the development of the immune system. Adaptive human immune responses are generally viewed as immature at birth. However little is known about innate immune capacity at birth. The TLR system plays an integral role as pattern recognition receptors in the innate immune response. It is also critical in initiating and regulating the adaptive immune response. Preterm birth is associated with increased risk of developing infections in early life and has been associated with increased risk of development of other chronic disorders in later life; however the underlying mechanisms are not at all well understood. Recently, late preterm neonates (34-36 weeks gestation vs full term, 37+ weeks) have been identified as having significantly greater risks of morbidity and mortality in the perinatal period than their full term counterparts. Hence, we focus on examination of TLR responses in late preterm and full term neonates to better understand immune potential and function in these populations. We examined cord blood cytokine and chemokine responses following stimulation with a broad range of TLR agonists. Our results show for the first time that late preterm neonates have reduced capacity to produce both pro- and anti-inflammatory cytokines following stimulation with a panel of TLR agonists. This reduced responsiveness was not due to a reduction in the number of responding cells, but instead appears to be mediated by a reduction in the intrinsic levels of expression of TLRs and associated adaptor proteins.

Because little is known about how the innate immune system develops throughout life, we next compared TLR responses in full term neonates to

children, adolescents and adults. We found that neonates had selective impairments in TLR responses, most notably in anti-inflammatory cytokine production and anti-viral immune responses compared to the other age groups.

Epigenetic modifications, such as the addition or removal of acetyl groups to histone proteins by histone acetyl transferase (HAT) and histone deactylase (HDAC) respectively, are able to modify the expression of genes. Hence, environmental stimuli have been shown to influence gene expression in part by modifying the level or activity of these epigenetic regulators. Currently there are no studies which have examined how epigenetic modifications may influence neonatal innate immune responses. Hence, we sought to determine how modulation of endogenous HDAC activity would affect neonatal innate immune responses. We found that inhibition of HDAC had both inhibitory and enhancing effects on cytokine expression depending on the TLR pathway activated, indicating that the endogenous HDAC expression does not have a global inhibitory impact on all TLR-dependent responses.

In summary, this body of work demonstrates that neonatal innate immune responses vary depending on gestational age, indicating that the final few weeks of gestation are crucial for maturation of responses to both bacteria and viruses. Neonates respond differently to TLR stimuli than do older individuals, further highlighting a maturation process of the innate immune system which continues throughout life. Finally, we have shown that environmental exposures may have powerful effects on immune responses in early life.

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List of Abbreviations

APC	Antigen presenting cell
AHR	Airway hyper-responsiveness
B cells	Bone marrow-derived lymphocytes
CBMC	Cord blood mononuclear cells
CD80	Costimulatory molecule involved in T cell activation
CD86	Costimulatory molecule involved in T cell activation
cDNA	DNA copy of RNA template
CXCL4	Platelet Factor 4
CXCL10	IFNγ-inducible protein of 10 kDaltons (IP-10)
CpG (ODN)	Oligodeoxynucleotides containing CpG dinucleotide motifs
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot assay
EDTA	Ethylene diamine tetra acetic acid
ETS	Environmental tobacco smoke
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HAT	Histone Acetylase
HDAC	Histone Deactylase
IFNγ	Interferon-gamma
Ig	Immunoglobulin (M, D, G, E, A)
IL	Interleukin-(1 to 33)
LPS	Lipopolysaccharide
MALDI	Matrix assisted laser desorption ionization
MHC I	Major histocompatibily antigen class I
MHC II	Major histocompatibily antigen class II
µg	Microgram
mg	Milligram
ml	Milliliter
ng	Nanogram

PBMC Peripheral blood mononuclear cells

PBS Phosphate buffered saline

pg Picogram

PGN Peptidoglycan, bacterial cell wall component

PHA Phytohemagglutinin mitogen

PolyI:C Polyriboinosinic polyribocytidylic acid (RNA analog)

PSF Penicillin G sodium, streptomycin sulfate, amphotericin B

RT Room temperature

RT-PCR Reverse transcription polymerase chain reaction

RPMI Roswell Park Memorial Institute medium

SAGE Study of Asthma Genes and the Environment

T cells Thymus-derived lymphocytes

Th1 / Th2 T helper 1 / T helper 2

TSA Trichostatin A

Type 1/Type 2 Immune responses involving T cells, B cells and APCs

INTRODUCTION

1.1 Innate Immunity: Overview and Recent Advances

The innate immune system is an integral component of an effective immune response and involves many complementary pathways and cell types (reviewed in (1)). It provides the first line of defense against invading pathogens and both initiates and instructs subsequent adaptive immune responses (2,3). Important characteristics of the innate immune response include: rapid induction of inflammation, cell recruitment, clearance of viral and bacterial infections (4). One of the key effector mechanisms of the innate immune system is the presence of highly evolutionarily conserved sensors, known as pattern recognition receptors (PRRs), which recognize invariant microbial patterns (5). However, vertebrates possess unique innate immune elements, such as NK cells, type 1 interferons, eosinophils and basophils, suggesting that over time the features of the innate immune system have become tailored to suit this phylum (2). Although the innate immune system is capable of responding to microbial products present in normal commensal microorganisms, it is imperative that the inflammatory signals are reserved for infectious agents. Thus, as was first introduced by Matzinger, the innate immune response also includes a mechanism of recognizing 'danger signals' which accompany invasive pathogens (6,7).

While the adaptive immune system has been studied extensively for decades, the innate immune system has only become better understood in recent

years. One of the major advances in the study of innate immunity was the identification of highly conserved PRRs (8).Toll-like receptors, which are reviewed below, are the best characterized family of pattern recognition receptors (8). They are membrane-associated receptors that are found both on the cell surface and within endocytic vesicles in a variety of cell types (9,10). In recent years knowledge of the innate immune system has become more sophisticated with the identification and characterization of several other families of evolutionarily conserved receptors, such as Nod-like receptors and RIG-like helicases (2). Collectively these families of PRRs are capable of responding to a wide range of microbial peptides as well as host factors (2).

The innate immune system is crucial not only for recognition of invading pathogens but also for the development of appropriate adaptive immune responses. PAMP-dependent activation of antigen presenting cells (APC), such as macrophages and dendritic cells, results in the upregulation of Class I and II major histocompatibility complex (MHC) gene expression and surface levels, as well costimulatory molecule expression, promoting subsequent peptide presentation to T and B cells (11). The activation of APCs via PRRs also leads to the production of a variety of cytokines which impact the type of adaptive immune response which ensues (12). Although the connections between various innate immune effector pathways and interactions with the adaptive immune response are not completely understood, recent advances in innate immune research have helped to improve knowledge in these areas.

1.2 Nod-like Receptors

Nuclear oligomerization domain(NOD) -like receptors (NLR) are a recently discovered family of pattern recognition receptors which are capable of responding to microbial patterns as well as self-derived peptides (13,14). These receptors are related to R (Resistance) proteins from plants and are a major component of cytosolic complexes, referred to as 'inflammasomes' (13,15). The general structure of NLRs consists of an N-terminal effector domain, a central nuclear oligomerization domain (NOD) and a C-terminal domain containing Leucine-Rich Repeats (LRRs) (16,17). Currently 23 mammalian NLR family members have been identified, although not all of the ligands for these receptors are known (13,14). NLRs can be divided into 3 groups based on their N-terminal effector domains: NOD, NALP (contain Pyrin domain) and NAIP (contain baculovirus-inhibitor-of–apoptosis repeats (BIR) (13,14). Emerging evidence suggests that different combinations of NLRs and signalling molecules can recognize a wide variety of cytosolic proteins, providing an increased spectrum of specificities within a small group of receptors (18). Some examples of well-characterized ligand-receptor pairs include: NOD-1 and NOD-2 which bind the muramyl dipeptide in peptidoglycan, NALP-3 which recognizes monosodium urate, the agent responsible for gout (18). Some of the major signalling pathways commonly employed by NLR members include the activation of RICK kinase, nuclear factor kappa-b (NFκB), p38, extracellular signal-regulated kinase (ERK), and caspase 1/5 (reviewed by (6). Recent studies have shown that NLRs can act synergistically with TLRs in response to pathogens in dendritic cells, enhancing

microbial-induced cytokine production (19,20). For example, Park *et al* found that RICK kinase is essential for providing optimal IFNγ response in macrophages following LPS, Listeria and muramyl dipeptide treatment (21). These findings collectively suggest that although NLRs and TLRs utilize different signalling pathways, their synergistic activity provides optimal protection against pathogens. The role of NLRs in autoimmune disorders is currently being actively studied (13). Some recent discoveries in this area include linkages between gain-of-function mutations in NOD2 and NALP3 and the Mendelian inflammatory disorders Muckle-Wells syndrome (familial cold autoinflammatory syndrome) and Blau syndrome(chronic infantile neurologic cutaneous and articular syndrome) respectively (22). Hence, while proinflammatory NLR responses are generally protective against microbial infection, mutations which prevent inhibition of these pathways can lead to maladaptive inflammation and tissue damage.

1.3 RIG-like Helicase Family

Another recently discovered family of cytosolic sensors involved in innate immune activation is the retinoic acid inducible gene 1 (RIG)-like helicase family, also referred to as the RLH family (23,24). Like the NLR family, these receptors are found in the cell cytoplasm and are important in providing protection against RNA viruses (25) . The best characterized members of this group of receptors are RIG-1 and melanoma differentiation-associated gene 5 (MDA5), which both contain two N-terminal caspase recruitment domains (CARDs), followed by a DExD/Hbox helicase domain (23). MDA5 is important in the recognition of

dsRNA, especially in picornovirus infections such encephalomyocarditis virus (ECMV) (26). RIG-1 is important in responding to ssRNA and has been shown to play a role in recognition of viral DNA derived from Epstein - Barr virus (EBV) (27). The end result of RLH activation by dsRNA is the induction of the expression of type 1 interferons and proinflammatory cytokines, which synergize with other innate immune signalling pathways mediated by TLRs and NLRs (6,25). In humans, a single nucleotide polymorphism (SNP) in the MDA5 gene is associated with type 1 diabetes, which some groups have speculated may occur after viral infection in some individuals (28). As is the case with NLRs, inappropriate activation of RLHs may contribute to an increase in other autoimmune diseases, such as systemic lupus erythematosus (29).

2.1 TLR Family: Structure, Cellular Distribution, Expression Patterns and Effector Responses

Toll-like receptors are highly evolutionarily conserved receptors which were identified in vertebrates following the discovery of Toll receptors in *Drosophila* (3). TLRs have been shown to play essential roles in detection of viral, bacterial and certain endogenous ligands (30,31). The study of TLRs in human immune responses has become a topic of great interest in recent years, leading to an improved understanding of the roles these receptors play in directing appropriate versus inappropriate immune responses (4,32). TLRs are comprised of leucine-rich repeats (LRRs), a transmembrane domain, and a cytoplasmic domain designated the Toll/interleukin-1 receptor (IL-1R) homology (TIR) domain (2). These receptors are found on a variety of cell types including

monocytes, macrophages, dendritic cells, lymphocytes, polymorphic leukocytes and epithelial cells (2). Currently there are 11 identified human TLRs; the PAMPs recognized by TLRs are most commonly derived from either bacteria or viral molecules (1,33). An overview of the well established TLR-ligand binding partners is outlined in Table 1.

2.2 TLR Signalling Pathways

Since the initial discovery of TLRs in vertebrates, the ligands recognized by these receptors have been intensively studied. TLRs are located either at the cell surface (TLR1,2,4,5,6 and 10), or are found in the cytoplasm associated with endocytic vesicles (TLR3,7,8, and 9) as depicted in Figure 1. In general, the extracellular TLRs are responsible for protection against PAMPs located in the extracellular environment (34). Some notable exceptions to this rule are the recognition of respiratory synctial virus F protein and endogenous heat shock proteins by TLR4 (34). TLR4 is one of the best studied TLRs and has been shown to be instrumental in providing protection against gram-negative bacteria through the recognition of lipopolysaccharide (LPS) (1). In order to bind LPS and activate signalling pathways, TLR4 requires LPS-binding protein, MD2 and CD14 (1). Signalling pathways following LPS recognition by TLR4 and adaptor proteins signals in a similar mechanism to the IL-1 receptor, hence the designation of Toll/IL-1 Receptor (TIR) domains which are found in signalling components shared by both pathways (35). For example, myeloid-differentiation factor 88

Table 1. Summary of well characterized TLR and ligand pairs in humans

TLR	Ligands	References
TLR1	Triacyl peptides	(33)
TLR2	Lipoprotein, Peptidoglycan, LTA, Lipoarbinomannan, Zymosan	(31), (32), (33)
TLR3	Poly I:C, dsRNA	
TLR4	LPS, RSV F Protein, Pseudomonas exoenzyme S, HSP(60,70 and 90), fibrinogen, B-defensins,	(30),(31), (110)
TLR5	Flagellin	(215)
TLR6	Peptidoglycan, Diacyl Peptides, Lipoprotein	(31)
TLR7	ssRNA, Imiquimod, R-848	(33), (66)
TLR8	ssRNA, 3M002, 3M011, R-848, imidazoquinolines	(46), (113)
TLR9	CpG ODN	(31)

(Myd88) contains a TIR domain and a death domain (DD) and is an important adaptor molecule involved in TLR and IL-1 Receptor signalling (35). MyD88 is an essential signalling component for signalling through all TLRs with the exception of TLR3 and in some cases TLR4 (35,36). Other signalling proteins involved in Toll and IL-1 receptor signalling include the IL-1 receptor associated kinase (IRAK) and another downstream protein, TBF receptor-associated factor 6 (TRAF6) (37). IRAKs are comprised of an N-terminal DD and a C-terminal serine/threonine kinase domain. IRAKs interact with MyD88 through the DD. IRAK4 is important in promoting the production of cytokine and chemokines for most TLR signalling pathways (37,38). In contrast, IRAK1 is specifically important in initiating type 1 interferon production (37,38). Following recognition of the appropriate ligands, the extracellular TLRs' signalling pathways lead to the activation and nuclear translocation of NFκB through the phosphorylation and ubiquination of inhibitory I-Kappa-b proteins that sequester NFκB (3,39). Following nuclear translocation, NFκB leads to transcription of a variety of proinflammatory cytokines and chemokines such as tumor necrosis factor α (TNFα), interleukin 1-β (IL-1β), IL-8, monocyte chemoattractant protein (MCP)-1, or CCL2, as well as immunoregulatory cytokines such as IL-10 (40). TLR signalling can also activate other transcription factors, such as interferon regulatory factor (IRF)-3 and IRF-7 (37), which are essential for the transcription

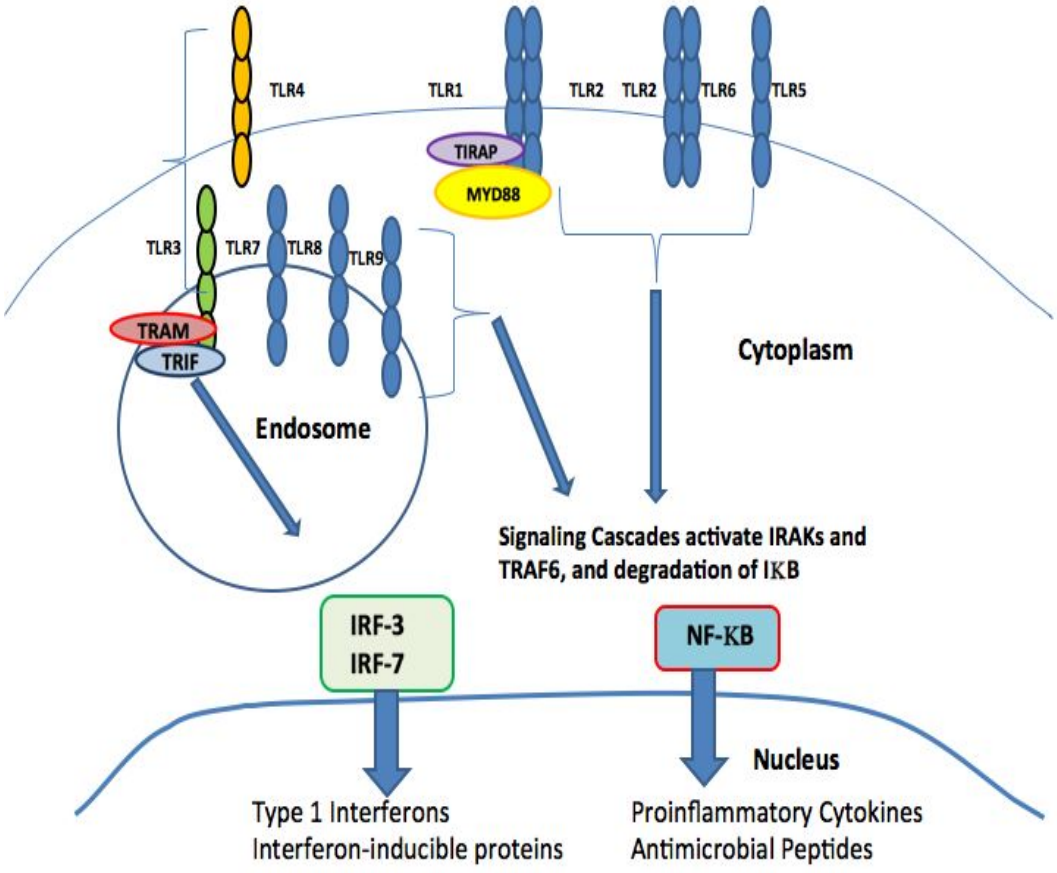

Figure 1. Overview of Signalling Pathways Initiated by TLR Activation in Humans (Artwork created by Stephanie Macpherson)

of type 1 interferons through TRAF-3 activation. Other surface TLRs signal in a similar mechanism to TLR4, however the mechanism of pathogen binding and certain downstream signalling molecules differs amongst TLRs (33). For example, TLR2 is able to respond to a broad range of PAMPs and has the ability to increase its repertoire through heterodimeric interactions with TLR1 and 6 (41).

The group of TLRs which are associated with the cytosolic endosomes, including TLR3,7,8 and 9, are mainly responsible for recognition of pathogen nucleic acids (42). For instance, TLR3 recognizes dsRNA (Poly I:C), and signals in a MyD88-independent fashion through the adaptor protein TIR domain-containing adapter inducing IFN factor (TRIF) exclusively (43). TRIF has also been implicated as an alternative adaptor in TLR4 signalling (42,44). TRIF contains N-terminal binding motifs which allow interaction with TRAF3 and TRAF6, the C-terminal domain contains a receptor interacting protein (RIP) homotypic binding motif which allows TRIF to interact with RIP1 and RIP3 (37,38). As with MyD88-dependent signalling, TRIF-dependent signalling can activate both NF-κB and IRF-7 in a TRAF6- and TRAF3- dependent fashion, respectively (37,38). TLR7 responds to single-stranded (ss) and double-stranded (ds) RNA, generating proinflammatory cytokines as well as type 1 IFNs (45). Plasmacytoid DCs, and to a lesser extent monocytes, express high levels of TLR7 and are important responders to TLR7 agonists (45). TLR8 is activated by ssRNAs, as well as synthetic purine compounds, some of which are able to activate TLR7/TLR8 simultaneously (46). TLR8 agonists yield strong responses in monocytes, myeloid DCs and monocyte-derived DCs (45). Some naturally

occurring viral nucleotides, such as ssRNA from influenza virus, are also able to activate both TLR7 and TLR8 (47). Signalling through both TLR7 and TLR8 occurs in the MyD88-dependent fashion; it remains to be determined if there are additional adaptor proteins required for agonist binding to these receptors (45,48). Although both TLR7 and 8 require MyD88, their downstream signalling occurs through different mechanisms. TLR8 signalling involves the activation of IRAK-4 and NF-κB, whereas TLR7 signals through a TRAF6 and IRF-7 dependent pathway (49). Interestingly, TLR7 agonists are also able to stimulate NALP3, leading to the production of IL-1β and IL-18 (50), illustrating the interplay of multiple innate immune recognition pathways by a common ligand. Unlike the other endosomal TLRs, TLR9 does not recognize viral RNA but instead recognizes unmethylated DNA sequences commonly found in bacterial and viral genomes (51). Interestingly, differences in the CpG motif class (A-D) elicit differential TLR9 responses, indicating that the same TLR can respond differentially depending on the nature of the ligand (52).

2.3 TLR Effector Functions

i) Direct Effects

Following the recognition of pathogens by TLRs the signalling pathways activated lead to a variety of effector functions which can confer protection against infections, or undesirable immune responses (such as chronic inflammation), for the host. For instance, the activation of TLRs rapidly leads to maturation and differentiation of human monocytes into macrophages (with increased phagocytic activity) and dendritic cells (with increased antigen-

presenting activity) (36,43). TLR signalling also induces the release of defensins from a variety of cell types. These antimicrobial peptides play important roles in destruction of bacterial and viral pathogens, providing additional protection against infectious agents (53).

Following activation of dendritic cells by TLRs these cells upregulate a variety of costimulatory molecules, such as CD80, CD83 and CD86 as well as the chemokine receptor CCR7, promoting migration to the lymph node (3). DCs also increase antigen presentation in the context of Class1 and II MHC by upregulating surface MHC trafficking, which combined with the upregulation of costimulatory molecules allows DCs to effectively activate T cells (40). Studies in MyD88-deficient mice indicate that TLR signalling is essential in generating antigen specific T cell responses (54). Although MyD88-deficient mice retain the ability to produce antigen-specific antibodies, B cells from these mice preferentially produce IgE, which is commonly associated with allergic disease (54). Recently, TLR signalling has been shown to be essential in the process of antigen sorting and effective presentation to CD4+ T cells (32). Collectively, these studies indicate a pivotal role of TLR activation in the development of adaptive immune responses.

Although cells associated with the innate immune system elicit the majority of TLR responses, these receptors can also have direct effects on cells of the adaptive immune system (55,56). One example is the use of CpG motifs are vaccine adjuvants; studies have shown that CpG motifs are able to directly activate B cells which express TLR9 and improve antibody production as well as

increasing the memory response (57). Regulatory T cells (Tregs) also express some TLRs, such as TLR2, TLR4 and TLR8; activation of these TLRs has been shown to activate the suppressive functions and proliferation of Tregs (49,58). Recently conventional effector T cells have also been shown to respond to TLR agonists (56). Studies in mice have shown that CD4+ T cells effector express a wide range of TLRs and can respond to CpG DNA and LPS in MyD88-dependent fashion (56,59). These findings suggest that in addition to the effects on innate immune cells, TLRs may play a pivotal role in mounting both humoral and cell-mediated immunity through the direct activation of cells of the adaptive immune system.

TLR polymorphisms highlight the importance of these receptors in the immune response. One of the best characterized examples is the Asp299Gly polymorphism of TLR4 which affects ligand binding (60). This polymorphism results in a markedly higher rate of infection with gram-negative bacteria and RSV (60). This polymorphism also correlates with a greater risk of developing asthma and allergies (61) , further supporting the role of TLR4 in mediating effective adaptive immune responses.

ii) **Indirect Effects**

Multiple TLR pathways lead to the secretion of proinflammatory and immunoregulatory cytokines and chemokines (4,31,62). Proinflammatory cytokines, such as IL-1β and TNFα induce endothelial cells to upregulate a variety of cell adhesion molecules, promoting the recruitment of a variety of cell types to the site of infection (63). For example, CC-chemokines such as CCL2

(MCP-1), are chemotactic for T cells, monocytes, basophils, DCs and NK cells (63). CXCL8 (IL-8), is also strongly upregulated following TLR activation and is important in the recruitment of neutrophils, and to a lesser extent T cells, basophils, eosinophils and NK cells (63). The first cell types recruited to the site of microbial recognition are neutrophils and monocytes, which phagocytose and degrade remaining microbes (4).

Dendritic cells bridge innate and adaptive immune responses (11). The type of TLR agonists encountered by DCs and other APCs can help to determine the type of adaptive immune response which ensues. Most studies suggest that TLR activation promotes the development of Type 1 CD4+ T cells, through the production of cytokines such as TNFα and IL-12 (64,65). Supporting this conclusion, MyD88-deficient mice have been shown to have selective defects in mounting Type-1, but not Type-2 immune responses (54,57). In addition, TLR8 and TLR9 have recently been used in vaccine development models to enhance Type 1 immunity and increase the duration of protective immunity (57,66,66). However, TLRs may also be able to induce type 2 biased immune responses. One example is the stimulation of TLR2 with Pam3Cys, which has been shown in some studies to promote type 2 immune responses and promote the development of asthma in a mouse model (67,68). In contrast, Patel et al found that Pam3Cys can ameliorate OVA-specific asthma and have suggested that it could be used as a novel therapeutic agent, suggesting that this receptor may not induce type 2 immune responses in all cases (69). While the majority of TLRs may not be involved in promoting type 2 immune responses, the type of cytokine milieu

present at the DC-epithelial interface has been shown to be important in determining adaptive immune response (70). Specifically, thymic stromal lymphopoietic (TSLP) production by epithelial cells has been shown to be instrumental in stimulating DCs and tissue mast cells to drive allergic inflammation (70). Currently epithelial cells are thought to be the main producers of TSLP, however the stimuli and circumstances that trigger TSLP production are not well defined (70). Developing an indepth understanding of the induction of TSLP production will be instrumental in determining how atopic symptoms develop. In addition to type 1 and type 2 immune responses, dendritic cells have also been implicated in the development of proinflammatory Th17 cells, via the production of IL-21, IL-23 and IL-6 (71). The type of cytokine profile produced by DCs and following TLR activation is determined in part by the DC subset stimulated (72,73). Waibler *et al* found that myeloid DCs, but not plasmacytoid DCs, produced IL-23 following stimulation with TLR3,4,7 and 9 ligands (73)(72). Th17 cells have recently been linked to several autoinflammatory disorders, therefore developing a better understanding of how these cells differentiate may allow for the development of therapeutic interventions in the future (71).

Not only can TLRs modulate the type of effector T cell response which develops but they can also render effector T cells temporarily resistant to T reg inhibition, promoting prolonged immune responses (58). Type 1 interferon produced following TLR activation also plays a role in modulating T and B cell responses (32). Type 1 interferon promotes T cell survival and stimulates B cell class switch and differentiation into plasma cells (32). The role of TLRs in driving

adaptive immune responses is actively being studied; recently TLRs have been linked to the pathogenesis of several autoimmune and allergic diseases (74). Hence, TLR activation and regulation may be essential in determining the balance of protective versus maladaptive immune activation.

2.4 Role of TLRs in Allergies and Asthma

In the past few decades the incidence of allergic disease, including asthma, has risen dramatically in Western nations (75,76). In North America asthma is the most common chronic condition in childhood and is the most common cause of hospital admission in children (77,78). Asthma is a multifactorial disease which is characterized by airway hyper-responsiveness (AHR) and variable airflow obstruction (79,80). The etiology of asthma is believed to involve a combination of genetic predisposition and environmental exposure (75,76). Atopic asthma is believed to be mediated by type 2 immune responses, including allergen-induced IgE production, recruitment of Th2 cells and cytokines such as IL-4, IL-5 and IL-13, as well as and eosinophil infiltration and inflammation within the lung (81). However, the immunologic mechanisms underpinning initiation and progression of allergic asthma remain poorly understood (61).

Several theories have been postulated to explain the rapid increase in allergic disease in Western nations. One of the most pertinent is the hygiene hypothesis. This theory suggests that the increase in allergic disease is primarily due to a reduction in the frequency of infections in early life due to improved vaccine, antibiotic interventions and a greater degree of household sanitation

(61,82,83). Hence, inappropriate instruction of the immune responses by reduced exposure to infections and microbial products in early life is proposed to be responsible for the development of atopic responses to innocuous substances (83). Several studies have found epidemiological evidence to support the hygiene hypothesis (83,84). However, uncovering mechanistic data to support this theory has been less successful. Several groups have studied TLRs in an attempt to determine if these receptors could be involved in the protection or underlying pathology in the development of atopic disease (85).

TLRs have been shown to be instrumental in the initiation innate and adaptive immune responses, as such inappropriate or hyporesponsive TLR activity may be involved in the initiation of allergic airway inflammation. TLRs are expressed not only on classical immune cells and structures, but are also expressed in tissue epithelium (81). Recently, epithelial tissue has been recognized as playing an active role in mediating immune responses (81) . Interaction of lung epithelium, the initial point of contact with the external environment, and resident immune cells in the lung may be instrumental in instructing subsequent immune responses (81). Thus, it is possible that through TLR activation the lung epithelium is able to instruct subsequent immune responses to environmental antigens and infectious agents.

Despite much research in the area, the role of TLRs in asthma is still not well defined. For instance, different epidemiologic studies indicate that TLR4 activation may be protective, have no effect or be detrimental in the pathogenesis of asthma (86). There is a relatively large body of research supporting the premise

that enhanced TLR activation in early life is protective against the development and maintenance of asthma. For instance, Gern *et al* reported that neonates capable of mounting greater IFNγ response to RSV (recognized by TLR4) at birth had a reduced incidence of wheeze in the first year of life (87,88). Wark *et al* found that epithelial cells from the lungs of asthmatics produced defective type 1 IFN responses following rhinovirus infection, suggesting that defective innate immune responses to viruses may play a role in the etiology and pathogenesis of asthma (89). This finding also supports the conclusion that immune responses at the epithelial interface of asthmatics are functionally distinct from healthy individuals.

One of the most studied interactions in the field of TLRs and human disease is that of endotoxin (LPS) and TLR4. The role of endotoxin in atopic disease has been somewhat controversial. Many studies support the conclusion that TLR4 activation via environmental endotoxin in early life is protective against development of allergic disease. High levels of environmental exposure to endotoxin (LPS) have been shown to reduce the incidence of allergic disease in children from both rural and urban environments (85,90). Roponen *et al* found that households with pets had greater concentrations of environmental endotoxin than those without pets (91). This exposure was associated with promoting type 1 responses in T cells following polyclonal activation of children's PBMCs (91). In order to mimic the effects of ambient exposures within the living environment, Ng et al tested how sterile House Dust Extracts (HDE) affect the subsequent immune responses to intranasal allergen (92). In this study, mice were either

given intranasal low-dose HDE in combination with ovalbumin on a daily basis or given vaccines containing HDE weekly. The results of these investigations indicated that the daily intranasal exposure to HDE in combination with ovalbumin induced tolerance to this antigen whereas weekly vaccination did not prevent airway sensitivity. In addition, the mechanism underlying the immunologic effects of HDE was found to be MyD88-dependent and could be duplicated using LPS, indicating the presence of TLR agonists in the ambient environment. This study highlights the importance of the frequency and timing of exposure in determining the type of immune response which ensues. In another mouse model of asthma, Hollingsworth *et al* exposed both wild-type and *tlr4* deficient mice to low levels of LPS; they found that *tlr4* deficient mice had significantly enhanced pulmonary allergic responses following repeated allergen challenge (86). Therapies, which introduce allergens in combination with the TLR4 agonist monophosphoryl lipid A (MPL), are approved for short course treatment of allergic rhinitis (93,94). This vaccine strategy was found to temporarily reverse type 2 immune responses in patients with seasonal allergy, supporting the production of allergen-specific IgG in place of IgE (93). The effectiveness of these therapies further supports the conclusion that TLR activation may be important in instructing appropriate immune responses to potential allergens.

Other TLRs may also play important roles in reducing the symptoms of pre-existing atopic disease by promoting type 1 immune responses. In a mouse model of asthma, Camateros *et al* found that administration of R-848 (recognized

by TLR7/8) after sensitization with ovalbumin inhibited acute symptoms of allergic asthma in a rat model, indicating a potential usage in the treatment of pre-existing allergic disease (95). New vaccines which involve the conjugation of CpG ODN (TLR9) to allergens are also currently being studied for use in the treatment of asthma (74).

Many genetic studies have examined TLR polymorphisms in an attempt to find an association between changes in TLR activity and the incidence of atopic disease. The underlying hypothesis of this class of studies is that polymorphisms in TLRs may either augment or reduce specific responses to infectious agents and environmental exposures, which could contribute to whether or not an individual develops atopy. The results of these studies have been diverse and have shown that TLR activation may play different roles at different stages of allergic disease (61), but as yet very few studies have found a conclusive link between TLR polymorphisms and atopic disease. The best studied polymorphism in human disease is *TLR4* Asp299Gly, is associated with a reduced systemic response to LPS associated with reduced production of the type 1 skewing IL-12, as well as reduced immunoregulatory IL-10 (96). A UK study examining the prevalence of this polymorphism in asthmatic families and healthy controls found no significant association, however individuals with asthma who also had the polymorphism in TLR4 had a higher incidence of severe atopy (61). Eder *et al* found that TLR2/-16934 polymorphism was associated with a reduced incidence of asthma compared to controls (3% vs 13%, P = .012), however this association was only applicable to children raised in rural environments and was not found for those

living in urban centers (97). A recent report from International Study of Asthma and Allergy in Childhood (ISAAC) in Europe found a relationship between polymorphisms in *TLR1, TLR6* and *TLR10* and asthma susceptibility (98). This report found that individuals carrying rare polymorphisms in these genes were much less likely than controls to have asthma (98). These polymorphisms were associated with increased expression of TLR1, TLR6 and TLR10 mRNA and production of TNFα, IL-12 and IFNγ compared to controls (98). These TLRs are all known heterodimeric binding partners of TLR2; the expression patterns of these TLRs may be instrumental in determining the type of immune response which develops.

In light of the evidence in support of the protective role TLRs play in preventing allergic disease it seems unlikely that TLRs are responsible for initiating type 2 responses to allergens. However, several reports suggest that TLRs may play an integral role in determining whether or not an individual develops atopic disease. For instance, deficiencies in TLR responses or expression levels have been found in patients with allergic disease (98,99). Regardless of the role of TLRs in shaping allergic responses, several reports have demonstrated that these receptors contribute to the inflammation associated with asthma exacerbations (100). For instance, viral infections inducing strong type 1 IFNγ responses are the most common cause of flare ups in asthmatics (81). Allergic asthmatics are more sensitive to the bronchoconstrictive effects of inhaled endotoxin than non-asthmatics, indicating that TLR activation may exacerbate pre-existing asthma (101). In addition, respiratory epithelium expressing TLRs

may contribute to damage and inflammation in the lung through the recognition of stress-related proteins such as heat-shock proteins (HSPs) (81). These studies demonstrate that in individuals who have pre-existing allergic asthma, the activity of TLRs may exacerbate the inflammation and bronchoconstriction associated with this disease. Hence, the timing, dose and route of TLR agonist exposure are important in determining how these ligands will affect the immune response. It is necessary to gain a clearer understanding of the functions of TLRs in the airways in humans and how different environmental exposures affect their activity. Once a clearer picture of how immune responses are initiated emerges it may be possible to develop interventions using TLRs to promote beneficial immune responses without exacerbating pre-existing lung disease.

3.1 Early Life and the Role of Innate Immune System: Shaping the future of immune responses

In recent years there has been an increased appreciation of the importance of early life exposures in shaping immunologic responses. These early years are also important in determining whether or not a child will develop asthma and allergies in later life (102,103). Previously it was believed that atopic disease could begin at any age (102). Now many well-powered longitudinal studies indicate that the majority of environmental factors that will influence—positively or negatively—the likelihood of subsequent development of asthma and allergies, occur predominately in the first few years of life. (76,104). At birth the anti-viral responses elicited from CBMCs have been shown to correlate with the incidence

of wheeze and lower respiratory tract infections in early life (78). Infants with recurrent wheeze in the first year of life have a greater incidence of wheeze later in life (88). Lung development occurring in early childhood shapes future outcomes in terms of asthma and lung function in later life (103). Poor lung function shortly after birth, which is often present following preterm birth, is also an indicator of reduced lung function in later life (78). Hence, factors which alter immune responses to pathogens and environmental stimuli in early life, such as reduced innate immune responses, could have important implications for future outcomes.

Familial history of atopy is a strong predictor of allergic disease (105). As such, several studies have examined immune responses in the neonate to determine if immune responses at birth are distinct in neonates with a familial history of asthma or allergy. Schaub *et al* found expression of FoxP3 and IL-10, factors associated with Treg activity, in CBMCs of neonates with atopic mothers was reduced (106). Leung *et al* found maternal atopic status was associated with increased levels of macrophage-derived chemokine (MDC), a chemokine which has been associated with promoting type 2 immune responses in cord blood serum (107). In addition Amoudrez *et al* demonstrated, albeit in a small sample, significantly reduced TLR2, TLR4 and CD14 expression on the surface of CBMCs of neonates whose mothers had a history of atopy (75). Following LPS and PGN stimulation, CBMCs from neonates of atopic mothers produced significantly less proinflammatory cytokines than those of nonatopic mothers (75). In contrast, Willwerth *et al* found that allergic status had no effect on

proliferative responses to common allergens in CBMCs (105). Hence, the role of familial allergic history on CBMCs is controversial. Clearer understanding of the mechanisms mediating neonatal priming and its potential role in development of protective vs allergic responses need to be better defined. Collectively these studies demonstrate the potential of *in utero* priming of immune responses in the fetus based on maternal exposures and immune responses. Therefore the fetal period may be important in shaping the immune responses which occur in early life. In addition, these studies suggest that reduced capacity to produce proinflammatory cytokines following TLR stimulation in early life, not necessarily a propensity to produce type 2 cytokines, may contribute to the development of atopy in late life.

Given that immune responses in infancy can influence the development of immune responses later in life it may be possible to promote protective immune responses through therapeutic interventions during this time. Arslanoglu *et al* recently reported that administration of a mixture of oligosaccharides found in human breast milk as a supplement to infants' formula may protect at risk infants from developing atopy in early life (108). This group selected healthy term infants with a familial history of allergic disease and conducted a randomized prospective study to determine the effects of supplementing with this 'prebiotic' mixture of oligosaccharides (108). Infants who received the oligosaccharide supplement in their formula in the first six months had significantly reduced incidence of atopic dermatitis, recurrent wheeze and urticaria compared to placebo (108). Interestingly, these oligosaccharides, which promote colonization of the GI tract

with lactobacillus and bifobacteria (probiotic microbes), also markedly reduced the incidence of upper respiratory tract infections, fever and antibiotic usage in the intervention group (108). The exact mechanisms which are involved in promoting these beneficial effects were not addressed by this study but may involve instruction of appropriate immune responses via interaction of TLRs and GI flora. These results indicate that supplementing formula of bottle-fed infants who are at high risk of developing atopy may lead to long-lasting protection from atopy as well as infectious episodes. Future studies will be necessary to determine if these results last into later childhood and if this approach can be duplicated in preterm neonates. TLR agonists, TLR9 (CpG DNA) in particular, are currently being tested as vaccine adjuvants in the clinical trials of adults (74). Treatment of neonates with these TLR agonists may be able to improve the protective effects of these vaccines or enhance type 1 responses. TLRs also have the potential to induce type 1 immune responses as a monotherapy or when combined with allergens (94,109). Future studies are required to determine if these therapies may be used safely in neonates to enhance vaccine efficacy and portentially prevent the development of allergic disease in high risk children born preterm birth or with a familial history of atopy.

Childhood may represent a window of opportunity to prevent the development of atopy, or to modify the course of disease to prevent chronic lifelong illness. Tulic *et al* demonstrated that children with atopy are more sensitive to the protective effects of endotoxin than atopic adults (110). In this study cells from the mucosa of atopic children or adults were stimulated with

allergens with or without LPS (110). LPS exposure was able to reduce allergen-induced type 2 responses and upregulate Th1 cytokines as well as IL-10 in the children but not adults (110). T cells from the mucosa of atopic children were found to be much more responsive to LPS stimulation than adults, emphasizing differential responses in children and adults. Currently, the maturation of the innate immune system throughout life is not well defined. Developing a clearer picture of how this maturation occurs is essential as it appears that early life may represent a window of opportunity to prevent allergic disease later in life.

3.2 Neonatal Immunity and Clinical Correlates

Although several reports suggest that early life exposures may be integral in educating immune responses later in life, few studies have examined how the immune system responds to various classes of stimuli at birth. Immune responsiveness at birth is hypothesized by many to be a predictor of future immune responses and clinical outcomes. In recent years neonatal immune capacity and ontogeny of responses have become a topic of active research. The neonatal period represents a time of increased susceptibility to infectious disease, which is the leading cause of death in neonates (111,112). Immaturity of the adaptive immune response has been implicated in the increased rate of mortality and morbidity in the neonatal period (112,113). As would be expected, due to both the lack of previous immune education by foreign antigens, and inherently compromised immune capacity, the adaptive immune response at birth is not fully

functional (114,115). For instance, neonates have reduced capacity to produce effective memory responses to vaccines, requiring a number of exposures to induce protective immunity (116). Type 1 immune responses in neonates are diminished in several ways (113). For example, reduced production of Th1 cytokines (IFNγ by T cells and IL-12 and TNFα by phagocytes), in addition neonatal macrophages are hyporesponsive to IFNγ stimulation (64,114). It has been hypothesized that the immune response may favor type 2 responses in order to maintain pregnancy while *in utero* and that this skewing extends into neonatal period (117). However, careful review of the available literature indicates that this so called Th2 bias is based on enhanced IL-10 and IL-6 production compared to other cytokines (such as TNFα). IL-10 and IL-6 are known to be produced by many cell types and the roles of these cytokines have not conclusively been linked to promoting Th2 immune responses. Hence, the labeling of neonatal immune responses as "Th2 biased" may be seen as an imprecise misnomer.

One potential contributor to the reduced proinflammatory cytokine production is adenosine, which was found to be present in high levels in cord blood compared to adult peripheral blood (118). Adenosine was shown to increase the accumulation of cyclic AMP which specifically inhibits TNFα and IL-12 production, while augmenting expression of IL-6 and IL-10 (118). However, not all studies report these defects in neonatal immune responses. Trivedi *et al* report that neonatal T cell proliferation following polyclonal activation was equivalent to adult cells (119). This group also found MHC-dependent T cell activation of neonatal cells induced levels of IFNγ, IL-4 and IL-10 equivalent to adults (119),

suggesting that neonatal T cells may be largely immunocompetent at birth and the challenges lie with APC function. .

A newer concept in the study of neonatal immunity is that the innate immune system may also be functionally distinct in neonates. The majority of current studies now support the conclusion that deficiencies in innate immune components, such as DCs and other APCs, may contribute significantly to diminished immune responses at birth (65). In a recent review Velilla *et al* outline the current literature regarding deficiencies in neonatal APCs compared to those from adults (112). One pertinent example of reduced innate immune capacity cited is the reduced capacity of neonatal DCs to sufficiently upregulate costimulatory molecules (such as CD40L, CD80/CD86) and Class II MHC, thus explaining why neonatal APCs are inefficient inducers of T cell responses at birth (112,120). Neonatal neutrophils have also been shown to be less effective at clearing bacteria and responding to LPS (121). One reason for the reduced responses observed in neutrophils was a reduced TLR response, in part due to reduced expression of MyD88 and p38 phosphorylation following TLR activation (121). Neonates also have impairments in neutrophil chemotaxis, maturation and transmigration into sites of inflammation (122). Cord blood mononuclear cells (CBMC), which are comprised of many cell types (including monocytes, macrophages and lymphocytes), have also been shown to have diminished responses to LPS compared to adult PBMCs despite similar levels of TLR4 expression (123). One potential mechanism contributing to the reduced innate immune responses observed in neonates is a reduced expression of MyD88 by

CBMCs, thus contributing to reduced TLR signaling compared to adults (123). CBMCs were hyporesponsive to TLR3 and TLR4 stimulation, producing significantly lower levels of TNFα, IL-12 and IFNα production compared to adult cells (120). Hence, reduced TLR responsiveness in the neonatal period may contribute to increased susceptibility of neonates to develop infections with a variety of gram-positive and negative bacteria as well as viruses (such as RSV) in the perinatal period (124).

However, not all immune responses are diminished in neonates. For instance, Levy *et al* found that IL-6 production was similar to adult levels following activation of cord blood with a variety of TLR agonists (64,113). TLR7 and TLR8 agonists were particularly effective at inducing strong cytokine responses in neonates, especially the synthetic agonists R-848 which was able to induce significantly greater production of TNFα than the other TLR agonists tested (64). As such, it is possible that TLR7/8 agonists could be utilized to enhance vaccine efficacy in neonates by skewing a desirable Type 1 immune response, which may reduce morbidity and mortality in the neonatal period (124). Gold *et al* found that TLR9-stimulation induced similar amounts of IFNα production in both cord blood and adult peripheral blood samples (111). Defects in the expression of costimulatory molecules on the surface of neonatal DCs could be mitigated by stimulating these cells with CpG-ODN (111). Krumbiegel *et al* report that co-administration of multiple TLR stimuli was able to induce maturation of neonatal monocyte-derived DC, whereas use of single TLR agonists was not effective (65). Combination of R-848/LPS/Poly I:C was required to

induce equivalent production of IL-12p70, IFNγ, TNFα in neonatal mDCs, whereas adult mDCs did not require synergistic stimulation. This treatment also improved neonatal mDCs ability to prime T cells to produce IFNγ (65), indicating that the synergistic activity of select TLRs may be useful as vaccine adjuvants in neonates. Responses to whole commensal bacteria in neonatal CBMCs were also found to induce IL-12 and TNFα at levels comparable to adult PBMCs, with a tendency to produce higher levels of IL-6 than adults (125). Responses to these commensal bacteria were found to be mediated through TLRs exclusively (125). Cytokine patterns elicited from CBMCs varied with the type of commensal organisms, suggesting that the colonization of the neonatal gut may be important in determining the type of immune response which ensues (125). Niers *et al* found that stimulation of neonatal DCs with the whole probiotic bacteria (such as Lactobacillus, Bifidobacterium etc) could skew Th1 polarizing responses, indicating that treatment with these bacteria in early life could help prevent maladaptive immune responses in early life (126). However, attempts to duplicate these protective effects *in vivo* have been largely unsuccessful in trials with human neonates (127). Collectively these studies indicate that TLR agonists may be able to improve the function of neonatal APCs and enhance protective immunity. Although the innate immune system in neonates may not behave in the same fashion as in adults, it appears that neonates are capable of mounting immune responses under the appropriate conditions. Further research is required to obtain an improved understanding of neonatal immune capacity and the intensity of responses to develop potential therapeutic interventions.

3.3 Effects of preterm birth on Immune capacity and outcomes

Premature delivery is defined as any delivery occurring prior to 37 weeks and zero days gestation (0/7) (128,129). This group can be further subdivided into early preterm (prior to 34 0/7 weeks) and late preterm (34 0/7 to 36 6/7 weeks gestation) (129). Preterm delivery is associated with increased short and, importantly, evidence of long term morbidity and mortality (128-130). The majority of studies examining outcomes following preterm birth to date have focused on extremely preterm (< 30 weeks), low birth weight infants who have very high risks of severe complications following delivery (131). In recent years, however, neonatologists have recognized that being born prior to 37 weeks, even if born near the cut off, leads to an increased risk of mortality and morbidity compared to their full term counterparts (128,132). In particular late preterm neonates, formerly referred to as 'near term', have been recently identified as a group of neonates deserving increased awareness (130).

The rate of infants delivered in the late preterm age group has increased in the past two decades by up to 37% in North America (129). Currently, late preterm deliveries account for 74% of all deliveries prior to 37 weeks in the US (133,134). Contrary to previous obstetrical guidelines, which assumed little or no increased risks for these 'great imposters', recent studies have determined that this population has significantly greater morbidity and mortality in early life than infants born at term (133,135). Shapiro-Mendoza *et al* found that late preterm neonates had a 7 times greater incidence of severe complications (such as respiratory distress, sepsis, and feeding problems) in their post-birth hospital stay

than those born after 38 weeks, suggesting that these neonates are not functionally mature at birth (133). These neonates are also up to 3 times more likely to be readmitted to hospital following delivery (133). Some of the common reasons for hospital visits in the perinatal period included: hyperbilirubinemia (71%), suspected infection (20%), and feeding difficulty (16%), problems that reflect developing physiologic and metabolic organ functions (132). These reports collectively suggest the need for late preterm neonates to be identified as being physiologically immature and requiring special attention in the neonatal period.

Not only are infants born prior to 37 weeks (considered as one homogenous group by many studies) physiologically immature, recent reports suggest that the immune response in these neonates is significantly impaired compared to neonates born at term (136,137). Not surprisingly, preterm neonates have a significantly greater risk of developing sepsis and infectious illnesses in the perinatal period (137). Early-onset sepsis is a major cause of death in very low birth weight infants (VLBWI) (138), as such several groups have been focused on identifying deficiencies in this group of neonates at birth. Hallwrith *et al* found that VLBWI had significantly reduced levels of HLA-DR in CBMC(136), possibly contributing to a reduced ability to mount effective adaptive immune responses. Monocytes from early preterm neonates (<30 weeks gestation) have also been found to have significantly diminished TLR4 levels compared to full term neonates; this reduced TLR expression correlates with reduced capacity of preterm monocytes to generate proinflammatory cytokines following LPS exposure (139). A report published by the same group found that the expression

of MyD88 and TLR4, was significantly diminished in all preterm neonates compared to full term neonates (140). Monocytes and the TLR system play important roles in initiating innate immune responses following microbial challenge, therefore reduced function of TLR pathways in these cells could contribute to the increased infection susceptibility observed in preterm neonates. Unfortunately, there is very limited information regarding the innate immune capacity of late preterm neonates specifically. Also, most epidemiologic studies examining postnatal outcomes in preterm neonates have been retrospective and have excluded late preterm neonates due to their presumed "near-normality" (129). Longitudinal prospective birth cohort studies which examine the postnatal period in all preterm neonates will be instrumental in identifying the immunologic nuances and functional consequences in this group. Long term epidemiology studies which are available have also found that preterm infants have increased risk of developing morbidity and mortality in childhood (141). Swamy *et al* found that preterm birth was not only associated with increased childhood mortality but was also a significant predictor of reduced academic performance, and reproductive capacity in adulthood (141).

Several reports have indicated that preterm birth is associated with an increased risk of developing asthma in later life (79,80). Kumar *et al* found that preterm birth (regardless of ethnicity, sex and maternal factors) is a risk factor for recurrent wheezing in early life (odds ratio [OR], 1.7; 95% CI, 1.2-2.6), with the greatest incidence in very preterm neonates (142). In another retrospective study, Gessner *et al* found that the risks of developing asthma were significantly greater

in children born preterm, but that the risks were reduced with each week of gestation (143). This study also found that preterm neonates who developed childhood asthma had a significantly greater risk of hospitalization for severe asthmatic exacerbations than children born at term. Grischkan *et al* found that infants born prior to 28 wks were twice as likely to have asthma in adulthood as those born at later gestational age (80). This study and others have found that the development of bronchopulmonary dysplasia, which occurs frequently in extremely preterm neonates requiring mechanical ventilation, was a significant risk factor for developing recurrent wheeze in later life (80,144,145). However, in this study there was no relationship between the asthmatic phenotype and atopy in the preterm born adults (80), indicating a potentially distinct mechanism of airway remodeling in these survivors of early preterm birth.

Studies examining outcomes of late preterm neonates in the postnatal period have found that these neonates are much more likely than term infants to develop respiratory distress, and pulmonary hypertension (129,146,147). The long term outcomes of infants which have these symptoms in the postnatal period are not well understood. It is possible that the combination of reduced immune capacity and physiologic immaturity of the lung in late preterm neonates could increase their risk of developing asthma in later life. Many studies suggest that certain viral infections in early life predispose infants to the development of asthma (79), which could contribute to the increased risk of asthma observed in preterm neonates.

In summary, the link between preterm birth and asthma has been duplicated by several studies. However, the mechanisms responsible for the increased asthma incidence remain unclear and may be distinct in very low birth weight infants and other preterm neonates. Future prospective birth cohort studies should include late preterm neonates and follow these neonates into the postnatal period in order to develop a more indepth understanding of the relationship between gestational age, immune capacity at birth and immunologic outcomes in early life.

Thesis Overview

Early life represents a critical period in education of the immune system. TLR responses which take place in the early years play a critical role in directing subsequent adaptive immune responses in later in life. However, relatively little is known about how broad innate immune capacity at birth. In addition, how gestational age affects the maturity of innate immune responses is not well understood. Recently late preterm neonates have been recognized as having increased risk of developing complications such as infections and respiratory distress in the post natal period. Currently there is very little known about innate immune capacity in this late preterm population compared to the full term counterparts.

It is well established clinically that susceptibility to infectious and allergic disease change over time. Current evidence suggests that the innate immune system plays an integral role in protecting against infectious agents and directing

adaptive immune responses which develop. Although the etiology of allergic diseases remains to be clearly elucidated, appropriate TLR signalling appears to be protective. The majority of studies have focused on inappropriate adaptive responses and how these develop throughout life. Very few studies have examined innate immune responses and how these develop over time.

In recent years there has been an increased appreciation of the role of environmental factors in determining how immune response develops. The role of epigenetics in modulating gene expression is beginning to become better understood however no studies have assessed the role of endogenous epigenetic regulation in mediating innate immune responses at birth.

In light of these gaps in knowledge the goals of my research were as follows:

i) Define innate immune capacity in late preterm neonates compared with those born full term

ii) Determine potential mechanisms underlying differential innate immune responses in late preterm compared to full term neonates

iii) Compare innate immune capacity in neonates with individuals at several points in later life to obtain a clear understanding of how innate immune response develops throughout life

iv) Determine how endogenous epigenetic modifiers regulate innate immune responses at birth

v) Examine global protein profile following TLR stimulation in preterm and full term neonate

Materials and Methods

Cord blood samples

This study was approved by the University of Manitoba, Faculty of Medicine Committee on the Use of Human Subjects in Research. Anonymous umbilical vein cord blood samples were obtained from the Women's Hospital, Winnipeg MB. Inclusion criteria for this study include labour and vaginal delivery (or emergency caesarean), no maternal history HIV or hepatitis, no evidence of chorioaminionitis or congenital abnormalities. Cord blood was collected from 111 full-term newborns (37-42 weeks gestational age) and 63 preterm samples ranging from 29-36 weeks of gestation (Appendix A). Cord blood was collected from the umbilical vein following delivery into sterile 50 mL tubes containing 3 mL of 2.7% EDTA. Samples were processed within 24 hours of delivery.

Cord Blood Mononuclear Cell Isolation

On average 30-50 mL of cord blood was collected on following delivery. Cord blood mononuclear cells (CBMC) were isolated using a ficol gradient. Briefly, the samples were diluted 2:1 with 0.85% sterile saline solution to improve cell yields. Blood was layered onto Histopaque-1077 Ficol (Sigma Aldrich Canada Ltd, Oakville Ontario, Canada). Cells collected from the buffy coat were washed twice in 0.85% sterile saline solution. Cells were resuspended promptly in complete RPMI media (containing RPMI-1640 supplemented with 10% heat inactivated FBS, 2 mM L-glutamine and 2 X 10^{-5} M 2-mercaptoethanol, and antibiotic-antimycotic (Life Technologies, Burlington Ontario, Canada)). Cell

counts were determined using a hemocytometer. Cell viability was greater than 95% in all samples as determined using trypan blue exclusion.

Short Term primary culture of CBMCs

Preliminary experiments using various cell concentrations demonstrated that optimal responses occurred at 3.5 X 10^{-5} cells/mL in 200 uL. Cells were cultured for 3 or 24 hours in 96-well U bottom plates (Nunc, Rosklide, Denmark). Prior to culture, an aliquot of cells were washed with saline, pelleted and stored at -80c for subsequent RNA analysis. Each sample had a control with no stimulation (media alone). Toll-like receptor ligands were used for stimulation at various concentrations. Toll-like receptor stimuli were used as follows: TLR1/2 (Pam3CSK4, Invivogen, San Diego CA), TLR2/6 (Peptidoglycan S. Aureus: Fluka Biochemica, Switzerland and Invivogen, San Diego CA), TLR3 (Poly I:C, Invivogen, San Diego CA), TLR4 Lipopolysaccharide (LPS) from E. Coli (Serotype 00011:B4, Sigma (St. Louis, MO)) , TLR7/8 (3M011, 3M Pharmaceuticals, St Paul MN), TLR7 (3M001 , 3M Pharmaceuticals, St. Paul MN), TLR8 (3M002,3M Pharmaceuticals, St. Paul MN) and TLR9 (CpG ODN 2216 (Invivogen, San Diego CA). Following culture, cell supernatants were harvested and stored at -20C while cell pellets were stored in at -80C RNA Later (Qiagen, Mississauga ON) for future analysis.

Cytokine and Chemokine ELISAs

Concentrations of cytokines and chemokines in cord blood supernatants following *in vitro* stimulation with TLR agonists were quantified using optimized

sandwich ELISA protocols. Matched pair anti-cytokine and biotylated antibodies for IL-6, IL-10 and TNFα ELISAs were obtained from BioLegend (San Diego CA). CCL2 (MCP-1) antibodies were purchased from Peprotech (Ottawa ON). IL-1b antibodies were obtained from Antibody Solutions (Mountain View CA).

Recombinant human cytokines and chemokines used as standards were purchased from BioLegend (for IL-6), Peprotech (IL-1b, TNFa), BD Pharmingen (IL-10 and MCP-1, PF4).

The general protocol used to detect human cytokines and chemokines is as follows:

1) Coating: Plates are coated overnight at 4C with 50 uL/well of coating antibody solution.
2) Blocking: Coating solution is removed from plate and plates are blocked for 2 hours with blocking buffer (75 uL/well) at 37ºC.
3) Sample Addition: Blocking solution is removed by washing plates four times with wash buffer. Samples were added at optimal concentrations and titrated (determined via optimization) along with the standard (recombinant cytokine of interest) to create a standard curve for each cytokine. Samples are incubated on plates overnight at 4C in a final volume of 50uL/well
4) Detection: Biotinylated detection antibodies were added following the removal of the sample solution. Plates were then incubated overnight at 4C in a final volume of 50uL/well.

5) Development: Following removal of detection antibody, Streptavidin-alkaline phosphatase (Jackson ImmunoResearch, Mississauga ON) was added at a concentration of 1:3000 (50uL/well) then incubated for 45 minutes at 37C. Plates were then treated with p-nitrophenol phosphate substrate tablets (Sigma Aldrich Canada Ltd).
6) Analysis: Plates were read between 405 and 690 nm (SOFTmax Pro 3.2.1 program, SpectraMax 190; Molecular Devices Corp, Sunnyvale, CA)

Detection limits were as follows: IL-1b, 19.5pg/mL; IL-6, 0.16 ng/mL; CCL2 0.78 ng/mL; IL-10 15.6 pg/mL, PF4 0.78 ng/mL, CXCL10, 39 pg/mL.

All cytokine and chemokine ELISA and ELISPOT analyses were examined over a series of a minimum of four dilutions. Standard curves were generated for each ELISA plate.

ELISPOT

The frequency of cord blood cells secreting IL-10 and IL-6 were enumerated using BD™ ELISPOT Human IL-10 Set (BD Biosciences, Mississauga, ON) and eBio-sciences Human IL-6 ELISPOT Ready-Set-Go™ Set according to the manufacturers' specifications (eBioscience, San Diego, CA**).** Briefly, plates were coated with anti-hIL6 or anti-hIL-10 capture antibodies for 2 hours at 37ºC, followed by blocking with complete RPMI for 2 hours. Cord blood mononuclear cells were cultured starting from 4 X 10^5 cells/well for the IL-10 assay (diluted 2-fold for four dilutions) and 8 X 10^4 cells/well for the IL-6 assay (diluted 3-fold for four dilutions). In triplicate, cells were stimulated with PGN (0.1ug/mL), LPS (0.5ng/mL), 3M002 (0.5ug/mL) or media alone.

Plates were incubated for 18 hours, washed and then incubated with 100 uL/well of biotinylated detection antibody for 2 hours at room temperature. Color was developed using the BD™ ELISPOT AEC Substrate Set (BD Biosciences, Mississauga, ON). Following spot development, the reaction was stopped by washing with distilled H_20. Plates were scanned using a Series 3B ImmunoSpot™ Analyzer (CTL Technologies, Cleaveland OH) and counted using ImmunoSpot™ Version 3.2 counting software (CTL Technologies, Cleveland OH).Variance in data typically obtained between triplicates was less than 5% deviation from mean values.

RNA Isolation and Q PCR

Total RNA was isolated from cord blood or peripheral-blood mononuclear cells by use of a standard phenol extraction method. Complementary DNA (cDNA) was synthesized from 4 ug total RNA using random hexamer primers and SuperScript^{TM} reverse transcriptase (Invitrogen Crop., Carlsbad,CA). The reaction mix (20 ul) was incubated at 25°C for 5 minutes, at 42°C for 1.5 hours for reverse transcription, and finally at 85°C for 5 minutes for reverse transcriptase inactivation. Quantitative real-time PCR of target cDNA was conducted for TLR-2, 4, 6, 7, 8, CD14, MyD88 and TRIF. Values were normalized and expressed as a relative fold change relative to 18s rRNA expression. All PCR primers were listed in table. Experiments were performed in 96-well plates in duplicate using SYBR Green Master Mix (Stratagene). Real-time PCR amplification was performed on an ABI 7500 Detection System. PCR conditions were 95ºCfor 10 min, and then 45 cycles at 95ºC for 15s, 60ºC for 30s and 72ºC for 1 min.

Table 2. Primer Sequences used for RT-PCR Experiments

Gene symbol	Forward primer (5'-3')	Reverse primer (5'-3')
TLR2	TCTGCAAGCTGCGGAAGATAAT	GCAGCTCTCAGATTTACCCAAAA
TLR4	GGACTGGGTAAGGAATGAGCTAGTA	CACACCGGGAATAAAGTCTCTGT
TLR6	GGATGATGGTGAATAGTACAGTCGTAA	CAGTTCCCCAGATGAAACATTG
TLR7	AAGCCCTTTCAGAAGTCCAAGTT	GGTGAGCTTGCGGGTTTGT
TLR8	CGGATCTGTAAGAGCTCCATCCT	TCAAGACCACATTTCTCAGAGTTTG
MyD88	TTGAGGAGGATTGCCAAAAG	CAGGGGGTCATCAAGTGTG
TRIF	ACTGTGTCATCCCCTTCCTG	ATCTGGGAGTGTTCGTCCAG
18s rRNA	CCGCAGCTAGGAATAATGGA	CCCTCTTAATCATGGCCTCA

Flow Cytometry

To determine relative proportions and absolute numbers of CD14+ cord blood leukocytes 100 uL fresh whole blood was incubated with a CD14-PE/CD45-FITC or isotype control cocktail (Beckman Coulter Canada Inc, Mississauga, ON) for 15 minutes at room temperature . Red blood cells were lysed using 500 uL of Optilyse C Solution (Coulter Canada Inc, Mississauga, ON). Following red blood cell lysis samples were diluted in 1X PBS and then fixed in 2% paraformaldehyde. Samples were analyzed using EPICS model 753 fluorescence-activated cell sorter (FACS; Coulter Electronics, Hialeah, FL). Gates were determined based on forward and side scatter properties and isotype

controls. Staining of samples in duplicate elicited a typical variances in the proportion of CD14+ cells of less than 5%.

For intracellular detection of IL-6 production, CBMC were stimulated with 100 ng/mL LPS in the presence of Golgi Plug (BD Bioscience, Missauga, ON) for 6 hours. Cells were fixed in 2% paraformaldehyde, permeabilized with 0.1% saponin and stained with 5 uL anti-CD14-FITC (BD Bioscience, Missauga, ON) and 20 uL anti-IL-6-PE (BD Bioscience, Missauga, ON) on ice for 30 minutes. Following staining cells were washed and analyzed using BD

Statistical Analyses

GraphPad Prism 5.0 software (GraphPad, San Diego CA) was used to conduct statistical analyses. When the distribution of data was determined to be Gaussian, the two-tailed unpaired *t* test was used to compare mean ± SEM values between groups. When data were non-Gaussian (not normally distributed) the Mann-Whitney nonparametric test was employed. P-Values of <0.05 were considered statistically significant.

Proteomics

Sample Preparation

In order to analyze secreted proteins via proteomic techniques, supernatants from CBMCs samples cultured for 24 hours were removed and frozen prior to processing. Total protein content in the samples was estimated using BCA (Bradford Assay). Preparation of samples involved protein reduction with DTT (10 mM 65 ^{0}C, 30 min), alkylation (50 mM iodoacetamide, room temperature 45

min) and trypsin digestion (1:50 enzyme/substrate ratio, incubated at 37°C overnight). Prior injection into chromatographic systems samples were diluted to provide ~4-5 μg injection of the digest (LC-MALDI MS) and ~100 μg (2D LC-ESI MS/MS). pH of the samples was adjusted prior injection to ~10 with ammonium formate and to ~3 with trifluoroacetic acid for LC-ESI and LC-MALDI analyses, respectively.

HPLC-MALDI MS

Digested supernatants and cytokine-spiked serum free media samples were fractionated using linear water-acetonitrile gradients (1% acetonitrile starting conditions) on a micro-Agilent 1100 Series system (Agilent Technologies, Wilmington, DE). The column effluent (3 μL/min) was mixed on-line with 2,5-dihydroxybenzoic acid MALDI matrix solution (0.5 μL/min, 150 mg/ml DHB in water/acetonitrile 1:1) and deposited by a computer-controlled robot onto a movable metal target at 0.5-min intervals. Both eluents contained 0.1% trifluoroacetic acid (TFA) as an ion-pairing modifier. Samples (5 μL) were injected directly a 300 μm × 150 mm (PepMap100, 3 μm; LC Packings-Dionex, Sunnyvale, CA). The gradients used were 0.75% acetonitrile per minute (230).

TOF Mass Spectrometry

Spots of the chromatographic fractions were analyzed by single mass spectrometry (MS) with *m/z* range 550-5000, and by tandem mass spectrometry (low-energy CID, MS/MS) in the Manitoba/Sciex prototype MALDI quadrupole/TOF (QqTOF) mass spectrometer. Proteins were identified using

standard peptide mass fingerprint approach (10 ppm mass accuracy) with additional retention time constraint (2 minutes prediction accuracy). (231)

2D RP HPLC-ESI MS/MS

Samples were applied to 1X100 XTerra column with Agilent 1100 Series HPLC System (Santa Clara, CA) and a linear water acetonitrile gradient (20 mM ammonium formate pH 10 in both eluents A and B, 1% acetonitrile/min, 150 µL/min flow rate). A concentrated 200 mM solution of ammonium formate at pH 10 was prepared Buffers A and B for first-dimension separation were prepared by a 1/10 dilution of this concentrated buffer with water and acetonitrile, respectively. Approximately 100 µg of the digest was fractionated in replicates runs, while UV detection at 214 nm was used to monitor the elution profile, which showed good reproducibility between these replicates. Forty 1-min fractions were collected (roughly 2.5 µg/fraction in each within 10-50 min elution window). Each was lyophilized and resuspended in 25 µL of buffer A (2% acetonitrile and 0.1% formic acid in water) solution for the second-dimension separation. A splitless nanoflow Tempo LC system (Eksigent, Dublin, CA) with 20-µL sample injection via a 300 µm × 5 mm PepMap100 precolumn and a 100 µm × 150 mm analytical column packed with 5-µm Luna C18 (Phenomenex, Torrance, CA) was used in the second-dimension separation prior to MS analysis. Both eluents A (2% acetonitrile in water) and B (98% acetonitrile) contained 0.1% formic acid as ion-pairing modifier. A 0.33% acetonitrile/min linear gradient (0-30% B) was used for peptide elution, providing a total 2-h run time per fraction in the second dimension. Add reference here (232)

TOF mass spectrometry

A QStar Elite mass spectrometer (Applied Biosystems, Foster City, CA) was used in standard MS/MS data-dependent acquisition mode. The 1-s survey MS spectra were collected (m/z 400-1500) followed by three MS/MS measurements on the most intense parent ions (80 counts/s threshold, +2-+4 charge state, m/z 100-1500 mass range for MS/MS), using the manufacturer's "smart exit" settings. Previously targeted parent ions were excluded from repetitive MS/MS acquisition for 60 s (50 mDa mass tolerance). Raw spectra files were treated using standard Mascot.dll script (Analyst QS2.0) to generate text files in mascot generic file format (MGF). These files containing the MS/MS spectra information for all 40 fractions were concatenated and submitted for protein identification by the X!Tandem (GPM) search engine. Standard QTOF settings were used for the search: 100 ppm and 0.4 Da mass tolerance for parent and fragment ions, respectively. Permitted amino acid modifications included constant carbamidomethylation of Cys, variable deamidation of Asn and Gln, the N-terminal loss of ammonia at Cys and Gln, Met oxidation, and N-terminal acetylation.

CHAPTER 1

TLR RESPONSIVENESS IS DEFICIENT IN LATE PRETERM NEONATES.

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CIHR National Training Program in Allergy and Asthma Research [1]Dept. Immunology[1], Pediatrics and Child Health[2] and Pathology[3] Univ. of Manitoba

ABSTRACT

Background: Late preterm neonates (34-36 weeks) have historically been grouped clinically with full term neonates; however this group is increasingly recognized as being at increased clinical risk, including, having increased risk of infection and respiratory distress at birth. Our objective was to assess how gestational age affects the pattern and intensity of innate immune responses at birth.

Methods: Cord blood samples were collected following vaginal delivery (only) from full-term (<37weeks, n=77), early (<33 weeks, n=8) and late preterm (34-36 weeks, n=40). Cord blood mononuclear cells (CBMC) were cultured with agonists of TLR1-9 for 24 hours. Expression of cytokines and chemokines in supernatants was measured via ELISA. The frequency of CBMC producing IL-6 and IL-10 was measured via ELISPOT analysis. Flow cytometry was utilized to quantify absolute number of CD14+ monocytes. Q- PCR was employed to assess the mRNA levels of various TLRs and related signaling molecules.

Results: We examined a broad range of responses to TLR ligands for both viral and bacterial antigens at different gestational ages. In general, proinflammatory markers (IL-1 β and CCL2), anti-inflammatory (IL-10) and global markers of TLR dependent activation (IL-6) were all markedly reduced in the late preterm group following TLR stimulation. Frequencies of IL-6 and IL-10 producing cells and the absolute number of CD14+ monocytes were not different in the preterm and full term groups. However, the expression of TLR2,4,6,7,8, CD14, MyD88

and TRIF mRNA was significantly reduced in the late preterm compared to the full term population.

Conclusion: Late preterm neonates demonstrate an impaired innate immune response to both bacterial and viral stimuli. Our data suggests that CBMC from late preterm neonates possess intrinsic deficiencies in TLR signaling pathways which could contribute to the hyporesponsiveness observed in this population.

PREAMBLE

Early life is an extremely influential period in the development of the immune system (102,103,148,149). The neonatal period represents a period which is instrumental in programming the immune system (150,151). Toll-like receptors (TLR) provide a link between early life environment, developing innate immune capacity, the types of adaptive immune responses which develop and ultimately the clinical phenotype displayed (85,152). Selective deficiencies in TLR responses in neonates compared to adults have been reported by several groups (65,118,121,123). However, certain TLR responses in cord blood cells are similar to adults, including TLR7/8 responses to R-848, suggesting that TLR responses are not uniformly deficient at birth (64,113).

The neonatal period is associated with increased susceptibility to bacterial and viral infection (137,153). Importantly, the risks of acquiring serious infections are significantly greater following preterm delivery (112,139,140,154). Preterm delivery is also associated with increased morbidity and mortality later in life, including an increased risk of developing respiratory distress and asthma

(102,128,130,155,155,156). The putative role of the immune system in this process remains speculative. Early preterm neonates (>33 weeks) have been the focus of the limited studies examining the immunologic consequences of premature delivery due to the high rate of severe complications in this group, however very recent research indicates that late preterm neonates have also been shown to have a much greater incidence of morbidity and mortality in the perinatal period (128). To our knowledge there is no published research which examines innate immune capacity in the late preterm population compared to full term neonates.

RATIONALE AND HYPOTHESIS

Immune function in the neonatal period and how its activity is influenced by gestational age are incompletely understood. This period represents a potential time to modify immune responses to prevent infection and reduce maladaptive immune responses, such as allergy and asthma, in later life (91,106) . The frequency of premature births has been steadily increasing in recent years (131,135). The majority of neonates born preterm fall into the late preterm category (weeks 34-36) (131,135,142,157), hence increased complications and hospital readmissions as well as chronic long term morbidity occurring in this population represents a large health care burden. The innate immune system is the primary immunologic defence in the neonatal period (124). We therefore hypothesized that the late preterm population has deficient capacity to mount innate immune responses at birth, contributing to increased risks for infections and hospital readmission in early life observed in this population.

RESULTS

Threshold concentrations of TLR ligands better demonstrate differences in neonatal TLR responsiveness than do widely used pharmacologic concentrations

The vast majority of published studies of TLR ligands utilize concentrations several orders of magnitude above typically encountered environmental levels (158). Typical concentrations utilized by many studies range between 100ng/mL-100 ug/mL (159); however we and others have shown that much lower concentrations of TLR ligands (0.5ng/mL-50ng/mL) elicit quantifiable production of cytokines and chemokines from PBMCs (159). To determine if CBMCs were responsive to low concentrations of TLR stimuli we initially compared TLR stimuli across a four order of magnitude range (Figure 1). The data in Figure 1 demonstrates that readily quantified production of most cytokines of interest is evident at much lower, putatively more environmentally relevant, TLR ligand concentrations than widely published. Secondly, as previously demonstrated for pediatric populations (8-10 years of age), the capacity to detect differences in distinct populations is generally enhanced at lower TLR concentrations (159). In general, the most statistically significant differences in cytokine production between preterm and full term neonates were observed at threshold levels of TLR stimuli. As the concentration of TLR agonists increased, the differences in these groups were less significant, demonstrating that threshold levels of TLR stimuli are best able to demonstrate functional differences in preterm and full term neonates. For example, upon stimulation of TLR8 using

0.5 ug/mL and 50 ug/mL of 3M-002 respectively, differences between preterm and full term neonates in production of IL-10 and IL-6 responses decreased in significance by 10-100 fold (p= 0.009 vs p=0.037 and p=0.0006 vs p=0.06 respectively). Thus, throughout the study we report comparisons using TLR stimulation at the lowest concentration that consistently elicited quantifiable responses in the neonatal populations of interest.

Differential expression of TLR stimulated proinflammatory cytokine and chemokine production in late preterm and full term neonates.

Neonate populations were stratified based on gestational age. The three groups used to study innate immune capacity in response to TLR ligation in cord blood mononuclear cells *ex vivo* were as follows: early preterm (29-33 weeks gestation, n=7), as a control for well established differences in innate immune capacity late preterm and full term neonates were tested for the production of proinflammatory mediators following TLR stimulation. Proinflammatory cytokines and chemokines such as IL-1β and CCL2 (MCP-1) are well recognized markers of TLR engagement (10,48,160). Responses shown are grouped by agonists derived from bacteria (TLR1,2,4,5,6) and virus associated (TLR 3,7,8,9) ligands. In response to bacterial TLR ligand activation, IL-1β production was largely unaffected by gestational age. In contrast, median MCP-1 production significantly reduced (1.5-3.6 fold lower (for the majority of bacterial agonists in the late preterm population (Fig 3A and 3B). Among viral ligand associated TLR, pro-inflammatory cytokine and chemokine production was even more profoundly

affected, with significant reductions in preterm responses evident for most ligands and proinflammatory markers examined.

Anti-inflammatory cytokine expression is impaired in late preterm neonates.

IL-10 modulates a variety of innate and adaptive immune responses (161,162), and plays a key role as an anti-inflammatory cytokine in a wide spectrum of immune responses, leading us to examine the capacity to generate IL-10 production following exposure to both viral and bacterial-related stimuli. Moreover, we hypothesized that the reduced proinflammatory cytokine production observed in the late preterm group was secondary to increased IL-10 expression. However, contrary to this initial hypothesis, IL-10 production was also significantly reduced in late preterm and the small group of 29-33 week old neonates available relative to the full term cohort (Fig 4). Importantly, while both bacterial and viral TLR responses were broadly reduced, responses for some TLRs were very similar, indicating the importance of considering each TLR selectively for translational use of such data.

Production of IL-6 is impaired in late preterm neonates.

In light of the frequently deficient pro and anti-inflammatory cytokine and chemokine production revealed in response to a broad panel of TLR stimuli, we

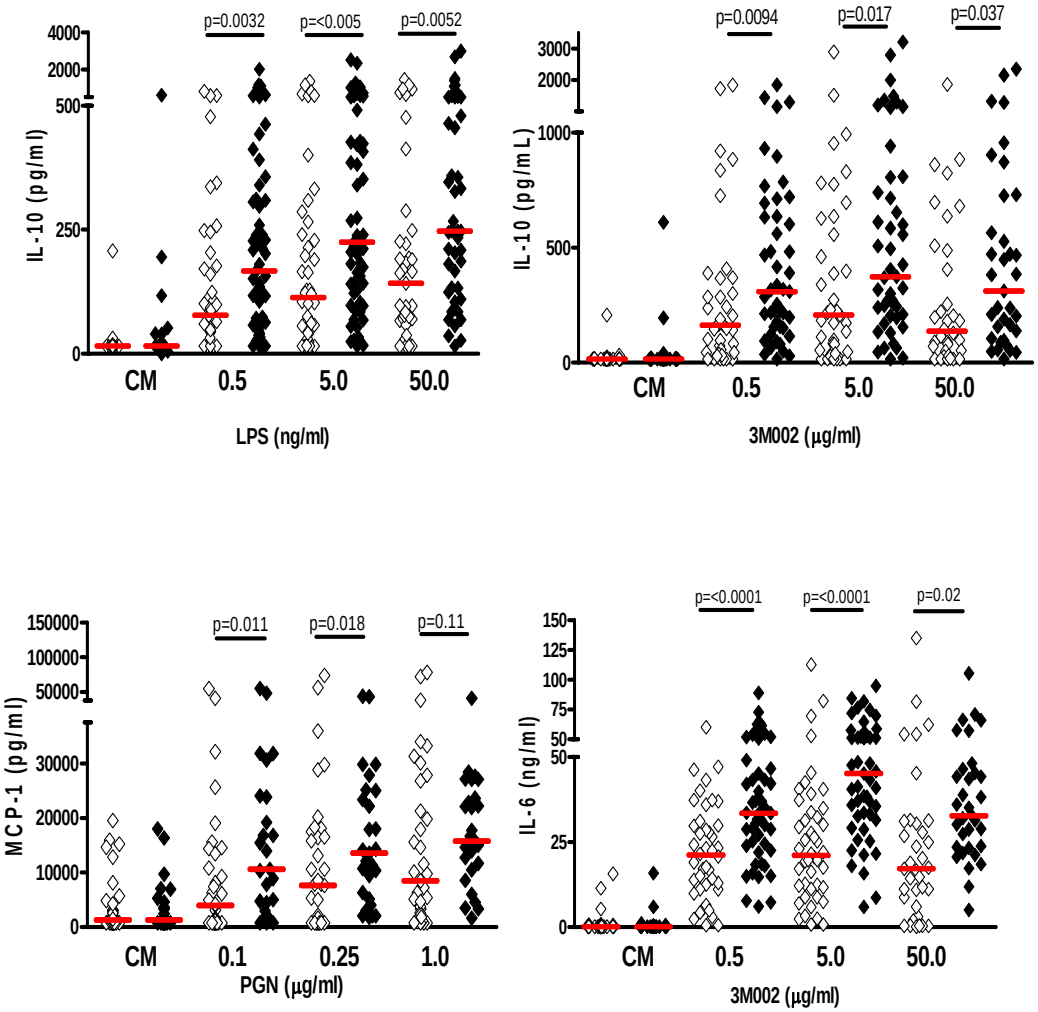

Figure 2: Threshold Concentrations of TLR agonists elicit differential cytokine production in preterm and full term neonates. CBMC from preterm neonates are shown in white and full term neonates represented in black. Median values of cytokines and chemokines are shown in each graph. The number of subjects represented ranges from 37-50 for the preterm group and 50-77 for the full term population.

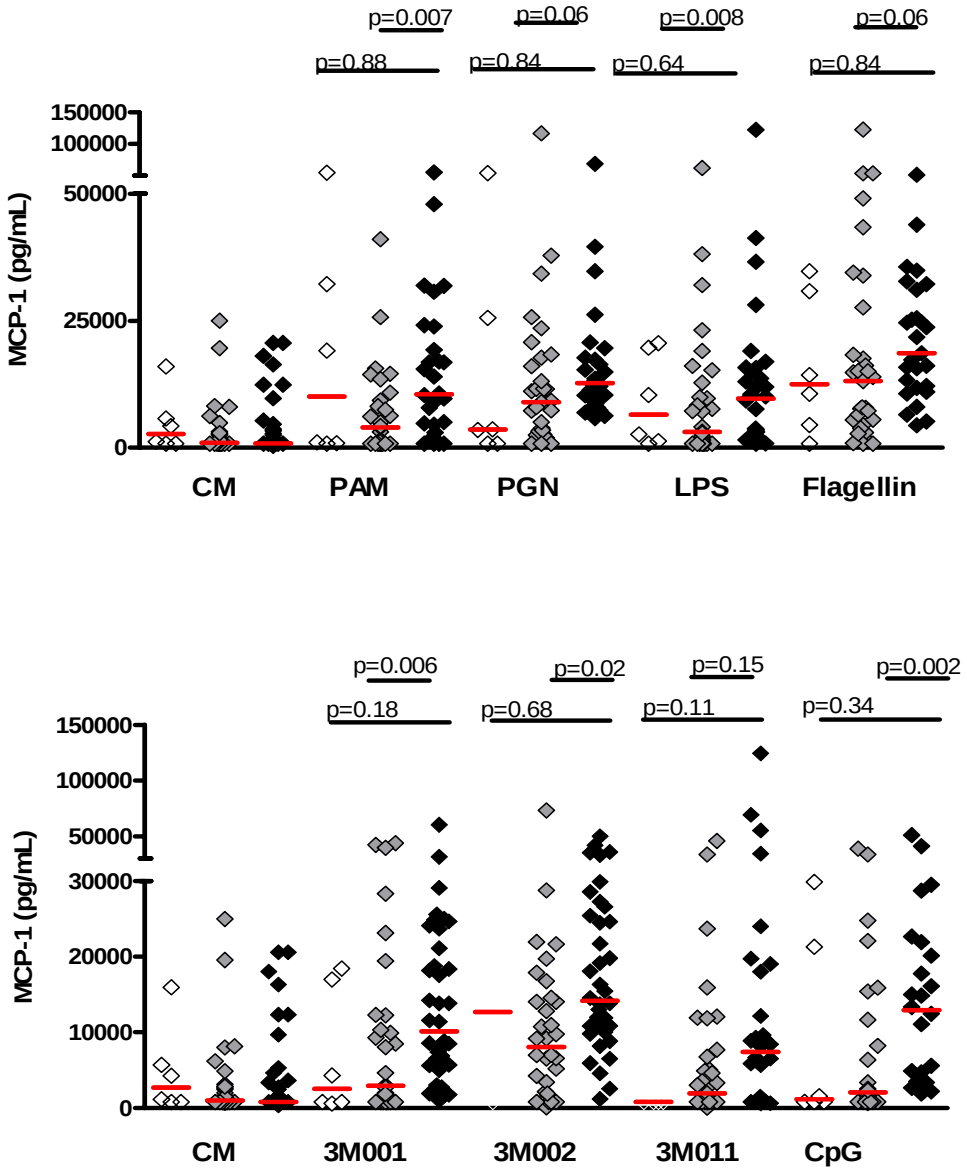

Figure 3A: Late preterm neonates have diminished proinflammatory cytokine and chemokine expression. Cord blood mononuclear cells were cultured for 24 hours in the presence of PAM3CysK, PGN, LPS, Flagellin, 3M001, 3M011, 3M002 or ODN. Supernatants were analyzed via ELISA for CCL2 levels. Early preterm neonates shown in white (n=8), late preterm in grey (n=30) and full term (n=31).

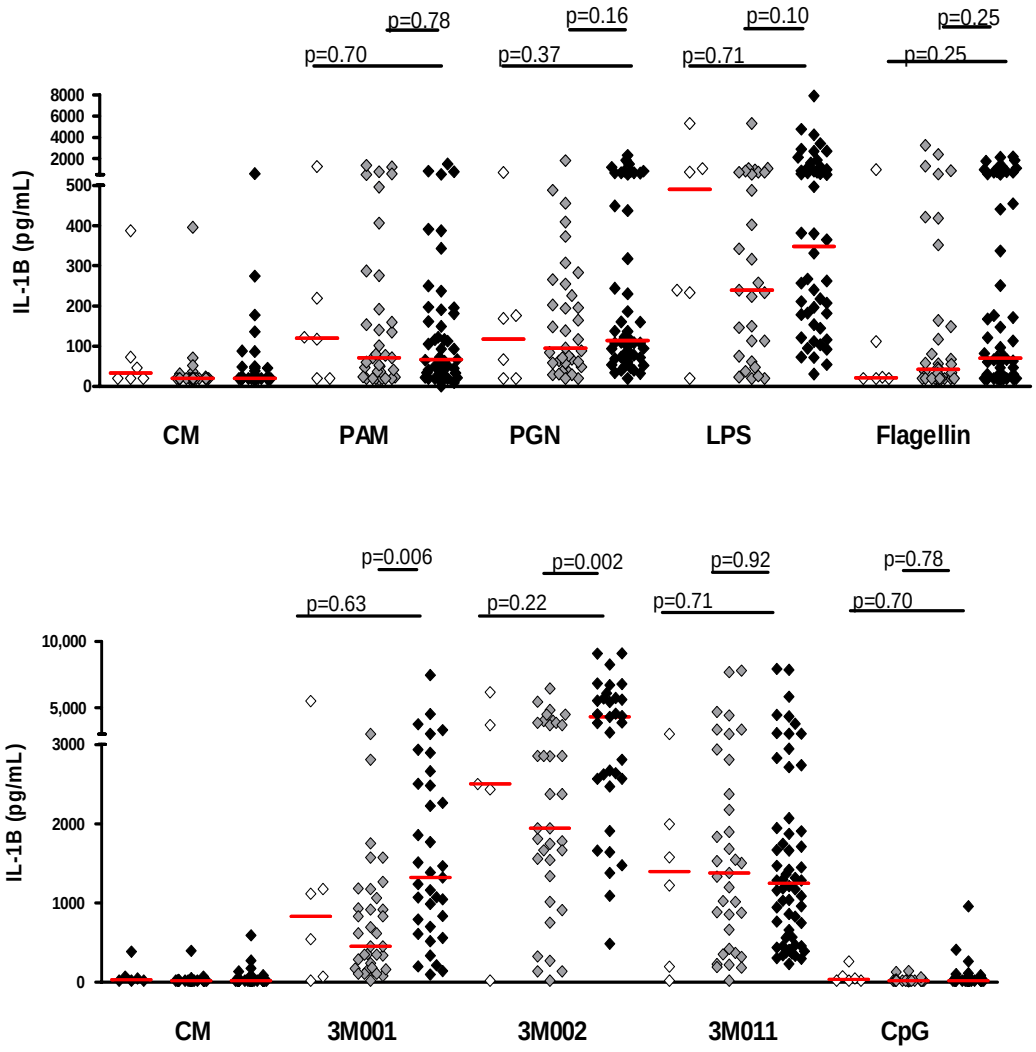

Figure 3B: Late preterm neonates have diminished proinflammatory cytokine and chemokine expression. As with CCL2, cord blood samples were cultured for 24 hours and median values of IL-1b are shown above (n=6 early preterm, n=35 late preterm and n=58 full term).

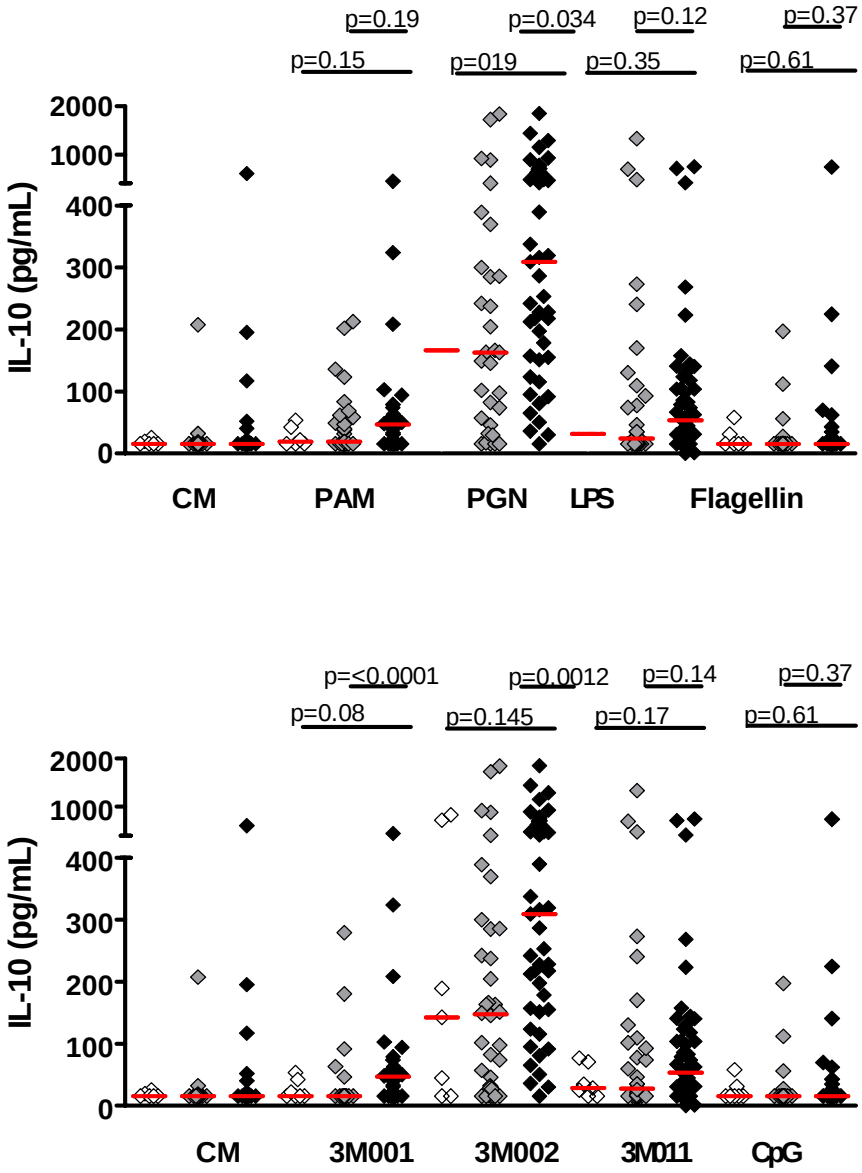

Figure 4: **Late preterm neonates have diminished IL-10 production.** Cord blood mononuclear cells were cultured for 24 hours in the presence of PAM3CysK, PGN, LPS, Flagellin, 3M001, 3M011, 3M002 or ODN. Supernatants were analyzed via ELISA. Early preterm neonates shown in white (n=7), late preterm in green (n=37) and full term in black (n=61)

sought to determine if a global defect in responsiveness was apparent in late pre-term neonates. TLR stimulated IL-6 production, a global indicator of APC activation, was examined. As illustrated in Fig 5, CBMC of full term neonates are capable of producing significantly greater levels of IL-6 than are late preterm infants following exposure to virtually all bacterial and viral stimuli tested. Thus, global TLR responsiveness in late preterm neonates is diminished compared to neonates born at term.

Modulators of Type 1 and Type 2 Immunity are undetectable in CBMCs

In addition to proinflammatory and immunoregulatory cytokines, we also tested CBMC to determine if well-known modulators of type 1 and type 2 immune responses were affected by gestational age. Interferon-regulatory protein 10 (IP-10 or CXCL10) is associated with promoting type 1 immune responses following viral infection and is important in NK cell recruitment (163). Hence, we examined production of this chemokine in 20 individuals following stimulation of TLR3,7,8 and 9 and found no detectable expression in CBMC (data not shown). This finding is in agreement with Aksoy *et al* who found IP-10 production to be deficient in cord blood cells (153).

TSLP has recently been identified as a key modulator of type 2 immune responses which is produced primarily by epithelial cells and may be induced by certain microbial products (164). However no reports to our knowledge have previously examined TSLP production in CBMCs. We therefore tested TSLP

production in supernatants following CBMC stimulation with TLR agonists. We were unable to detect TSLP in CBMC supernatants in the 10 individuals tested (data not shown). PF4 (Platelet Factor 4, CXCL4) is another chemokine involved in the promoting type 2 immune responses which is produced by a variety of cell types, including platelets (163). We examined production of this chemokine following TLR stimulation, however basal levels of PF4 in cord blood and culture were very high (Fig. 6). We were unable to detect differences in PF4 production in untreated and TLR stimulated samples (Fig. 6).

Monocytes are the primary cytokine producing cells in CBMC following TLR activation

Following our observation of significant functional impairment of late preterm neonatal innate immune capacity, we sought to determine if these differences could be attributed to a reduction of responding cells in the late preterm group. We employed flow cytometry and intracellular staining to determine which cells were responsible for cytokine production following TLR stimulation. As recently shown by Yerkovich *et al* (165), we found that CD14+ monocytes were responsible for virtually all detectable intracellular IL-6 following LPS stimulation in CBMC and PBMC from children (Figure 5).

The absolute number of CD14+ cells in late preterm and full term cord blood does not differ significantly

We next hypothesized that the late preterm population may have a reduced number of CD14+ monocytes, which could account for the observed defects in

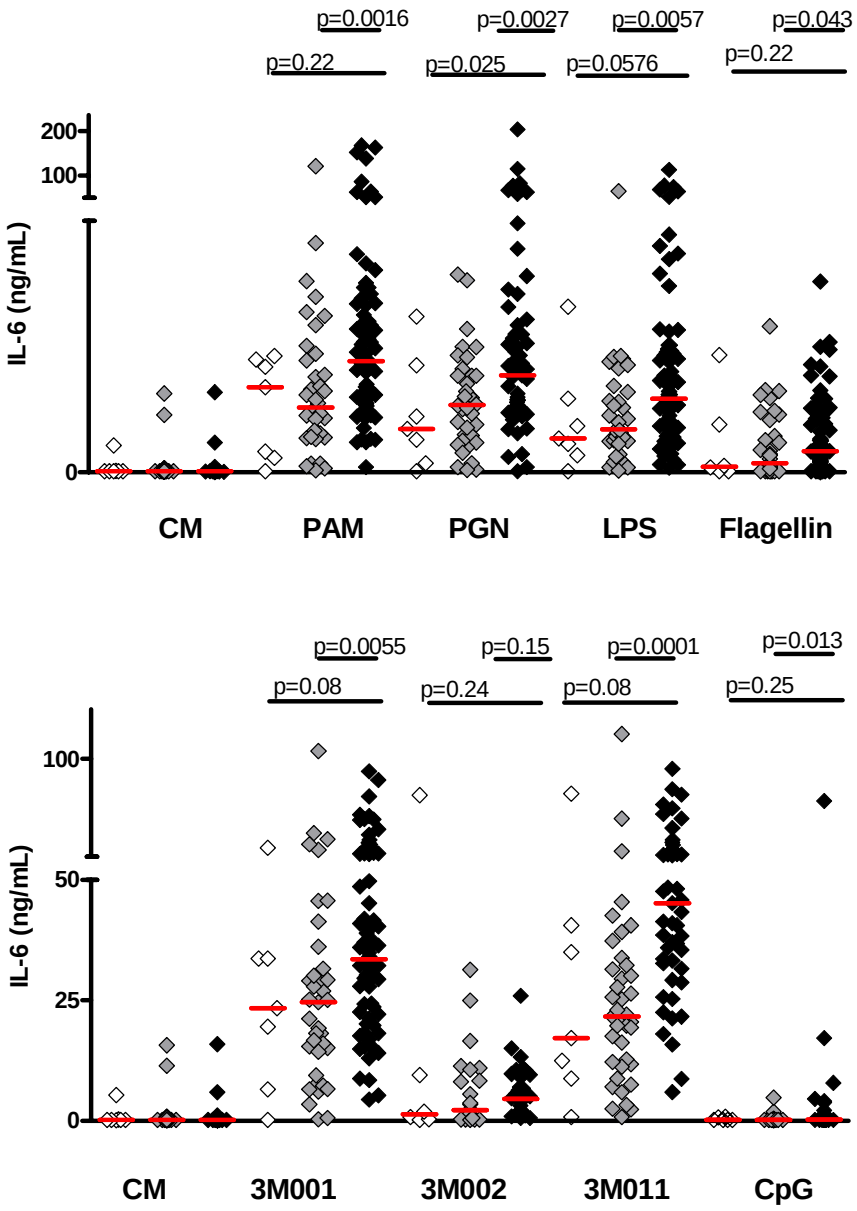

Figure 5: **IL-6 production is markedly diminished in preterm neonates.** Cord blood mononuclear cells were cultured for 24 hours in the presence of PAM3CysK, PGN, LPS, Flagellin, 3M001, 3M011, 3M002 or ODN.

pro and anti-inflammatory cytokine production in the late preterm population. Hence, we determined the absolute number of CD14+CD45+ monocytes in whole blood of both late preterm and full term neonates was determined by flow cytometry (Fig 7). Both late preterm subjects (n=7) and full term neonates (n=10) had similar absolute monocyte counts, arguing that the reduction in cytokine production was not due to a reduced number of cells available for cytokine production.

The frequency of IL-6 and IL-10-producing cells does not account for functional differences in TLR responsiveness

An alternative explanation for reduced cytokine levels is that there are simply fewer cells in the preterm cord blood responding to TLR stimulation. Thus, we completed ELISpot analysis of IL-6 and IL-10 following TLR2/6,4 and 8 activation. We selected IL-6 and IL-10 as differences in the levels of these cytokines were the most striking. As demonstrated in Fig 8, the frequency of cells producing IL-6 and IL-10 in late preterm (n=8) compared to full term (n= 6) neonates was not significantly different (variation among triplicate wells was less than 5%). Thus, confirming that the quantitative differences in IL-6 and IL-10 production are not due to a reduction in the number of cells responding to TLR activation in late preterm neonates compared to their full term counterparts.

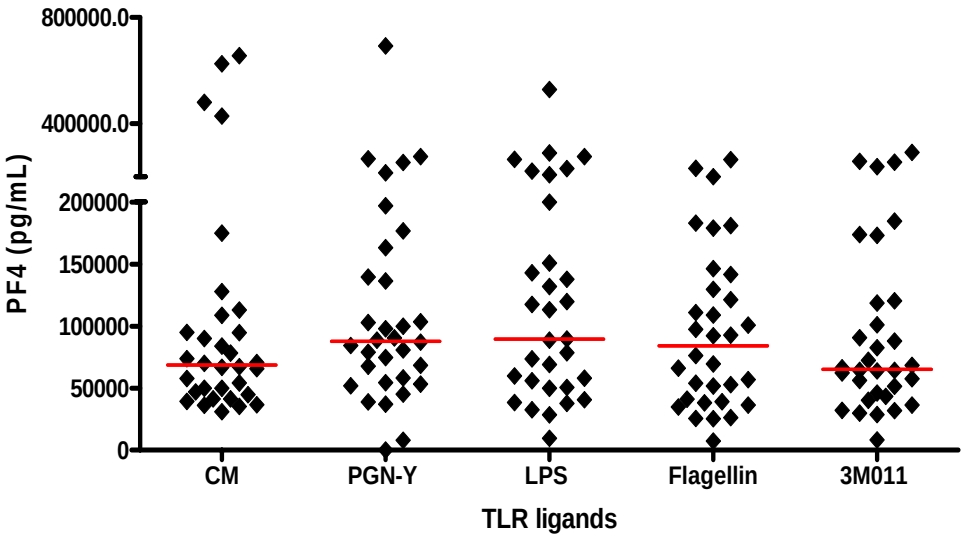

Figure 6. PF4 levels are elevated in CBMC supernatants in the absence of TLR stimuli. We tested levels of PF4 in media alone and following TLR ligation in 15 full term and 15 preterm neonates. As shown above, the levels of PF4 in the media alone were high and did not change significantly upon TLR ligation in either group.

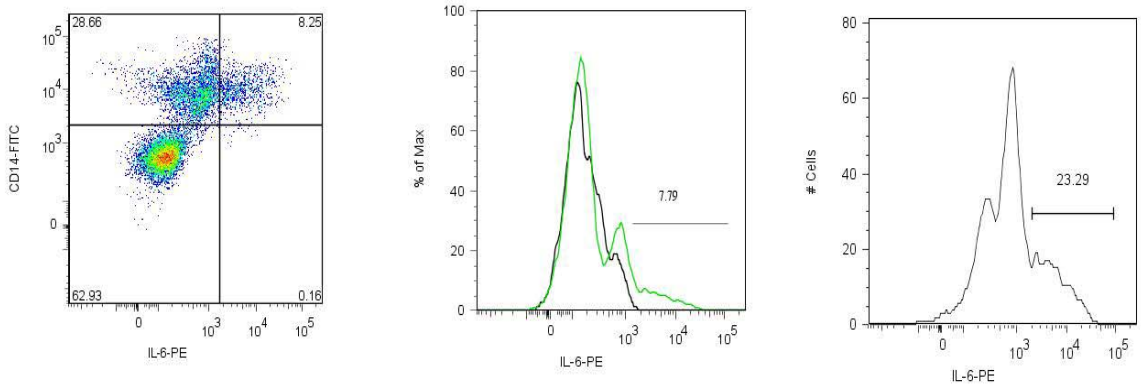

Figure 7: Monocytes are the major cytokine producing cells among CBMC following TLR4 stimulation. Representative graphs of CBMC (n=4) are shown. Cells were stimulated for 6 hours with 0.5 ng/mL of LPS in the presence of GolgiPlug. Frequencies of IL-6+ cells are shown above. Plot A demonstrates that CD14+ monocytes are the main cell type responsible for IL-6 production following LPS activation. The frequency of IL-6+ cells in the total cell population is shown in plot B, the frequency of IL-6 positive monocytes in shown in plot C.

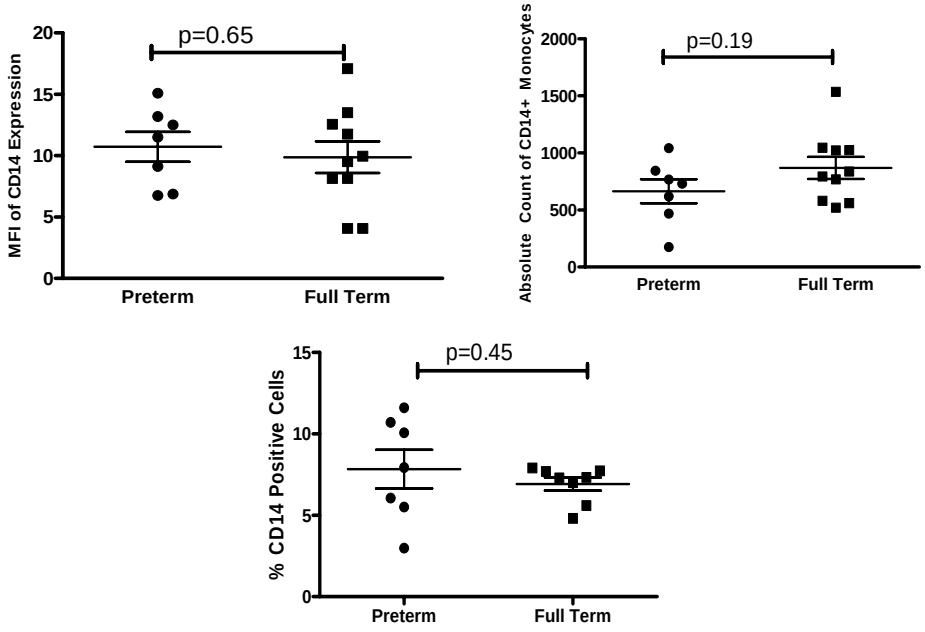

Figure 8: Absolute number of CD14+CD45+ cells are is not significantly different in late preterm and full term neonates. Whole blood from neonates was stained within 24 hours following vaginal delivery. Preterm n=7 (median gestational age: 35 weeks), Full term, n=10 (median gestational age was 40 weeks).

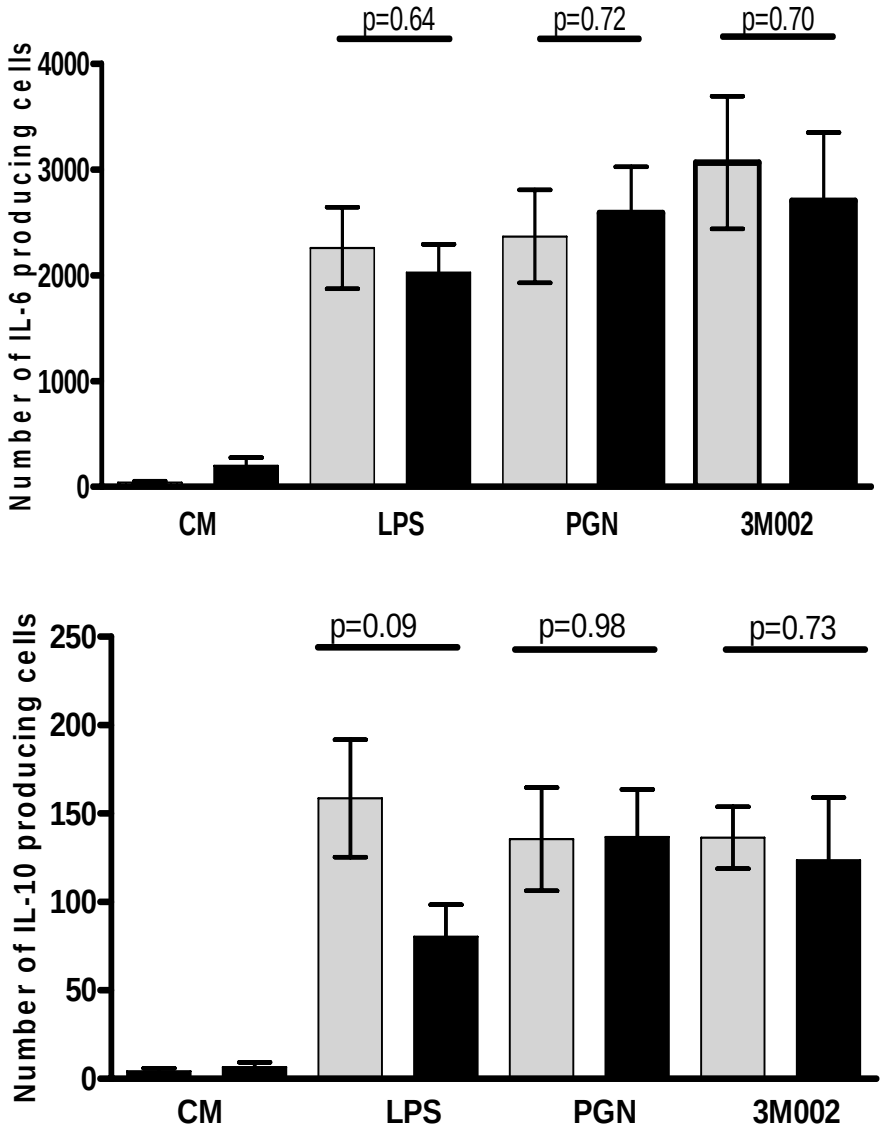

Figure 9. The frequency of IL-6 and IL-10 producing cells was not significantly different in preterm and full term neonates. ELISPOT immunoassays were performed on CBMC stimulated with LPS (5ng/mL), PGN (0.25ug/mL) and 3M002 (5 ug/mL). Late preterm samples shown in grey (n=8) and full term samples are shown in black (n=6).

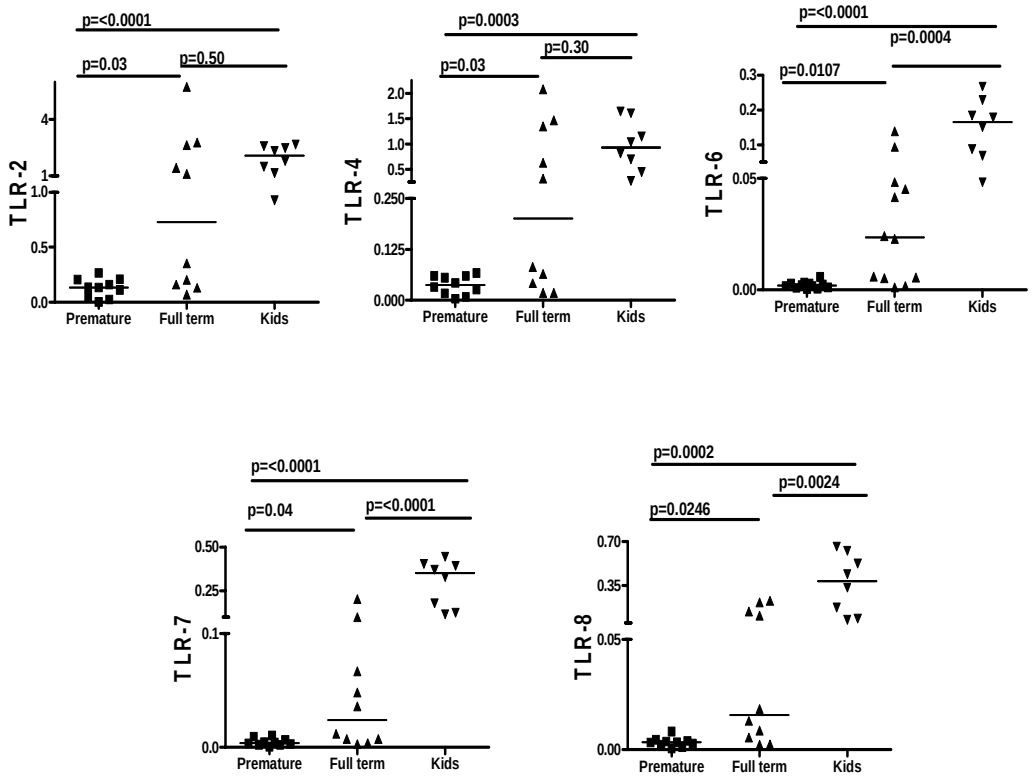

Figure 10: Late Preterm cord blood cells express significantly lower levels ofTLR mRNA. Basal TLR mRNA levels were measured in CBMC from late preterm (n=10, median gestational age is 35 weeks), full term (n=10, median gestational age is 39 weeks) or PBMCs from 12-13 year old children (n=8) via Q-PCR. Values are expressed as ratios relative to 18S RNA.(Q-PCR experiments performed by Jungang Xie)

Late preterm neonates have diminished basal mRNA levels of TLRs and adaptor molecules

An alternative mechanism that may underlie the hyporesponsiveness commonly seen above is a quantitative reduction in TLR receptor expression and/or signaling molecule capacity. To acquire a broader understanding of the effects of gestational age on the developing innate immune capacity we examined expression of TLR2,4,6,7 and 8 in late preterm (n=10) and full term neonates (n=10) compared to 8-12-13 year old healthy children as controls (n=8). Figure 10 demonstrates that TLR expression directly ex vivo is substantially reduced in the late preterm population relative to that seen in either full term neonates ($p = 0.01$-0.04) or older children ($p = \leq 0.0001$-0.0003). Differences in median TLR mRNA expression ranged from 4.5-10X lower in late preterm than full term neonates. To better assess the breadth of deficiency in TLR receptor expression, we also utilized the same paired samples to examine basal mRNA expression of MyD88 and TRIF as key representative signalling molecules. Figure 11 demonstrates that while there are no statistically significant differences evident in this size cohort between full term infants and 12-13 year old children in TRIF and MyD88, the expression of these adaptors is markedly reduced in the late preterm population relative to the full term and children.

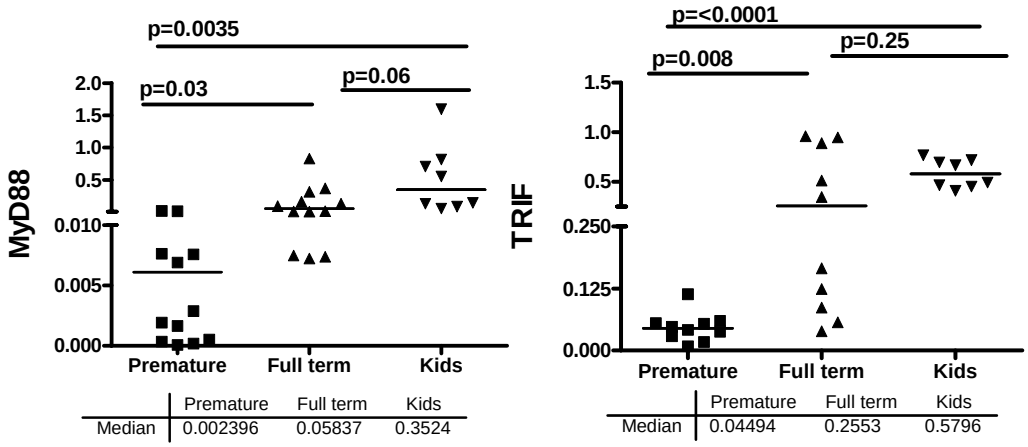

Figure 11: **Expression of MyD88 and TRIF is diminished in late preterm neonates.** mRNA values measured in CBMC from late preterm (n=10), full term (n=10) or PBMCs from children (n=8) via qRT PCR. Values are expressed as a ratio compared to 18S RNA. Median values are shown. Late pre-term infants exhibit striking reduction in both TRIF and MyD88 mRNA levels.

Discussion

The post-natal period represents a time of increased susceptibility to infection as well as a potential window for instructing subsequent immune responses in later life (87,165,166). In the neonatal period the innate immune system is particularly crucial in providing protection from pathogens, as the adaptive immune responses is not fully functional (114,124,166). Several studies have reported marked deficiencies in neonatal innate immune responses compared to adults (reviewed by (115)), potentially contributing to the enhanced susceptibility to infectious illness in the neonatal period. Few studies have examined how innate immune capacity is affected by gestational age; the few available studies have focused almost exclusively on extremely preterm neonates. Preterm birth is associated with an increased risk of developing severe complications such as respiratory distress, sepsis and necrotizing enterocolitis in the postnatal period (91,138,141,167). Recent reports suggest that neonates born near term (referred to as late preterm), have previously unrecognized risks of morbidity and mortality in early life compared to neonates born full term (128,133). Until recently, clinicians treated late preterm neonates as functionally mature; however recent evidence from epidemiologic studies suggests that clinical outcomes in this expanding population are distinct from those of fully mature infants.(133,168). However, underlying mechanisms to explain these clinical findings remain speculative. Currently there are few studies which examine the immunologic consequences of preterm birth, and none which focus

on the late preterm population. Hence, the goal of our study was to examine broad innate immune capacity in late preterm compared to full term neonates.

Our study is the first to clearly demonstrate marked differences in the innate immune capacity of late preterm neonates, born 1-3 weeks before term, and those infants born at and after 37 weeks. In this report we chose to focus on neonatal TLR responses in cord blood. TLRs are recognized as integral mediators of innate immune responses to a variety of bacterial and viral pathogens and hence play important roles in protecting neonates against infection (124). In comparison to previous studies examining innate immune capacity at birth, our study included a relatively large population of subjects (typically <30 vs. 135) and a broader range of TLR responses. Therefore, these results provide a clearer picture of the immune response in both preterm and full term neonates.

Importantly, we included not only bacterial agonists, but also viral stimuli. We demonstrate for the first time that late preterm neonates have significant impairments in many TLR responses to both viral and bacterial compared to neonates born at term. Interestingly, we show that the deficiencies in TLR responses observed in the late preterm population are not caused by a reduction in TLR responsive cells, but instead appear to be mediated by a reduction in the expression of TLR family.

Previously, Forester-Waldl *et al* demonstrated that extremely preterm neonates (born less than 30 weeks gestation) are markedly less responsive to LPS than full term neonates (139). This group also found that TLR4, MyD88 and IRF5

levels increased with gestational age (169), indicating that reduced TLR responses in extremely preterm neonates could account for the increased susceptibility to infection observed in these neonates. As a point of reference we also examined a small subset of early preterm neonates (n=8), however due to the small sample size the majority of cytokines we examined did not differ significantly amongst the early preterm population and full term neonates. Hence, we chose to focus mainly the majority of our study on late preterm neonates as little is known about the immune response in this group.

In this study we focused on neonates who had been exposed to labor and vaginal delivery, omitting infants delivered by cesarean section. Yerkovich *et al* found no difference in TLR-induced cytokine production and mode of delivery ((165). However, other groups have found significant differences in the innate immune response of infants exposed to vaginal flora compared to those delivered via C-section (170). In order to eliminate this possible confounder we included only infants born via vaginal delivery. In addition, serum factors in neonates such as adenosine have been implicated in modulating cytokine responses in cord blood (113). Our study focused on cytokine responses in washed cord blood mononuclear cells, as opposed to modulators in cord blood serum. Hence, we used a well established protocol to isolate and wash CBMCs and supplemented the culture media with fetal calf serum (FCS) as opposed to using autologous human plasma.

Several groups have reported that full term neonates may be able to produce levels of proinflammatory cytokines similar to adults following TLR

engagement (171). However, few studies have examined the ability of preterm neonates to produce proinflammatory mediators. Here we report for the first time that late preterm neonates have significantly impaired ability to produce the proinflammatory mediators IL-1β and MCP-1 following stimulation with certain TLR agonists. Interestingly, agonists of TLR7 and TLR8 elicited the most striking differences in both IL-1β and MCP-1 between the late preterm and full term groups (Fig 3). As reported by Levy *et al* we found that TLR7 and TLR8 agonists were able to induce higher cytokine levels than the majority of other TLRs tested (113). However, the markedly reduced responsiveness to these agonists observed in the late preterm population could have important consequences on immune responses in infancy. Late preterm neonates also produced significantly diminished MCP-1 following activation LPS and PAM (Fig 3). The other TLR agonists examined did not elicit significant differences in IL-1β and MCP-1 levels between late preterm and full term neonates, indicating selective impairments in proinflammatory mediator expression in late preterm neonates. Proinflammatory mediators, such as IL-1β and MCP-1, are essential in maintaining protection form bacteria and viruses infection (172). These mediators are able to recruit and activate proinflammatory cells to mediate rapid clearance of infections (172). Hence, reduced capacity to upregulate proinflammatory mediators could contribute to the reduced capacity of preterm neonates to clear infection effectively.

Upon determining that late preterm neonates had significantly impaired ability to generate proinflammatory cytokines following TLR activation we tested

levels of IL-10 if production of this cytokine differed amongst full term and late preterm infants. Contrary to our initial hypothesis, we found significantly diminished IL-10 production for all of the TLR agonists in the late preterm population (Fig 4). Following TLR activation IL-10 is upregulated in a MyD88-dependent fashion (173), this cytokine has the ability to then modulate the ensuing inflammatory response. IL-10 is produced by many different cell types, including monocytes, macrophages, DCs, Tregs and effector T cells depending on the type of pathogen encountered (reviewed by (174)). IL-10 has been shown to mediate its anti-inflammatory effects mainly by acting on innate immune effectors, such as monocytes and macrophages, reducing expression of proinflammatory mediators and costimulatory molecules in these cells (Couper et al, (173) . Through its inhibitory effects on macrophages and DCs, IL-10 is able to reduce the activation of Th1 and NK cells, hence promoting the resolution of inflammation (175). The inability of preterm neonates to upregulate this cytokine following TLR activation could have important consequences. For instance, colonization of the gut occurring in the perinatal period could induce significant inflammation and pathology without the tolerogenic effects of adequate IL-10 production. Necrotizing enterocolitis occurs more frequently in preterm neonates, potentially due to reduced ability to modulate TLR responses in gut ((176). Hence, exposure of preterm neonates to microbes in the perinatal period may lead to pathological conditions as a result of prolonged expression of inflammatory mediators.

We next sought to test levels of IL-6 following broad activation of TLRs in neonates at varying gestational ages. Several studies have reported the CBMCs from full term neonates are capable of producing levels of IL-6 equivalent to adult cells (177). IL-6 has been shown to play an integral role in the acute phase of responses to bacteria and viruses (178). IL-6 has also been shown to be useful as a marker of global monocyte activation, as these cells produce it rapidly following activation (179). IL-6-deficient mice were shown to have severely compromised ability to recruit neutrophils and clear bacterial and viral infections (180,181). More recently IL-6 was also shown to play a role in the transition from an innate to an adaptive immune response (180). IL-6 has also been shown to promote the development of memory T-cells following viral infection (181). These studies highlight the role of this cytokine as a key modulator of immune responses following pathogen exposure. Although full term neonates may be capable of producing adequate levels of IL-6 following TLR activation, we demonstrate here that late preterm neonates have significantly reduced capacity to produce this cytokine following TLR stimulation (Fig 5). This trend was observed for all of the bacterial and viral TLR agonists (with the exception of 3M011 which elicited little IL-6 from either group). This markedly diminished capacity of late preterm neonates to produce sufficient levels of IL-6 could contribute not only to an increased susceptibility to infection in early life, but also impaired adaptive immunity and memory responses.

In addition to increased susceptibility to infections in early life, preterm neonates have been shown to have increased incidence of asthma in later in life

(141). Previous studies have indicated that cord blood responses may predict future outcomes in terms of early life wheeze (88). Therefore we also tested mediators of type 1 and type 2 immune responses to see if the preterm and full term groups of neonates had detectable differences in the levels of these mediators following TLR activation. Recently TSLP has been identified as an important mediator of type 2 immune responses; however the stimuli which induce its expression and the cells capable of producing this mediator have not been completely elucidated (182). We therefore tested CBMC supernatant in an effort to determine if this mediator was present. We were unable to detect TSLP in cord blood supernatants or serum following stimulation of TLRs with a panel of stimuli (data not shown). We also examined PF4, a type 2 related chemokine (163). Although we were able to detect PF4 in CBMC supernatants the levels did not differ significantly in untreated and treated samples, indicating that TLR activation was not responsible for the PF4 generated. In addition to the type 2 mediators we also tested CBMCs for the production of IP-10, a type 1 related chemokine (163). We and others have shown previously that IP-10 is produced in children and adults following activation of TLR (unpublished data), however we were unable to detect this mediator in any of the cord blood samples regardless of gestational age (data not shown). Collectively these results indicate that mediators of adaptive immunity expressed in children and adults are deficient at birth.

In order to determine why late preterm neonates had significantly reduced capacity to generate proinflammatory and immunoregulatory cytokines following TLR activation we first wanted to determine which cells were primarily

responsible for generating these cytokines in our CBMC samples. Intracellular staining experiments confirmed that CD14+ monocytes were responsible for the majority of cytokine production in our samples, in agreement with work published recently (Fig 7) (165). Monocytes are important players in early immune responses to pathogens; they have the ability to produce large amounts of cytokine following TLR activation (183,184). In addition, these cells are able to differentiate into myeloid DCs or macrophages following exposure to TLR stimuli (183). Following the identification of monocytes as the primary cytokine-producing cells in CBMCs we next examined the absolute numbers of CD14+ monocytes in the late preterm and full term populations. As demonstrated in Fig 8, we found no significant difference in the absolute numbers of expression levels of CD14 in whole cord blood in preterm and full term subjects. These results suggested that differences in the number of monocytes in preterm and full term populations are not responsible for the functional differences observed following TLR activation.

We employed the ELISPOT immunoassay to further elucidate the mechanism underlying the functional differences in TLR responses. This assay has previously been demonstrated to provide a very sensitive and accurate measure of the frequency of cells producing a cytokine of interest in peripheral blood mononuclear cells (185). For these analyses we chose to focus on the expression of IL-6 and IL-10, two cytokines which elicited the most significant differences in the late preterm and full term groups. In agreement with our flow results showing no impact of gestational age on the numbers of monocytes in cord

blood, we found no significant difference in the number of cytokine-producing cells using ELISPOT analysis.

The next approach we employed to determine the mechanism underlying the hyporesponsiveness to TLR stimulation in late preterm neonates was to examine the basal expression of TLRs and associated adaptor molecules in the preterm and full term populations. As demonstrated in Fig 10 we found significantly reduced expression of all TLRs examined in the late preterm group compared to their full term counterparts. In addition, expression of MyD88 and TRIF, two downstream adaptors of TLRs were also markedly reduced in the late preterm group (Fig 11). These results indicate that the reduced capacity of late preterm neonates to produce proinflammatory and immunoregulatory cytokines could be due to a reduced level of basal TLR expression. Therefore, the last few weeks of gestation may be critically important in the development of an effective innate immune response.

Reduced responses to bacterial and viral stimuli in early life could have a major effect on the ensuing immune responses in infancy and childhood. For instance, Gern *et al* found that reduced responses to RSV in cord blood cells were predictive of the development of wheezing in early childhood (88). RSV infects up to 50% of infants generally leading to self-limiting infection (186). However, studies in children indicate that reduced global TLR4 responses could predict individuals who develop severe symptoms following RSV infection (186). It seems plausible that reduced capacity of preterm neonates to respond to viral

stimuli could contribute to the increased susceptibility of these infants to wheeze in early life.

Interestingly, some epidemiologic studies suggest that sepsis in the neonatal period may be protective against the development of allergic disease in childhood, in agreement with the hygiene hypothesis (80,167). Similarly, TLR activation has been shown previously to enhance immune maturation in early life (70). Therefore, strategies to augment type 1 immune responses through TLRs may be able to safely mimic the protective effects of early life infections. To conclude, our study was the first to focus on immunologic differences in late preterm and full neonates. Here we report that late preterm neonates have diminished TLR responsiveness to a wide range of bacterial and viral components. This hyporesponsiveness may be mediated by reduced basal TLR expression as well as reduced expression of the downstream adaptors MyD88 and TRIF. It remains to be determined if these deficiencies are directly related to the increased rate of infectious disease and maladaptive immune responses found to accompany preterm birth.

CHAPTER 2

INNATE IMMUNE CAPACITY THROUGHOUT LIFE

Acknowledgements: Yuriy Lissitisyn[1], Isha Ostopowich[1], Ganesh Srinivasan[2], Allan Becker[2] and Kent T HayGlass[1]

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ABSTRACT

Rationale: The expression and functional capacity of the innate immune response throughout life has not been clearly elucidated. Differential responsiveness to TLR ligands throughout life could help to explain certain susceptibilities to acute and chronic diseases and how these change with age.

Results: TLR responses at birth generally are significantly less robust than responses generated in childhood, adolescence and adulthood. However, some responses such as LPS-induced IL-6 are equivalent to adults at birth. In comparison, responses amongst post-neonatal groups, proinflammatory mediators were upregulated in children and teens compared to other age groups. However, the response to viral surrogates was greatest in adults.

Conclusions/Implications: The capacity to mount TLR responses is not static throughout life. In the neonatal period the majority of TLR responses are reduced compared to later age groups, which may underlie the increased susceptibility to infection seen in early life.

PREAMBLE

Recently, we and others have found selective deficiencies in TLR responses in neonates compared to adults (120,123,139,154,166). However, some TLR responses appear to be intact in the neonatal period (64,125). Collectively these reports suggest that TLR responses may be differentially modulated in neonates and adults. The development of appropriate adaptive immune responses to many pathogenic and commensal microbes takes place in early life (85,102,187). As postulated by the hygiene hypothesis, differential exposures and responsiveness to microbial products in early life may be essential in determining whether or not an individual develops atopy (76). The preschool years may represent a time in which interventions may be most effective in preventing inappropriate immune responses as the reversibility of atopic disease decreases over time (102,187). Although several studies have compared neonatal immune responses to adults, little is known about the natural progression of innate immune capacity at multiple time points over human lifespan. The goal of our study was to compare cytokine and chemokine responses to several TLR stimuli at multiple developmental time points (including neonates, children, adolescents and adults), to obtain a clearer picture of how the innate immune response develops and functions over time

RATIONALE AND HYPOTHESIS

Susceptibility to various types of infections, as well as the prevalence of allergic conditions, autoimmunity and a range of other immune dysfunctions, changes throughout life. The factors influencing these immunologic phenomena are not well understood. Increasing evidence suggests that differential innate immune responses, including those mediated by TLRs, play an integral role in directing adaptive immune response. Very few studies have examined innate immune capacity throughout life. The timing and type of TLR activation may play important roles in determining how the immune response develops over time. Obtaining a clearer picture of how TLR responses differ in neonates, children, teens and adults will shed light on how the innate immune system is affected by aging from early life on to old age. Here we hypothesize that innate immune capacity is not static throughout life and becomes progressively more efficient throughout life.

RESULTS

Proinflammatory mediators are selectively diminished in neonates

In this study we sought to determine how TLR responsiveness changes throughout human life. In contrast to the limited studies conducted previously, we examined a number of cytokines and chemokines in a large number of individuals of each age range. We tested neonatal cord blood from full term neonates delivered vaginally, and peripheral blood from an ongoing cohort of healthy

children (7-8 years old) and pubescent young adults (12-13 years old) as well as healthy adults (18-35). We used the same threshold concentrations of all TLR ligands as well as the same detection methods to maintain consistency throughout our study. In contrast to previous studies which typically focused exclusively on LPS, we examined several TLR agonists (LPS, PGN, 3M011) as well as a broad range of cytokine readouts.

The first mediators we examined following TLR activation were those associated with promoting inflammation and recruitment of various cell types during infection. The first cytokine examined was IL-1β, a well known proinflammatory mediator produced following TLR activation. In general, IL-1β production followed the trend of increasing levels with increasing age following PGN and 3M011 (bacterial and viral surrogate respectively) exposure (Fig 12). The production of IL-1β was significantly weaker in the neonatal population compared to children, adolescents and adults following stimulation with these ligands. In contrast, TLR4 activation via LPS elicited the strongest IL-1β responses in children, who were found to produce significantly greater levels than all other age groups.

CCL2 (MCP-1) is a proinflammatory chemokine which is widely upregulated following activation of TLR2-9 (188). It is essential in the recruitment of monocytes and other CCR4+ cells to the site of infection (188). Interestingly, we found that CCL2 levels were significantly upregulated in children compared to other age groups following PGN and LPS stimulation (Fig

13). Following 3M011 stimulation, children and teens had similar median levels of CCL2, both of which were significantly greater than those detected in neonates or adults. Neonates had similar CCL2 levels to adults and teens following PGN, with significantly higher levels than adults following LPS exposure. The reduced expression of proinflammatory mediators in the neonatal period suggests a reduced capacity to initiate an effective adaptive response to bacterial and viral pathogens.

IL-6, another cytokine associated with the acute inflammatory process, has been examined by several groups following TLR activation in neonates compared to adults. The majority of studies report equivalent, if not enhanced IL-6 production in cord blood compared with adults following LPS stimulation (64,153,165). In support of previous studies we found that neonates produced similar IL-6 levels to adults following PGN stimulation, however this was not the case for all TLR stimuli. For instance, following stimulation with LPS we found significantly enhanced IL-6 levels in cord blood supernatants compared to adults. Children and teens also had significantly higher IL-6 levels than adults following LPS stimulation. In contrast, neonates had markedly reduced capacity to generate IL-6 following 3M011 stimulation compared to the other age groups examined. Collectively, these results suggest that global TLR activation is generally reduced in neonates compared to older individuals, with the most significant defects to viral surrogates.

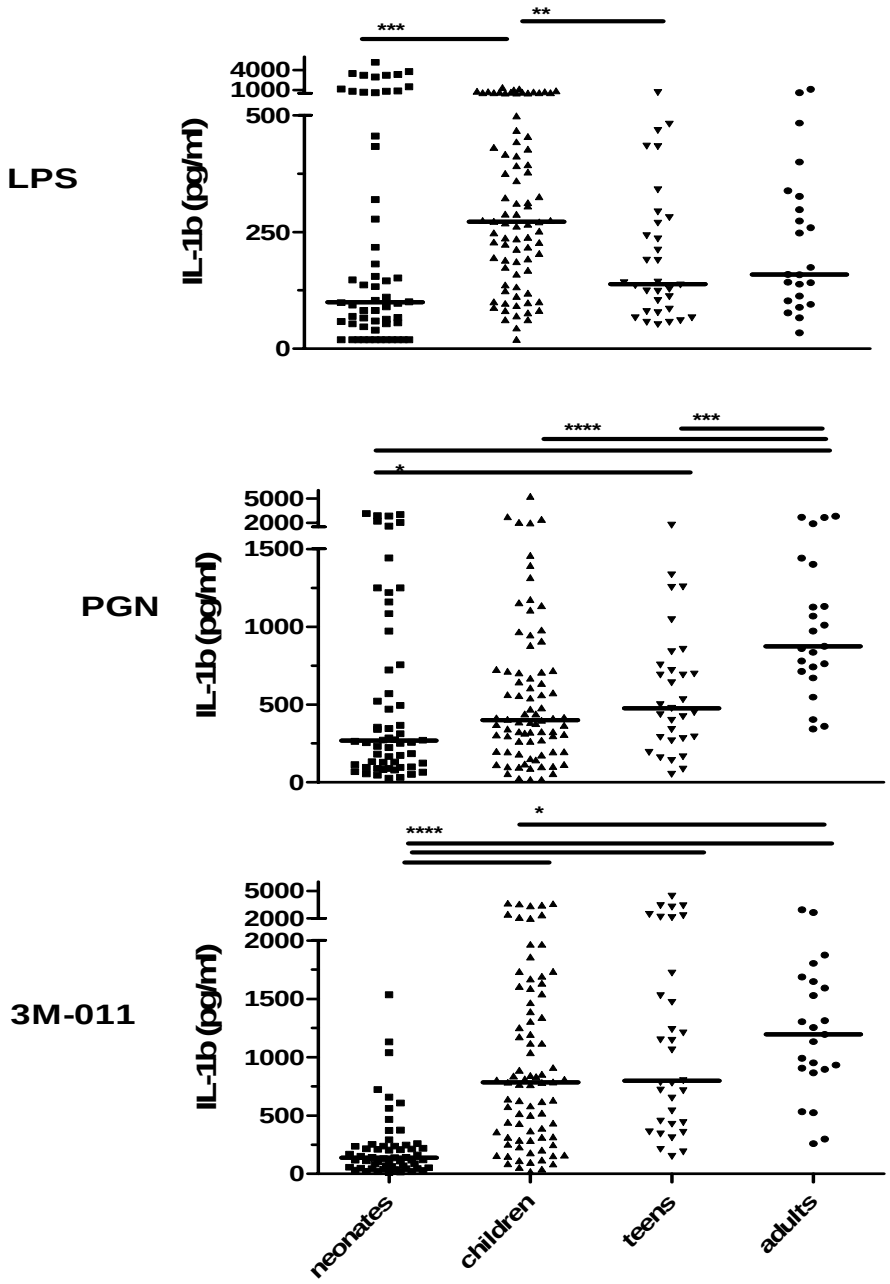


Figure 12. IL-1β Production Increases with age. CBMC or PBMC were cultured with 0.1 ug/mL PGN (Fluka), 0.5 ug/mL of 3M011 or 0.5 ug/mL LPS for 24 hours. Supernatants were tested for IL-1β via ELISA. ****=P-value less than or equal to 0.0001, *** = less than 0.001, **= less than 0.01, *=less than 0.05.

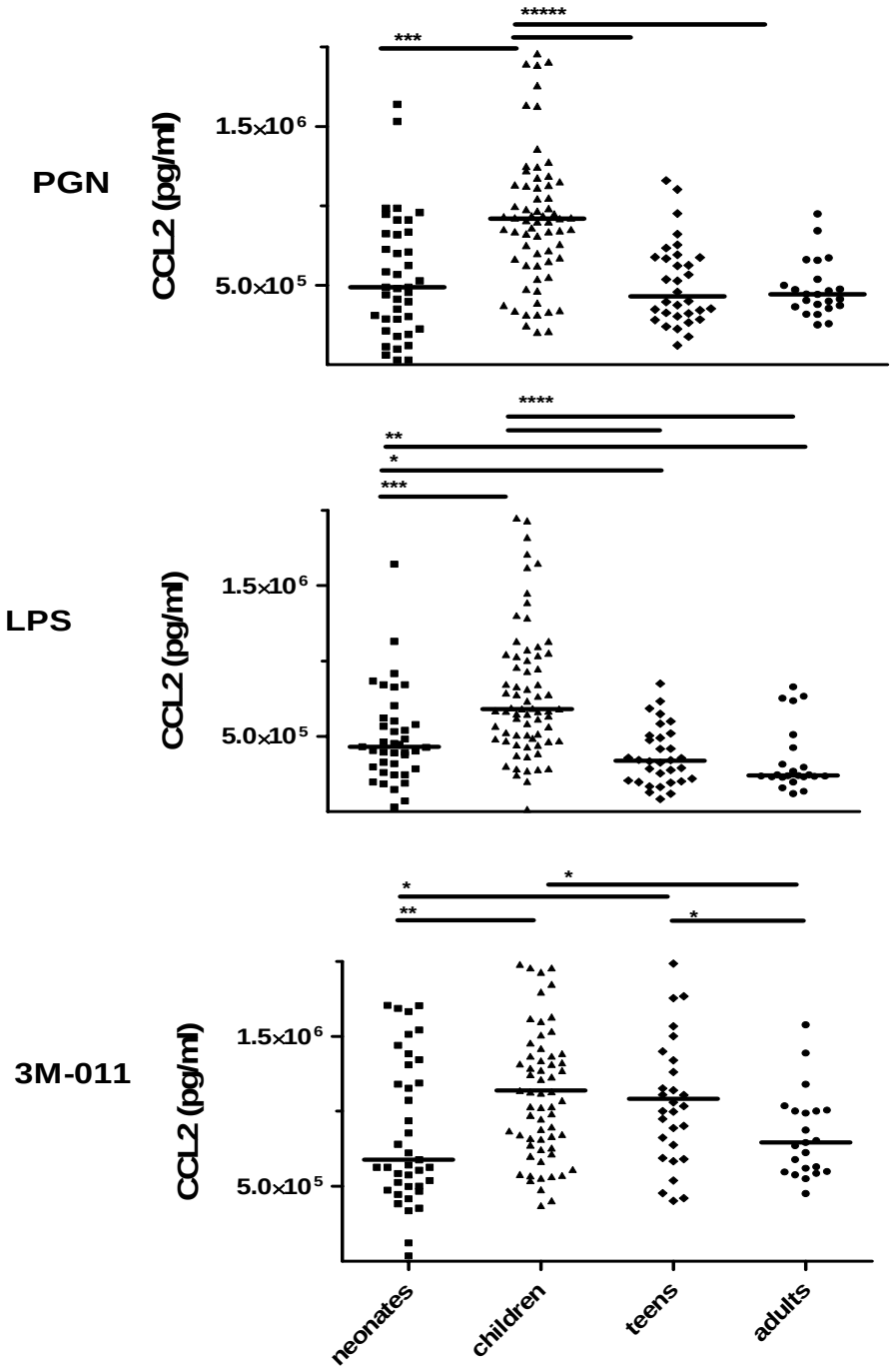


Figure 13. CCL2 production peaks in childhood. CBMC or PBMC were cultured with 0.1 ug/mL PGN (Fluka), 0.5 ug/mL of 3M011 or 0.5 ug/mL LPS for 24 hours. Supernatants were tested for CCL2 via ELISA. ****=P-value less than or equal to 0.0001, *** = less than 0.001, **= less than 0.01, *=less than 0.05

IL-10 production is deficient at birth

Next we examined the production of the anti-inflammatory cytokine IL-10 in the various populations. Analysis of our populations demonstrated an overall deficiency in the ability to produce this immunoregulatory cytokine at birth compared to other age groups across all stimuli (Fig 15). Other populations demonstrated different trends in IL-10 production. 3M011 elicited similar IL-10 levels amongst children, teens and adults. Although the median levels of IL-10 were not the same amongst children, adolescent and adults following exposure to bacterial agonists, the levels measured were all markedly higher in these populations than in the neonates.

Type 1 and Type 2 Mediators are Significantly Diminished at Birth

TLR responses have been shown to play important roles in instructing the development of adaptive immune responses (2,54,85). We next sought to determine how the production of chemokines involved in skewing type 1 or type 2 immune responses change over time. CXCL10 (IP-10) is involved in the type 1 response following exposure to TLR3,7,8 and 9 agonists (153,161), hence we examined the production of CXCL10 following exposure to CpG ODN (TLR9) and 3M011 exposure. We were only able to compare children and neonates in the case of CpG ODN. In the subset of neonates tested (n=30), no CXCL10 was detected following ODN or 3M011, whereas all of the children and

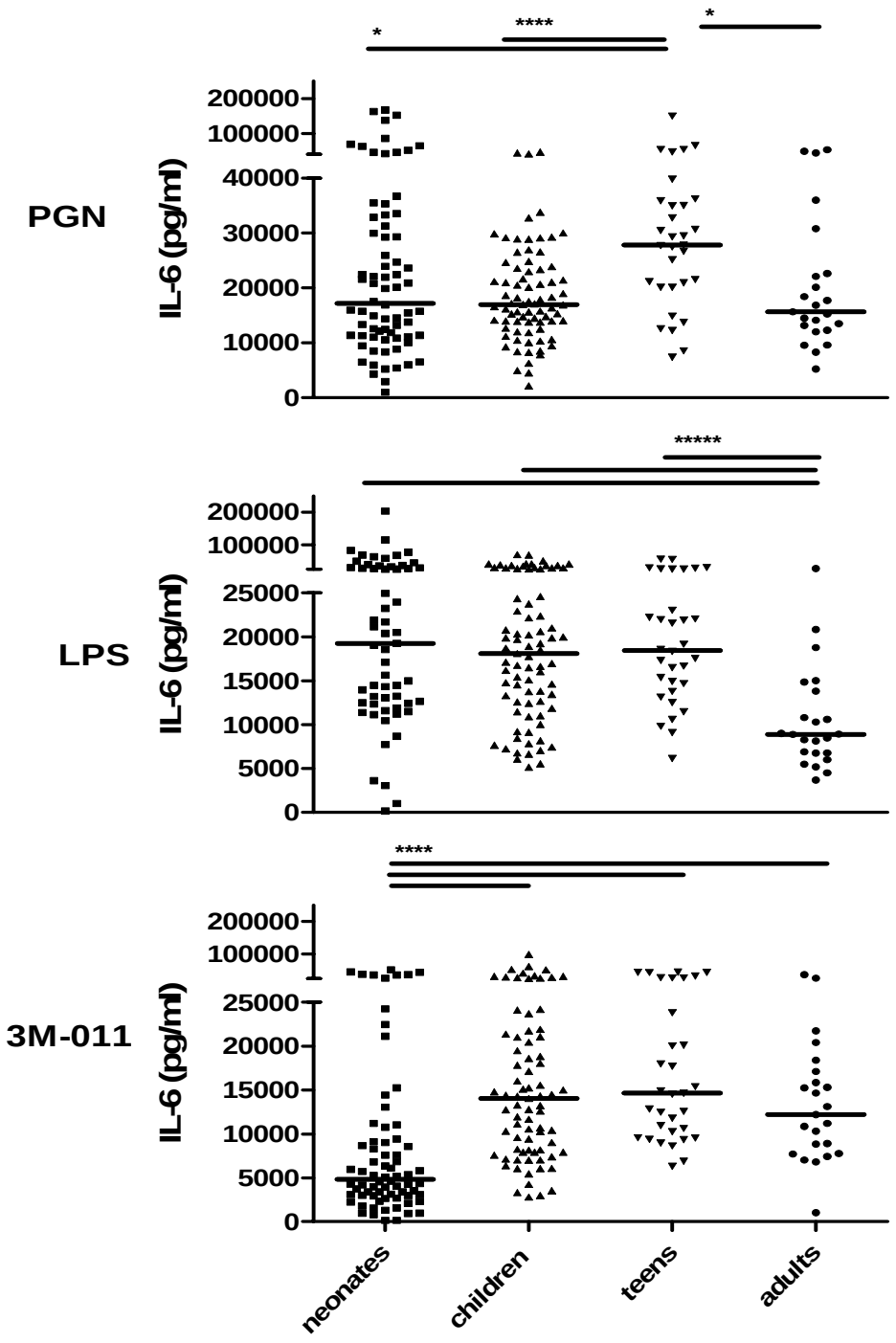


Figure 14. Anti-viral IL-6 response is diminished at birth. CBMC or PBMC were cultured with 0.1 ug/mL PGN (Fluka), 0.5 ug/mL of 3M011 or 0.5 ug/mL LPS for 24 hours. Supernatants were tested for IL-6 via ELISA. ****=P-value less than or equal to 0.0001, *** = less than 0.001, **= less than 0.01, *=less than 0.05

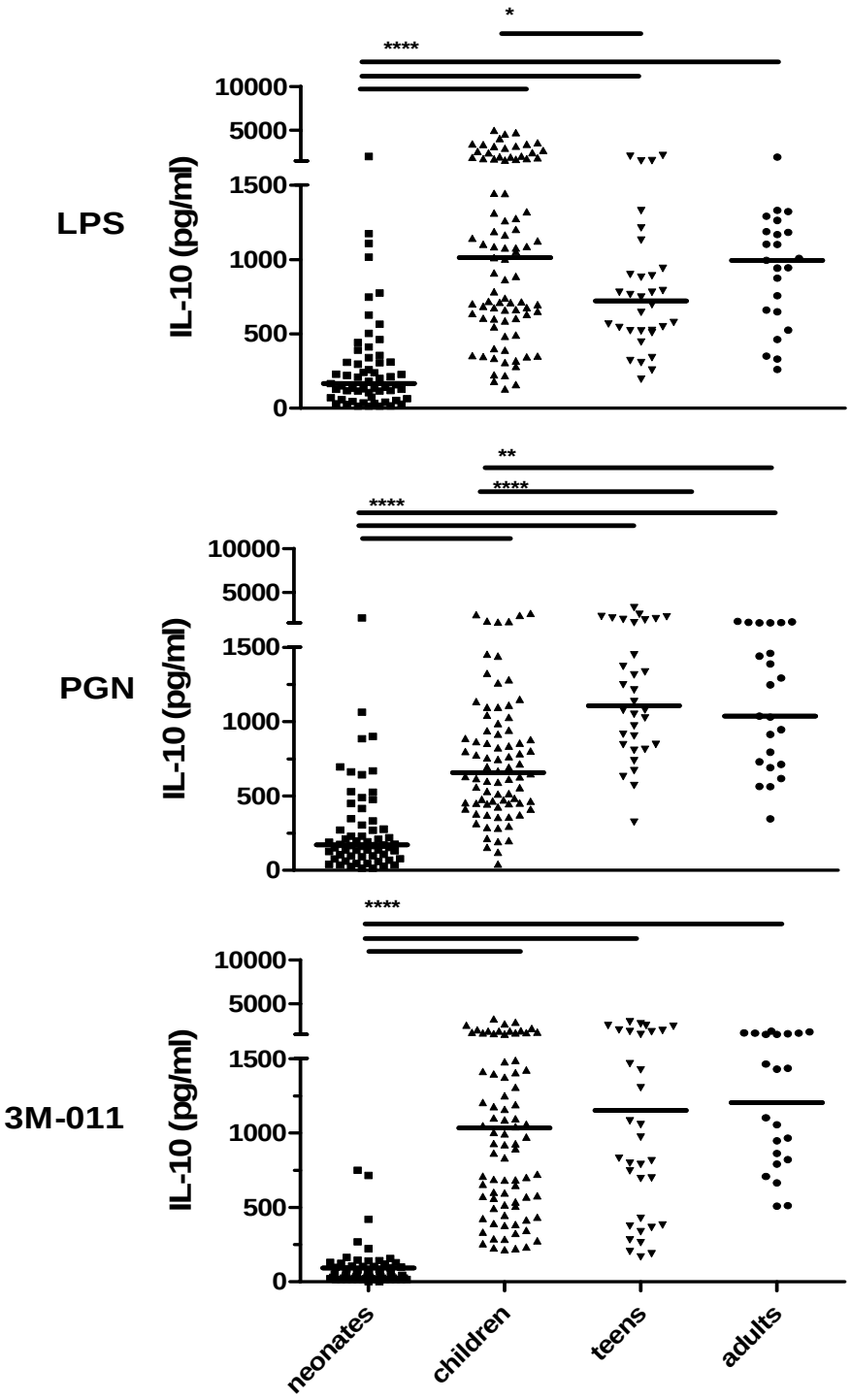


Figure 15. IL-10 production is markedly reduced in neonates. CBMC or PBMC were cultured with 0.1 ug/mL PGN (Fluka), 0.5 ug/mL of 3M011 or 0.5 ug/mL LPS for 24 hours. Supernatants were tested for IL-10 via ELISA ****=P-value less than or equal to 0.0001, *** = less than 0.001, **= less than 0.01, *=less than 0.05

adults tested produced measureable levels of CXCL10. The levels of CXCL10 were significantly greater in the adult population compared to children following 3M011 exposure; this finding suggests that Th-1 skewing immune responses are not fully developed until adulthood.

CCL22 (MDC) is a chemokine associated with promoting type 2 immune responses (188). Our previous studies demonstrated that PGN and LPS are strong inducers of CCL22, we therefore tested the production of CCL22 following stimulation with these agonists. As shown in Figure 17, CCL22 was detectable in the neonatal population; however the levels generated were again markedly reduced compared to all other age groups for both stimuli. Following LPS exposure, the adolescents produced significantly greater levels of CCL22 the children and adults. Following PGN stimulation, the children and teens produced similar levels of CCL22 whereas the levels produced by adults were significantly lower. In summary, both Th-1 and Th-2 skewing chemokine production is reduced in neonates, suggesting an overall reduced capacity to stimulate adaptive immune responses at birth. In addition, Th2-skewing immune responses may be favored in childhood and early adolescence whereas adults have an increased propensity to promote Th1 responses.

DISCUSSION

The goal of our study was to develop a clearer picture of how innate immune system capacity changes throughout life by examining a wide range of

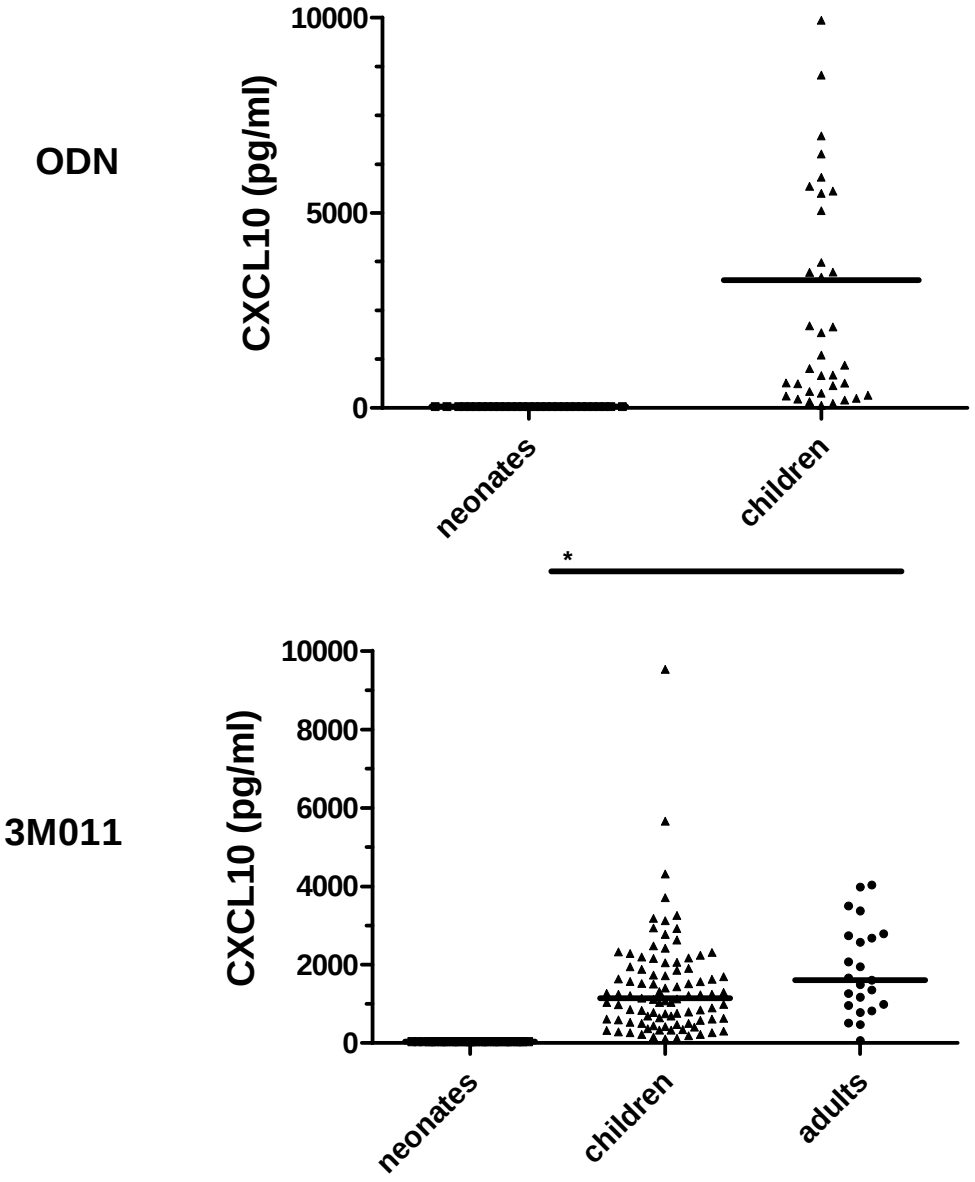


Figure 16. CXCL10 production is impaired at birth. CBMC and PBMC were stimulated with 3 ug/mL CpG ODN or 5 ug/mL 3M011 for 24h. Supernatants were tested for CXCL10 via ELISA. *=$p \leq 0.05$

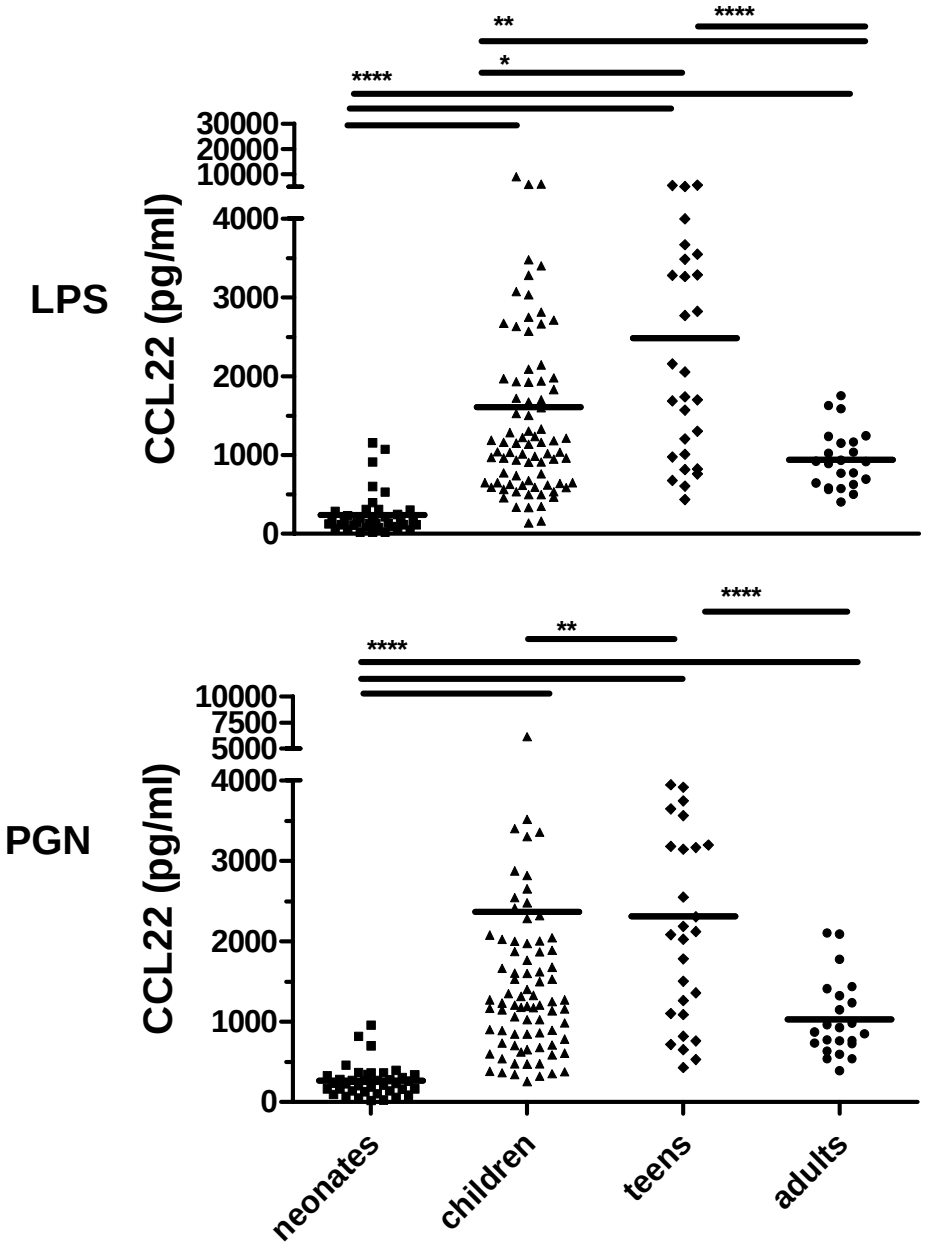


Figure 17. CCL22 production is upregulated in children and teens. CBMC and PBMC were stimulated with 5 ng/mL LPS or 1 ug/mL PGN for 24h. Supernatants were tested for CCL22 via ELISA. *=$p<0.05$

time points, TLR stimuli and cytokine readouts. Our results indicate that TLR responses to both bacterial and viral agonists are very different at various developmental stages. Specifically, neonates have reduced to cytokine responses to both bacterial and viral stimuli while children and adolescents have a heightened capacity to mount inflammatory responses to bacterial stimuli.

Previous studies have generally focused on differences between the innate immune response in neonates versus adults. One study recently published by Yerkovich *et al* examined the cytokine responses of infants in the postnatal period compared to children, adolescents and adults following LPS exposure only (165). This study focused on immune response in the post-natal period, finding that the initial immune response in cord blood are transient and are reduced in the early post natal period with a gradual increase over time ((165). Here we extend these results and contribute novel information by examining a broader range of TLR responses in a much larger population.

Neonates are Hyporesponsive to Select TLR Stimuli

The immune response at birth is generally believed to be immature compared to that of children and adults. Neonates are more susceptible to developing bacterial sepsis and viral infections than other age groups (106,123,140). For instance, Group B Streptococcus, a microbe which is found in the normal mucosal flora of adults is a major cause of sepsis in the perinatal period (189). Infancy is also associated with an increased risk of developing viral infections, the majority of which are caused by RSV (190). The mechanisms underlying this increased

susceptibility to infections by bacteria and viruses are incompletely understood, however recent reports suggest that the innate immune system at birth may not function effectively in response to certain potential pathogens (112,120,121,131). **S**everal reports demonstrate that neonatal APCs have a reduced capacity to upregulate costimulatory molecules and are less effective at initiating T cell responses (reviewed by (112)).

In general, our results support the conclusion that TLR responsiveness is reduced in the neonatal period, with the most significant deficits observed in IL-10 and CXCL10. Chelvarajan *et al* used a mouse model to study neonatal innate immune responses and found that monocytes from neonatal mice produced significantly higher IL-10 than those of adult mice following LPS and PGN exposure (191). Yerkovich *et al* recently reported that LPS combined with IFNγ induced similar levels of IL-10 in CBMC and PBMC from children and adults (165). In contrast, we found consistently that CBMC had strikingly diminished capacity to induce IL-10 compared to all other groups. IL-10 is known to be an important regulatory cytokine which is able to reduce inflammation and promote Treg responses (62). The reduced capacity of neonates to generate IL-10 following exposure to bacterial and viral products could contribute to the increased rate of inflammatory conditions such as sepsis and necrotizing enterocolitis which occur at a much higher frequency in neonates than older children and adults (108,121).

Previously Aksoy *et al* found that CBMC were unable to generate levels of CXCL10 and type 1 interferons comparable to those produced by adult PBMC

following activation with viral and bacterial agonists (153). Our results support these findings as we were unable to detect production of CXCL10 in any of the cord blood samples tested following stimulation with ligands for TLR3,7,8 and 9 (Fig 16). These findings suggest a potential defect in anti-viral immunity in neonates compared to older individuals.

We found a different trend when examining the production of proinflammatory mediators in the neonatal population. Production of both CCL2 and IL-1β elicited similar patterns in neonates. Following PGN and LPS stimulation, levels of CCL2 and IL-1β were not significantly different from the other populations tested (with the exception of the child population which produced the highest levels of both mediators) (Fig 12 and 13). Interestingly, the production of CCL2 and IL-1β was markedly reduced in the neonatal population following 3M011 stimulation.

Neonatal production of IL-6 following TLR stimulation has been reported by several groups, with contradictory findings. For instance, Levy *et al* reported that neonates produced higher IL-6 than adults following TLR2 and TLR4 stimulation (64). In contrast, several other studies have found neonatal production of IL-6 is significantly reduced compared to adults (140,191). In our study, we found that neonatal IL-6 production was not significantly different from adults following LPS and PGN stimulation, whereas 3M011 induced a markedly diminished IL-6 response in neonates compared to other age groups (Fig 14). IL-6 is an important early response cytokine following infection, as such reduced

capacity to generate IL-6 following TLR7/8 activation early in infection, further contributing to a reduced anti-viral response in neonates.

Finally, we found that production of the type-2 skewing cytokine CCL22 (MDC) is dramatically reduced in the neonatal population compared to other age groups. Taken together with the reduced expression of the type-1 related CXCL10, our results suggest that both type 1 and type 2 immune responses may be reduced in neonates compared to older individuals. These results are somewhat in opposition to previous studies which report a supposed type-2 biasing in neonates (64,113,118), which was primarily based on a reported increase in IL-10 and IL-6, and instead suggests that adaptive immune responses (both type 1 and type 2) may be globally diminished in neonates.

Children are highly responsive to LPS Stimulation

Several studies have shown that the preschool years represent a critical period in the education of the immune system (102,187). This time period is instrumental in determining whether or not an individual will develop lifelong hypersensitivities (76,110,187). However, the mechanisms involved in shaping immune responses in childhood are not well understood.

Here we report several important differences in TLR responsiveness in the child population compared to other age groups. For instance, we found that children produced the highest levels of proinflammatory mediators (IL-1β and CCL2) for all 3 TLR ligands tested (Fig 12 and 13). In addition, LPS-induced IL-

10 was the highest in children compared to all other group (Fig 15). Our results support previous work by Tulic *et al* in which LPS responses in the nasal mucosa of children and adults with allergic rhinitis was tested (110). Tulic *et al* found that nasal mucosa of children was far more responsive to stimulation with LPS than adults (110). In addition, when nasal explants from children was treated with allergen and LPS simultaneously, LPS was able to reduce the production of type 2 cytokines as well as increasing cell proliferation and proinflammatory cytokine production in the children only (110). Hence, childhood may represent a window in which the protective effects of LPS, and other TLR agonists, are able to provide the most benefit in terms of preventing the development of atopy.

In terms of the type 1 and type 2 balance, our results suggest that children may have a propensity to generate enhanced type 2 responses compared to adults. As shown in Fig 16 and 17, children produced significantly higher levels of CCL22 than adults, with the opposite trend for CXCL10 production. In support of our findings, Kawamoto *et al* found that cytokine profiles, type 1 versus type 2, varied with age, with a tendency towards a greater proportion of Th1 cells and cytokines in adults compared to children (192).

Response to PGN is augmented in adolescence

Very few studies to date have examined innate immune responses in adolescence compared with other time points. In this study we found several marked differences in TLR responses in this age group compared to the other groups tested. For instance, the adolescent group had the highest production of

PGN-induced IL-6, IL-10 and CCL22 (Fig 14,15 and 17). In addition, the LPS-induced CCL22 production was greatest in the teenage population. In general, the responses to LPS were less robust in teens than children, suggesting that TLR4-mediated reactions may become less intense as individuals progress into adulthood. In contrast, the responses to 3M011 more closely resembled those elicited by children than adults, suggesting that anti-viral responses are not fully developed until adulthood.

Adults generate reduced proinflammatory mediators following TLR2 and TLR4 stimulation

In this study our adult population in general was equally or less responsive to TLR stimuli compared with children and teens. The TLR responses generated by adults were still markedly more robust than the neonatal population for the majority of cytokines tested. Some of the most interesting differences noted include the reduced production of IL-6, CCL2 in adults compared to children and teens following LPS and PGN stimulation (Fig 12 and 13). However, IL-1β and CXCL10 responses were higher in adults following PGN and 3M011 exposure. These results suggest that cytokine responses are differentially regulated in adults and younger individuals. It is possible that by adulthood, tolerance to the proinflammatory nature of LPS has been acquired to long term environmental exposure. Our results are similar to a study conducted in mice which found that adults produced significantly less proinflammatory cytokines than children (193). Interestingly, the level of IL-10 in adults is similar to those in children and teens,

suggesting the anti-inflammatory nature of this cytokine is not responsible for the reduced proinflammatory responses observed in adulthood.

The major limitation of this study, as for others addressing such questions, is the cross sectional nature of this report. In order to determine how differential TLR responses affect clinical outcomes future prospective birth cohort studies which begin in the neonatal period will be essential. However, this type of study will obviously take many years to perform. Hence, cross-sectional studies can provide a more timely analysis of how TLR responses change over time. We have a concerted effort to enhance the validity of our study by keeping our comparisons amongst different individuals as consistent as possibly by maintaining the same protocols for culturing, stimulating and cytokine detection for all samples. In addition, we have obtained data from a large number of individuals yielding a high-powered statistical analysis.

Our study provides evidence of important differences in TLR functional capacity at various time points in development. We have identified a general reduction in neonatal responsiveness to TLR agonists, especially those resembling viruses. The impairment in neonatal TLR responses could be due in part to a reduction in the expression of TLRs and associated signalling molecules as we have recently found a significant reduction in the mRNA expression of TLR4,6,7 and 8 in neonates compared to young adults (see Fig 10, Chapter 1).

In addition, the enhanced proinflammatory response to LPS and PGN in children and teens suggests that these stimuli may be useful in developing

strategies to prevent asthma and allergies in these age groups. The adult population seems to have ‘fine-tuned’ the TLR responses with a general reduction in TLR responsiveness, with the exception of 3M011 responses. This study demonstrates that the innate immune system continues to develop throughout life, indicating the need to tailor therapies for each phase of development.

CHAPTER 3

EPIGENETIC MODIFIERS PLAY A ROLE IN REGULATING NEONATAL INNATE IMMUNITY

Acknowledgements: Ruey-Chiy Su[1], Jungang Xie[1], Isha Ostopowich[1], Ganesh Srinivasan[2] , and Kent T HayGlass[1]

CIHR National Training Program in Allergy and Asthma Research [1]Dept. Immunology[1] and Pediatrics and Child Health[2] Univ. of Manitoba

PREAMBLE

Recently there has been an increased appreciation of the importance of environmental and developmental signals in the determining gene expression (194). Epigenetic modifications have been identified as an important regulatory mechanism involved in integrating intra- and extracellular signals to determine the phenotype expressed at the cellular level (195). Epigenetics involves changes in the accessibility of chromatin to transcriptional machinery via a variety of covalent modifications to DNA (ie methylation) and histone proteins (ie acetylation, phosphorylation, ubiquitination) (196,197). Given the central regulatory roles epigenetic modifications play in differentiation and gene expression, it is not surprising these modifications play important roles in influencing the types of innate and adaptive immune response which develop following exposure to microbial and environmental stimuli (195,198). Environmental cues modulate histone acetylation at specific gene loci. For example, TLR stimuli, such as LPS, increase histone acetylation in alveolar macrophages (199). In a murine model, Bode *et al* demonstrated that global HDAC inhibition by trichostatin A (TSA) following LPS stimulation negatively impacted expression of several cytokines and costimulatory molecules, indicating the importance of endogenous HDAC activity in modulating innate immune responses (200). We previously demonstrated the importance of histone acetylation in modulating the balance of immune responses by studying helper Th1 and Th2 recall responses in human PBMC (198). We showed that inhibition of endogenous HDAC activity (ie hyperacetylation) specifically enhanced Th2-

associated recall responses by upregulating Th2 specific transcription factors such as GATA-3 (198). Our results suggest that endogenous HDAC activity may be important in modulating the balance of Th1 and Th2 responses by specifically inhibiting overexpression of Th2 recall responses. Collectively these studies indicate the importance of HDAC:HAT balance in maintaining appropriate innate and adaptive immune responses.

Given the importance of early life exposures in determining susceptibility to a variety of chronic conditions in later life (194,201), we chose to focus on the role of epigenetic modifications in neonatal innate immune responses. In this report we examine the roles of histone acetylation on TLR-induced cytokine production in neonates. We also determine whether or not endogenous HDAC and HAT activity is affected by the gestational age of newborns.

RATIONALE & HYPOTHESES

Early life exposure to viral infections and noxious environmental stimuli, such as tobacco smoke, has long term consequences for children (76,87,102,202). In addition, following premature birth (> 37 weeks gestation), neonates have significantly greater risks of developing complications (both acute and chronic) than infants born at term (140,141,156). There are no reports to our knowledge which have examined endogenous HDAC/HAT activity in neonates, or the role of epigenetic modifications in regulation of neonatal innate or adaptive immune responses. Here, we focus on the role of histone acetylation (the balance between HDAC and HAT activity) in modulating neonatal immune responses as these

modifications represent a major mechanism of epigenetic regulation. Noakes *et al* demonstrated that *in utero* exposure to maternal smoking has the ability to modify innate immune responses at birth (203). Furthermore, CSC is shown to affect endogenous HDAC activity in *in vitro* experiments. Here we hypothesized that treatment of neonatal cells with histone deactylase inhibitors, such as the synthetic compound Trichostatin A (TSA) or cigarette smoke condensate (CSC) modify TLR-induced expression of cytokines. Characterizing the levels of endogenous HDAC and HAT activity in the cord blood cells of newborns will contribute to our understanding of the heterogeneity of epigenetic baseline among neonates of various gestational age ranges. In addition to measuring endogenous HDAC/HAT activity, we tested the hypotheses that experimentally altering the HDAC/HAT balance during TLR-mediated innate immune activation (using TSA or cig smoke condensate) would alter the nature and intensity of cytokine expression and that this alteration in neonatal immune responses is linked to altering the expression levels of TLR mRNA.

RESULTS

The ratio of HDAC:HAT is significantly higher in PBMCs than cord blood

Our initial goal was to assess endogenous HDAC/HAT activity directly ex vivo in CBMC from preterm neonates (>37 weeks, n=7) and full term (n= 33) compared to young adult controls (age 12-13 years, n=20). As shown in Figure 18, total HDAC activity increased substantially after birth. Interestingly, full term neonates also had significantly greater HDAC activity ($p<0.0001$) than those born

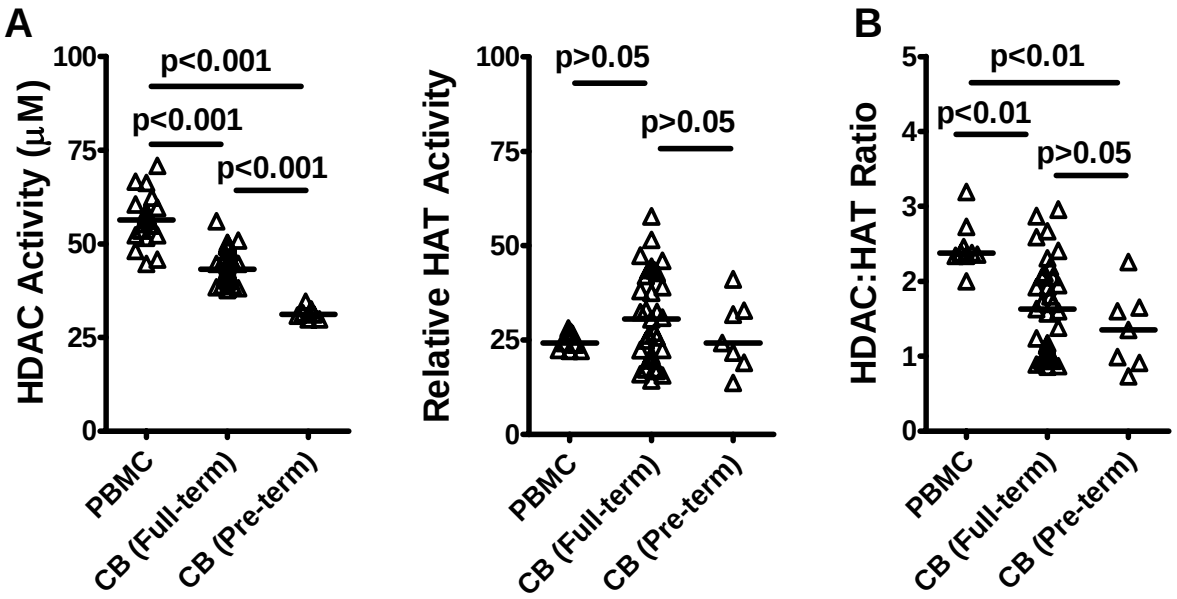


Figure 18. HDAC Levels Increase with Age while HAT do not exhibit significant differences. HDAC and HAT activity was measured directly ex vivo in cord blood of preterm neonates (n=7), full term neonates (n=33) and peripheral blood of adolescents age 13-14. (n=20). Median values are depicted above.

** R Su performed the HDAC and HAT assays.

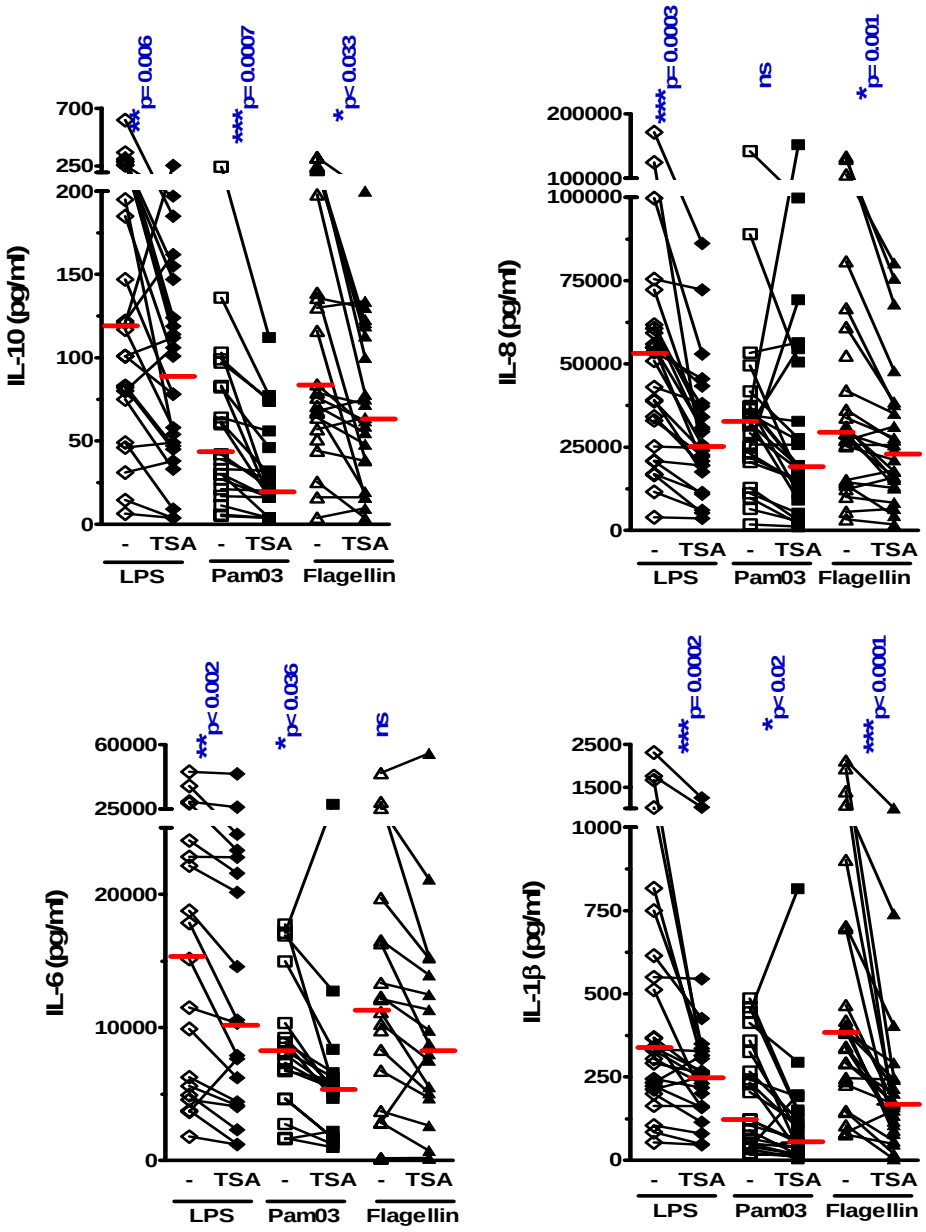


Figure 19. Global HDAC Inhibition Reduces TLR-Induced Cytokine Production in Neonates.Cord blood cells from full term neonates (n= 25) were treated with TSA beginning 45 min prior to culture with select TLR agonists for an additional 24 hours. Supernatants were tested for cytokine production via ELISA. Differences between TLR-ligand plus and minus TSA are depicted.
****Culture performed by S Macpherson, ELISAs by R Su and S Macpherson**

preterm. In contrast, relative HAT activity did not differ significantly between pre-term and full term neonates or between neonates and adolescents. Hence, suggesting that HDAC activity is not fully active until the last few weeks of *in utero* development.

HDAC Inhibition leads reduction of select TLR-ligand induced cytokine production in cord blood

Following measurement of endogenous HDAC/HAT activity, we determined the effect of modulating these levels on cytokine responses following TLR activation. We first tested a broad range of TLR responses by stimulating mononucleated cord blood cells with a variety of TLR stimuli (ie LPS, PGN-Y,3M001, 3M002, Pam3, Flagellin) following pretreatment with an HDAC inhibitor, TSA. Following 24h stimulation, cytokine responses to TLR stimulation were assessed using ELISA. Figure 19 shows cytokine responses that were most significantly impacted by TSA treatment. Although TSA is generally believed to enhance histone acetylation, which leads to increased gene expression of the associated gene loci, we found that inhibition of histone deacetylase activity has an inhibitory effect on the majority of TLR responses we measured. In contrast, IL-6 and IL-8 responses elicited by PGN and 3M002 were significantly increased following HDAC inhibition (data not shown). These data show that the level of endogenous HDAC activity plays a regulatory role in neonatal innate immune responses.

Cigarette Smoke Condensate has an Inhibitory Impact on Select TLR Responses

We next wanted to determine how a common environmental compound might mediate changes in the innate immune response of neonates. To do this we studied the effects of tobacco smoke through the use of commercially available cigarette smoke condensate, which contains over 4000 chemical components (204). Prior to beginning our experimentation we performed viability assays to determine the impact of CSC on cell viability. As shown in Figure 20, none of the concentrations tested had significant impact on cell viability under the conditions tested (ie 24h culture). We also tested CSC to determine its possible impact on HDAC activity. Although CSC contains many compounds which may have multiple effects on cellular processes, we found that application of these extracts to cord blood cells had a general inhibitory effect on HDAC activity. We selected 10ug/mL to use in future experiments as this concentration had no detectable impact on viability yet exhibited maximal impact ton HDAC activity. Although the inhibitory effects on HDAC observed are not as specific as TSA which only acts on HDAC, we wanted to determine how this complex mixture of chemicals may affect global cytokine production in neonates following TLR stimulation. We found that application of CSC had a significant inhibitory effect on cytokine production following stimulation of TLR1/2,4 and 5 (Figure 21). In contrast, 3M002 and PGN-mediated cytokine production (ie IL-6 and IL-8) was significantly increased following CSC exposure.

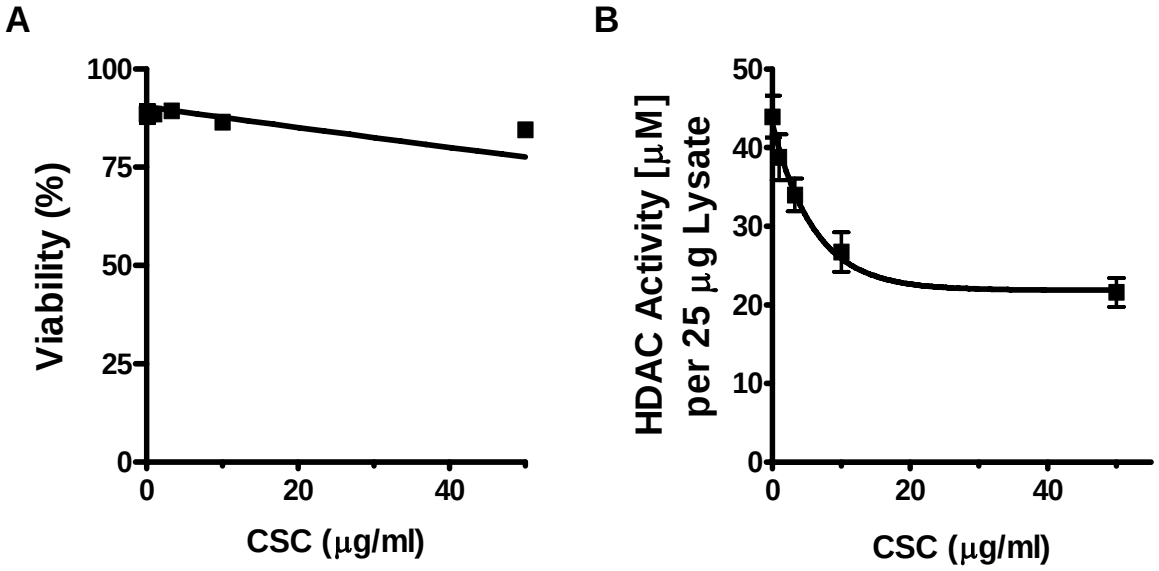


Figure 20. Acute exposure to cigarette smoke condensates results in reduced cell viability and cellular endogenous HDAC activity. Cord blood cells were exposed to increment amounts of cigarette smoke condensates (CSC) for 24h at 37^{o}C. Cellular viability was then examined using Guava instrument, which distinguishes apoptotic cells and dead cells, from viable cells. CSC significantly reduced cellular viability at concentrations greater than 50ug/ml. When the effects of acute CSC exposure on cellular HDAC activity were measured, we found that CSC reduced endogenous HDAC activity in a dose-dependent manner and the effects plateau at concentrations ranging from 10-20 ug/ml.

**Performed by R Su and S Macpherson

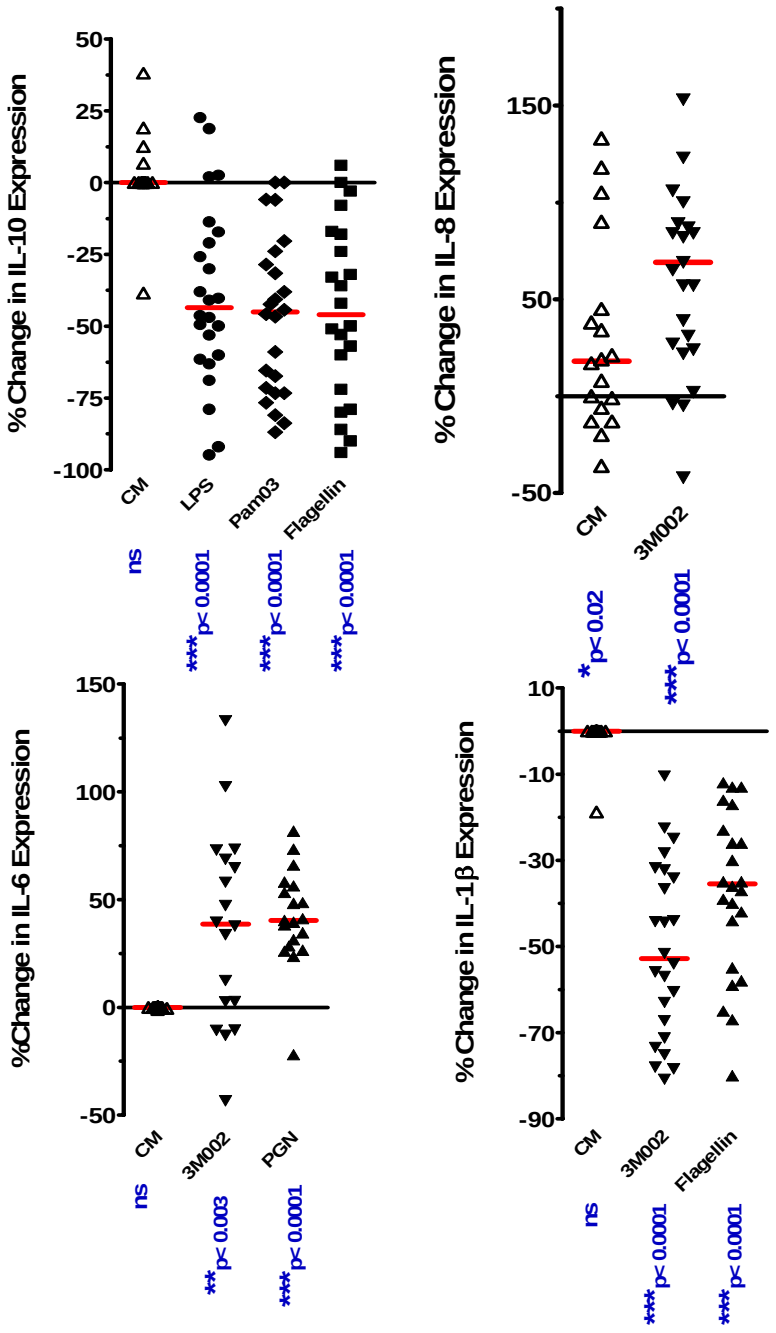


Figure 21. Effects of exposure to cigarette smoke condensates on cytokine responses to TLR-stimulation. Cord blood cells from full term neonates (n=25) were stimulated with TLR agonists with or without cigarette smoke condensate (CSC) pretreatment. Following 24 hour incubation, supernatants were tested for cytokine levels via ELISA.

**Performed by R Su and S Macpherson

TSA Inhibits LPS Mediated Upregulation of TLR4 expression

In order to determine how HDAC inhibition mediates the observed effects on TLR-induced cytokine production we examined TLR expression following exposure to TSA or CSC. When treated CBMC with TSA or CSC alone there was no significant effect on TLR4 mRNA expression, although the trend was an increase in TLR4 expression (Figure 22). In contrast, when we added TSA in the presence of LPS, the levels of TLR4 were significantly lower than following LPS treatment alone (similar trend with CSC although it did not reach significance, $p=0.12$). These preliminary results indicated a potential mechanism through which HDAC inhibition (via TSA or CSC) leads to reduced TLR-induced cytokine levels.

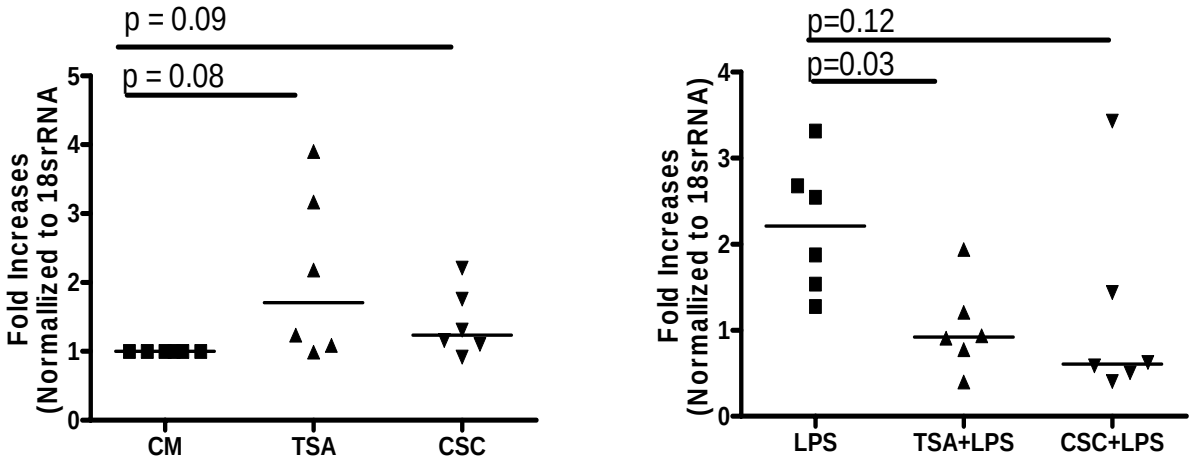


Figure 22. HDAC Inhibition leads to reduced expression of TLR4 following exposure to LPS. Cord blood cells from full term neonates (n=6) were cultured with or without LPS in the presence of TSA for 24 hours at 37C. Supernatants were harvested and cells were stored for RNA Isolation. Median values are shown as a fold increase compared with TLR4 levels in culture media (untreated).

Jungang Xie performed Q PCR.

DISCUSSION

Early life represents an impressionable period in development; hence pathogen and environmental exposures occurring during these formative years play important roles in influencing later susceptibility to chronic disease (194). This "developmental plasticity" in the perinatal is mediated by interaction of the genome and epigenome with environmental factors (194). Epigenetic modifications are responsible for linking environmental exposures to changes in gene expression (199). For the first time we have shown that HDAC activity is reduced in the neonatal population compared to young adults. In addition, preterm neonates have significantly less HDAC activity than their full term counterparts (Fig 20), suggesting that the activity of this group of epigenetic modifiers increases with age. We report here that inhibiting endogenous HDAC activity with TSA has significant impacts on neonatal innate immune capacity. Specifically, TSA had a marked inhibitory effect on pro- and anti-inflammatory cytokine production following PAM, LPS and Flagellin activation (Figure 19). These results initially seem somewhat counter-intuitive as TSA is a global inhibitor of histone deactylases, which are generally believed to reduce expression of genes; hence TSA generally promotes accessibility of genes (197,200). However, we have previously demonstrated that TSA treatment can inhibit specific cellular responses, such as Th-1 cytokine responses (198). Bode *et al* also reported a reduction in IL-12p40 and TNFα production following TLR stimulation in human and murine macrophages (200). Hence, although HDAC

inhibition may enhance cellular histone acetylation, TSA does not act as a global 'enhancer' of all genes. In line with previous studies, our results indicate that endogenous HDAC activity is associated with promoting expression of a subset of cytokine genes following TLR stimulation in the human neonate.

In this study we also examined the effects of cigarette smoke condensate on neonatal immune responses. Exposure to maternal cigarette smoke has been shown to reduce TLR-induced cytokine production at birth (203), and has been associated with increased incidence of infections and wheeze in early life (202,205). However, the mechanism through which tobacco smoke exposure impacts TLR responses is not known. In this study we examined how acute exposure to cigarette smoke condensates would impact both endogenous HDAC/HAT activity and cytokine production following TLR activation. We found that CSC had an inhibitory effect on endogenous HDAC activity and select TLR-induced cytokine production (Fig 20, 21). We have extended these findings by examining how inhibition of HDAC activity may have specific consequences in neonatal innate immune regulation. Cigarette smoke exposure may reduce neonatal innate immune capacity through inhibition of endogenous HDAC activity. One limitation of our study is that we do not have information on maternal smoking history throughout pregnancy, which could have an additive effect on reduction in TLR-induced cytokine production if the fetus was chronically exposed to cigarette smoke throughout the duration of the pregnancy. However, our results do demonstrate the ability of environmental factors, such as

CSC, to modulate the endogenous balance of epigenetic modifiers, hence impacting on gene expression.

In order to assess how TSA and CSC mediate their effects on TLR-induced cytokine production in neonates we examined how these epigenetic modifiers affect TLR mRNA expression. As indicated by Figure 22, LPS treatment lead to increased expression of TLR4 after 24 hours (2-fold higher than untreated control). When TSA or CSC was added to cells these mediators did not have a significant effect on TLR4 expression. In contrast, when cells were treated with LPS in the presence of either TSA the expression of TLR4 was significantly diminished compared to LPS alone ($p=0.03$, comparable to baseline TLR4 levels). The same trend was observed following LPS and CSC treatment although the change in TLR4 expression did not reach significance ($p=0.12$). These results suggest that HDAC inhibition could cause a reduction in the expression levels of TLR4, which could contribute to the reduced cytokine levels observed. Future experiments which examine changes in other TLRs and associated adaptor molecules will provide more insight into the effects of HDAC inhibition on TLR signalling pathways.

In conclusion, we are the first to demonstrate that epigenetic modifications represent an important regulatory element in neonatal innate immune responses. Also, the balance of endogenous epigenetic regulators, HDAC and HAT, can be modulated by environmental exposures such as tobacco smoke. Although genetic predisposition to several diseases, such as asthma and allergies, has been shown by several studies, it is now evident that environmental exposures also play an

integral part in determining disease susceptibility. Early life represents a potential window of disease modification; as such developing a better understanding of how the immune system is modulated in neonates may help to develop strategies which reduce the incidence of disease by modifying environmental exposures. Our data do not directly demonstrate a causal relationship between environmental stimuli in early life and future disease susceptibility. Future longitudinal studies which study prenatal and perinatal environmental exposures are required to determine causal relationships.

CHAPTER 4

IDENTIFICATION OF NOVEL BIOMARKERS IN THE CORD BLOOD SECRETOME

Acknowledgements: Oleg Krokhin[1], John Wilkins[1], Ganesh Srinivasan[2] , and Kent T HayGlass[3]

Manitoba Centre for Proteomics and Systems Biology[1]Pediatrics and Child Health[2] , CIHR National Training Program in Allergy and Asthma Research [3,] Dept. Immunology [3] Univ. of Manitoba

PREAMBLE

In recent years proteomics has become a powerful analytical tool for the analysis of biological fluids and tissues (206). Proteomic analysis has can be used as a high throughput process which can determine the identity of proteins of sufficiently high quantity in a given biological sample, and hence has the capacity to be useful in the study of many human diseases (206). The identification of biomarkers for disease in biological fluids, such as urine, using proteomic techniques is an increasingly expanding area of study (207). In contrast, analysis of secreted proteins (which are involved in cell-to-cell communication) in culture supernatants remains relatively undeveloped (207). A potential reason for the lack of progress in the study of the 'secretome' of a given cell population is that the analysis of these samples generates several technical challenges. For instance, many proteins of interest are expressed in relatively low levels making them difficult to analyze (207). In addition, culturing conditions, such as the common use of fetal calf serum in culture media, or the presence of high levels of endogenous proteins such as albumin, complicate analytical techniques (207). There are a limited number of studies on secreted proteins in cell lines which have used labour intensive traditional analytical approaches to protein identification, such as 2D gel electrophoresis (208). Here we have utilized an alternative approach to proteomic analysis by using two dimensional HPLC-MS-MS which also incorporates sequence specific retention time calculations in an attempt to develop a method of novel protein identification in a complex biological sample.

RATIONALE

Communication between immune cells requires the production and secretion of proteins into the extracellular environment (209); however, a broad understanding of the profile of proteins involved in mediating immune responses remains elusive. Currently very few studies have examined protein profiles secreted by human immune cells and none to date have focused on neonatal innate immune responses. Using proteomic analyses, Karamessinis *et al* found that cord blood serum of neonates with intrauterine growth restriction expressed several proteins which were not present in cord blood serum of neonates with normal weight (210). This suggests that proteomic techniques may reveal novel candidate proteins involved in pathologic mechanisms *in utero*. The goal of our study was to develop an assay to obtain a broad overview the entire protein profile expressed by neonates following TLR stimulation. In addition, we wanted to subsequently compare protein profiles in both preterm and full term neonates following TLR activation in an attempt to identify novel protein candidates which are either abnormally expressed or deficient in the preterm population compared to their full term counterparts. These results would provide a clearer understanding of innate immune capacity in the neonatal period and allow the identification of novel candidate proteins which are differentially expressed by preterm and full term infants.

RESEARCH APPROACH & RESULTS

Autologous Cord Blood Serum Inhibits Cytokine Responses

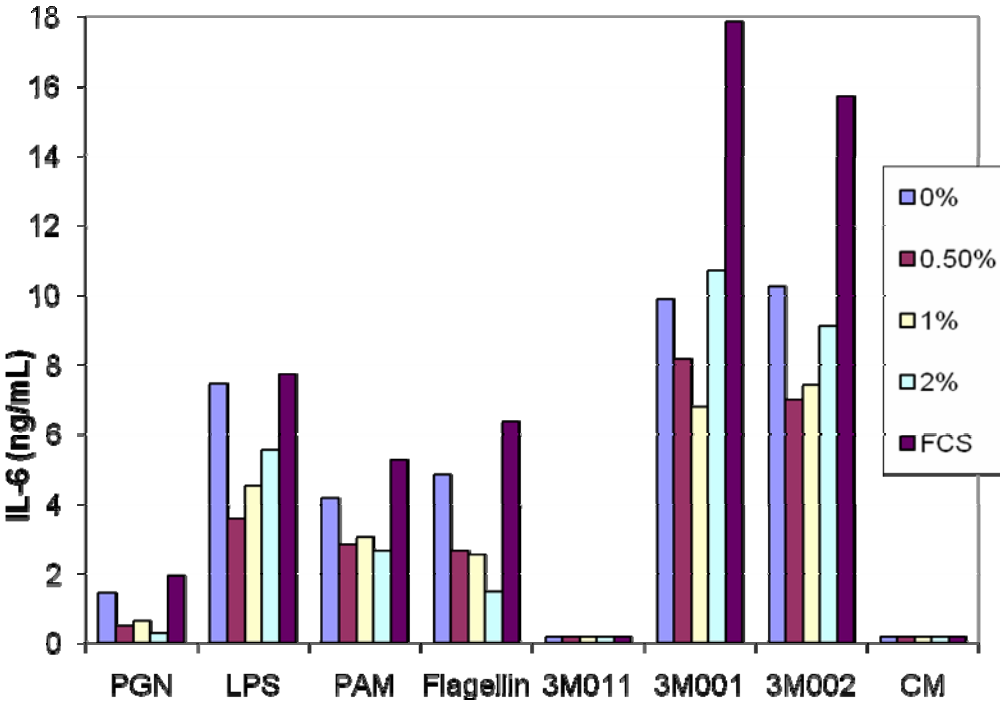
One of the challenges of secreted protein analysis is the development of culture conditions which support effective protein analysis while maintaining cell viability. In preliminary experiments it was determined that fetal calf serum, which is typically used as an essential nutritional supplement in culture media, interferes significantly with proteomic analysis as the majority of the immunologic proteins of interest are in relatively low abundance (ie pg/mL) relative to FCS which is present at 10,000 to >1,000,000 fold excess. To avoid this conflict with FCS, we used ELISA to test cytokine concentrations in media which was either not supplemented with any serum or was supplemented with heat inactivated autologous plasma at varying concentrations and determine the levels of cytokine production upon TLR activation relative to concentrations generated upon culture with fetal calf serum. We chose to test the efficacy of human serum in promoting cytokine responses for two reasons, first there are several types of affinity columns available which can deplete the 20 most abundant human serum proteins in culture supernatants (hence these would not interfere as significantly with protein detection), and secondly, previous studies have shown that autologous human serum can promote cell expansion in culture. However, we found that addition of autologous serum to CBMC culture led to reduced cytokine production compared to both serum free and FBS supplemented media for the majority of TLR responses tested (Fig 23).

In addition, these experiments demonstrated that serum-free media was a better choice for future experimentation as it would avoid the need to deplete serum proteins while still allowing for measurable cytokine production in short

term culture. We also tested cell viability in the serum free condition using a trypan blue assay, the cell viability was found to the same (95%) in the FCS supplemented and serum free conditions following 24 hour culture.

Recombinant cytokines were undetectable in serum free media using HPLC/MALDI-MS

The next step in our experimentation was to determine whether or not we could detect proteins of interest (ie cytokines and chemokines, as positive controls) at concentrations which would be present under typical culture conditions. To do this we first spiked serum-free media with IL-6 (1 ng/mL) and IL-10 at (500 pg/mL) to see if we could detect these proteins using tandem HPLC and MALDI-MS. Unfortunately, we were not able to detect physiological concentrations of either cytokine using these method. One possible explanation for our inability to detect these proteins was that the presence of phenol red (a pH indicator found in most culture media) was interfering with the mass spectrometry process due to its high concentration and retention time in the HPLC column. Hence, we repeated the previous experiment using phenol-red free media spiked with the same cytokines. To control for possible loss of the spiked recombinant cytokines by adsorption to the plastic walls of the tubes, we also added lactalbumin hydrosylate, a carrier peptide to the cytokine mixture in an attempt to improve detection. Unfortunately we were still unable to detect these cytokines using HPLC/MALDI-MS, most likely due to the absence of sufficient carrier proteins which would be present in complex cell supernatants.



Fi gure 23. IL-6 production is detectable in serum free media. These results are representative of 9 full term neonates which were tested for IL-6 expression. CBMC were cultured under various conditions (ie different concentrations of autologous plasma or FCS) for 24 hours, supernatants were harvested and cytokine expression measured via ELISA.

Analysis of cord blood proteome following LPS stimulation

Following the unsuccessful initial experiments using protein free culture media spiked with recombinant cytokines, we concluded that the lack of other carrier proteins in the sample was preventing the detection of these low abundance proteins. In order to correct for this we tested a biological cord blood supernatant which contains a wide range of peptides. We conducted an analysis of supernatant from CBMC cultured with or without LPS (50 ng/mL) for 24 hours. Using protein mass finger print and the sequence specific retention calculator, we were able to identify 24 proteins which were present only in the LPS-stimulated sample as shown in Table 3. Using a NCBI Protein query we identified the cellular localization of the identified proteins. The majority of the proteins identified were of cytoplasmic origin; however we were able to identify the expression of Interleukin-8 in the LPS-stimulated sample.

2D HPLC ESI-MS/MS analysis increases the sensitivity of protein detection in cord blood supernatants samples

Although we were able to identify many proteins using tandem HPLC and MALDI-MS, few of the identified proteins were believed to be involved in modulating immune responses. Therefore, in the next set of experiments we took a different approach to analyzing our cord blood supernatants in order to identify a greater number of potentially interesting proteins. The approach we used was to separate peptides first again using reverse phase liquid chromatography at pH 10 followed by a second dimension separation at pH 2 with electrospray ionization and tandem mass spectrometry analysis (ESI-MS/MS) (232). In these experiments

we were able to identify over 900 proteins in the CBMC supernatant, of those 200 were produced in the presence of LPS which were not observed in the unstimulated sample (Appendix B). As shown in Figure 24, the majority of the proteins identified were from intracellular origin (ie nucleus, cytoplasm and cell membrane). However, we were able to identify 13 proteins which were secreted following LPS stimulation only (Table 4). Included in the group of secreted proteins were several proteins which are known to play important roles in innate immune responses such as CXCL1, IL-6, Heat Shock Protein (HSP) family members, and Cardiotrophin-like cytokine (IL-6 family).

Table 3. Proteins Detected Following LPS Stimulation of CBMC using HPLC/MALDI-MS. Proteins are listed based on decreasing log (e) values (a measure of the statistical certainty of the protein identity, higher values indicating a higher degree certainty)

Name	MW (kDa)	Log (e)	Localization
Fibrinogen gamma chain precursor	45.9	-52	Serum
Glyceraldehyde 3-phosphate dehydrogenase	36	-24.7	Cytoplasm
Trioesphosphate isomerase	26.7	-16.3	Cytoplasm
Rho GDP-dissociation inhibitor 2	23	-10.3	Cytoplasm
Annexin A1	38.7	-9	Cytoplasm
Unc-112 related protein 2 (Kindlin-3)	75.9	-3.6	Membrane
Eosinophil cationic protein precursor	18.4	-3.4	Cytoplasm
Catalase	59.7	-3.2	Cytoplasm
Talin 2	271.6	-2.7	Cytoplasm
Interleukin-8 precursor (IL-8) (CXCL8)	11.1	-2.2	Secreted
Calreticulin precursor	48.1	-2	Cytoplasm
Histone H3.3	15.3	-1.9	Nucleus
Von Ebner minor salivary gland protein) (VEMSGP)	52.4	-1.8	Secreted
High mobility group protein 2	24	-1.8	Nucleus
Heat Shock Related 70 kDa Protein 2	70	-1.6	Intracellular/Secreted
Protein S100-P	10.4	-1.5	Cytoplasm/Nucleus
Antibacterial protein FALL-39 precursor	19.3	-1.4	Cytoplasm (granules)
Serum deprivation response protein	47.1	-1.4	Membrane
Histone 2A Family J Isoform 1	16.6	-1.3	Nucleus
Integrin Beta 3 Precursor	87	-1.3	Membrane
Ras suppressor protein 1	31.5	-1.3	Cytoplasm
ATP Synthase, Mitrochondrial Precursor	59.7	-1.2	Cytoplasm
Resistin precursor	11.4	-1.1	Secreted

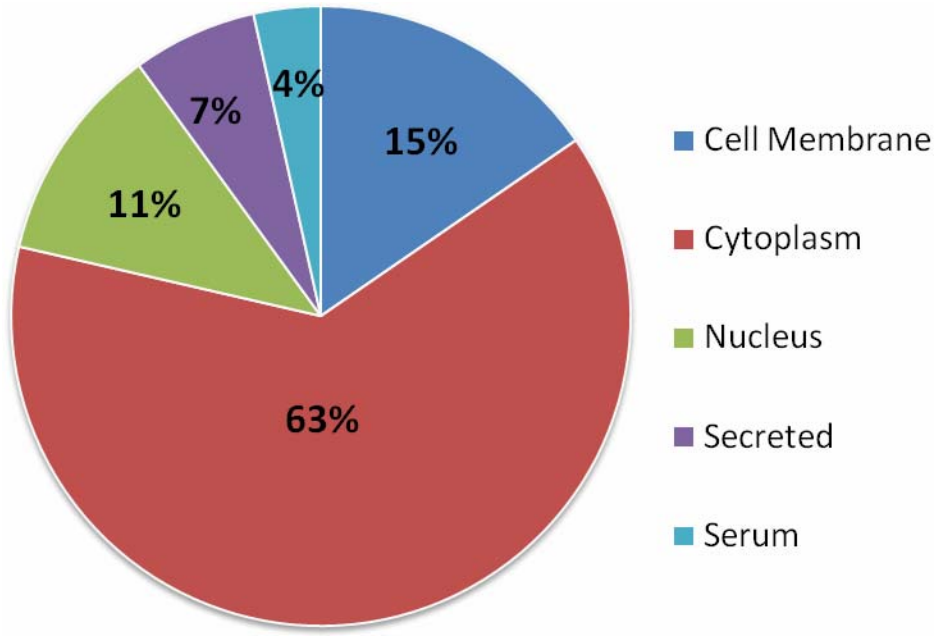


Figure 24. Localization of CBMC proteins identified following HPLC MALDI-MS. Using NCBI protein database searching the subcellular localization of all identified proteins was determined. The approximate proportion of proteins from each location is shown here.

Table 4. Secreted Proteins Identified via 2D HPLC ESI-MS/MS in CBMC Supernatants following LPS Stimulation.

Name	MW (kDa)	Log (e)
Growth regulated protein alpha precursor (CXCL1)	11.3	-15.7
Neudesin precursor (Neuron-derived neurotrophic factor) (Secreted protein of unknown function)	18.8	-2.5
MSFL2541	20.4	-1.2
Neuroendocrine protein 7B2 precursor (Secretory granule endocrine protein I) (Secretogranin V) (Pituitary polypeptide).	23.7	-1.4
Interleukin-6 (IL-6) (Interferon beta-2)	23.7	-25.2
Inhibin beta A chain precursor (Activin beta-A chain)	47.4	-2.5
Histidine-rich glycoprotein precursor	59.5	-2.7
Cardiotrophin-like cytokine; neurotrophin-1/B-cell stimulating factor-3.	25.2	-1.1
Transcobalamin I precursor (TCI) (TC I).	48.2	-1.6
Immunoglobulin heavy chain C gene	35.9	-41
Heat shock 70 kDa protein 1 (HSP70.1)	70	-146.1
Heat Shock Protein 75 kDa	80.1	-13.4
Heat Shock Protein 90-alpha (HSP 86).	98.1	-162.8

DISCUSSION

In recent years the use of proteomic techniques has emerged as a powerful tool in the study of global protein expression in biological and physiological processes (212). The study of proteins secreted by a given cell or tissue, known as secretomics, is a field which will allow for an improved understanding of which proteins are important in modulating physiologic processes and may help to identify therapeutic targets in human diseases (211). In this study our goal was to analyze global protein expression in neonates following exposure to TLR agonists in an attempt to develop a better understanding of the immunologic modulators involved in neonatal innate immune responses. We used a different approach from the majority of studies examining secreted proteins from human tissue which utilize 2D gel electrophoresis to separate proteins, followed by MALDI-TOF-MS (212). Our approach offers an alternative to the more traditional methods using 2D gel electrophoresis which have several limitations including a lower sensitivity, sample throughput and dynamic range (209). In our protocol we attempted to maximize protein detection using either HPLC MALDI-MS or ESI-MS/MS in combination with a novel sequence specific retention calculator. Currently there are no studies which have examined global secreted protein expression in neonates following TLR stimulation. In our first experiment using HPLC and MALDI-MS we identified IL-8, a chemokine which we and others have detected in CBMC supernatants at relatively high concentrations (ie 5-50 ng/mL) following LPS stimulation (140). In our second experiment using ESI-MS/MS we were also able to confirm the presence of IL-6 which we and others

have found to be highly upregulated by LPS stimulation in cord blood cells (140,165,189,213). In addition to IL-6, we also identified Cardiotrophin-like cytokine (also known as B cell stimulating factor 3), a recently identified member of the IL-6 family (214). Cardiotrophin-like cytokine has been shown to activate B cells *in vivo* and *in vitro* and has not previously been reported in neonates. We also identified CXCL1 (also known as Neutrophil activating peptide 2/Melanoma growth-related oncogene-alpha) which is a pro-inflammatory chemokine which participates in innate immune response to pathogens (215). CXCL1 is produced by macrophages and epithelial cells and has chemoattractant effect on neutrophils, contributing to initial response to pathogens in humans (216). Krolak *et al* reported the presence of CXCL1 in cord blood serum of human neonates, with significantly greater levels in preterm neonates than their full term counterparts (217). The sample tested in our case was from a full term neonate, however it would be interesting to quantify the levels of this chemokine following exposure to TLR ligands to determine how much this marker is upregulated compared to basal levels.

In addition to the aforementioned cytokines and chemokines our analyses also identified several heat shock protein (HSP) family members. In general, HSP are regarded as intracellular proteins which are upregulated during cell stress and function as danger signals (218,219). Recent evidence suggests that during the immune response to an incoming pathogen HSP may be released into the extracellular milieu (218,219). Several reports suggest that HSP have the capacity to modulate immune responses via several mechanisms including interaction with

TLRs and other cell surface receptors (219,220). In our sample we detected Hsp70, which was recently found to act as a vaccine adjuvant in vivo in human monocyte-derived DCs (mDCs) (220). The activity of HSP70 was mediated by IkappaB-alpha/NF-kappaB and ERK1/2 pathways in human DCs. Some reports suggest that heat shock proteins may be able to bind to pathogen-related proteins such as LPS and CpG ODN, allowing increased activation of TLR-mediated signaling pathways resulting in increased cytokine production (218). It would be beneficial to confirm and quantify the expression of HSPs in CBMC supernatants using ELISA techniques with and without subsequent TLR activation. To our knowledge there are no current reports which have examined the expression of these mediators following activation of CBMC. It is possible that HSP may play an important role in modulating innate immune responses in human neonates.

In this report we have demonstrated that proteomic techniques have the capacity to identify novel proteins which may be essential in modulating innate immune responses at birth. However, we were unable to detect many of the cytokines and chemokines which are known to be present in cord blood supernatants following stimulation with LPS. One possible explanation was that the expression of these proteins was below the limit of detection for proteomic analysis as preformed here. However in the case of IL-10 for instance we have found an average of approximately 300 pg/mL in cord blood supernatants following LPS stimulation, which should be detectable using ESI-MS/MS and our protocol. It is possible as well that the high level of contaminating proteins (of intracellular origin) impeded the detection of these lower abundance proteins. In

future experiments our goal is to increase the detection of immune-related proteins while minimizing contamination with dead or lysed cells. The detection of many proteins of intracellular origin in both the LPS stimulated and untreated samples suggests that there was some contamination in our samples.

We have planned an experiment using a concentration of cells which is 4X higher than our typical culture conditions in an attempt to increase the quantity of cytokines and chemokines present in the supernatants. One potential downfall of this approach is that we may also have more dead cells contaminating our samples. To avoid this, prior to removing supernatants the culture plates were centrifuged to prevent the accidental acquisition of cells when collecting supernatants. In addition, using flow cytometry to analyze cell pellets for signs of apoptosis as well as supernatants prior to proteomic analysis would give us a better understanding of how our samples have become contaminated (ie due to lysing of cells which are present in the supernatants).

To conclude, in this report we have demonstrated the potential use of proteomic approaches to study global protein expression in cord blood supernatants. These techniques may be applied in the future to compare protein profiles of neonates born preterm with their full term counterparts.

GENERAL DISCUSSION

The innate immune system plays a critical role in determining the quality, quantity and type of adaptive immune responses (221). The importance of the TLR system of PRR has been demonstrated in many animal models as well as human diseases that involve mutations which reduce the function of these receptors. For instance, a polymorphism in TLR4 which reduces its ability to bind RSV is associated with high rate of RSV infections and risk of severe bronchiolitis (186). By interacting with microbial agonists in the environment, TLRs are likely to play an important role in determining whether or not an individual will develop allergic disease (8,222). Priming of the immune response may begin in utero (194). For instance, infants born to mothers with atopy have reduced expression of select TLRs at birth (223). In addition, reduced TLR responses at birth have been shown to predict future susceptibility to asthma (88), arguing for the importance of early immune capacity in determining future outcomes. That said, most studies of human innate immune capacity in early life are hampered by limited size and breadth of experimental readouts. Hence, our focus was to develop a clearer understanding of TLR responses at birth and how they develop throughout gestation.

In the past few decades medical advances have significantly reduced neonatal mortality rates, leading to a much higher proportion of preterm born neonates surviving into adulthood (224). Recent clinical studies demonstrate that late preterm birth is associated with an increased risk of developing complications in early life, including an increased risk of developing infections in the perinatal

period (128,133,225). Although the number of infants delivered prior to 32 weeks has significantly dropped since 1990, the proportion of neonates delivered in the late preterm period has increased by at least 25% (226). Here we have demonstrated for the first time that late preterm neonates have reduced capacity to respond to a variety of bacterial and viral stimuli compared to their term counterparts. We have also shown that late preterm neonates have reduced basal expression of TLRs and associated adaptor proteins. The reduced capacity to elicit appropriate innate immune responses at birth could contribute to the increased rate of viral infections, wheezing and asthma which have all been shown to be increased in preterm born infants (133,141,168).

Our study is the first to demonstrate the markedly reduced capacity of late preterm neonates to respond to viral agonists. Future analyses which examine responses to whole viruses such as RSV and HMPV (which commonly cause respiratory infections in neonates) would provide a more in depth understanding of how preterm and full term neonates respond to intact pathogens. Another area of study which was not addressed in our analyses is the potential differences in Treg cells in neonates at various developmental time points. Regulatory T cells are an important source of IL-10 (227), one of the most highly deficient cytokines in the late preterm population. Tregs have also been shown to be essential in directing appropriate immune responses and may be defective in atopic individuals (227). Hence, reduced Treg activity following TLR activation at birth could contribute to the increased risks of developing asthma in late preterm neonates. In the current study we did not observe a difference in the number of

IL-10-producing cells following TLR activation; however these cells may be less responsive in late preterm neonates. It would be interesting to determine the proportion of Treg cells in CBMC of preterm and full term neonates.

One potential intervention to augment innate immune responses in the neonatal period and prevent the development of allergic disease is the use of daily prebiotic supplementation in an attempt to stimulate beneficial TLR responses through the digestive system (108,125). Arslanoglu *et al* conducted a randomized double-blind placebo controlled study to test the potential beneficial effects a prebiotic oligosaccharide found in breast milk in a group of formula fed neonates with a familial history of atopy (108). Administration of the prebiotic supplement daily in formula led to a significant reduction in the incidence of atopic dermatitis, recurrent wheeze and respiratory infectious disease in the first six months of life (108). This study suggests that prebiotic supplementation may be a useful therapeutic intervention to stimulate appropriate, protective immune responses and hence may be beneficial as a therapy for preterm neonates.

One limitation of our study is our focus on immune responses in cord blood without a subsequent prospective analysis of how these cord blood responses affect long-term immune responses. Future prospective birth cohort studies which following infants born at a wide range of gestational ages will provide a clearer picture of how preterm birth affects future immunologic outcomes.

Ultimately future studies will need to determine if immune responses at birth are predictive of clinical phenotypes later in life, paying attention to the contribution of gestational age as well as parental history. A recent report by Saghafian-Hedengren *et al* concluded that cord blood monocytes from neonates with a maternal history of atopy had a reduced capacity to respond to bacterial TLR agonists compared to neonates with non-atopic mothers (228), suggesting that preterm birth and atopic family history may have a synergistic effect on inhibition of TLR responses at birth. We and others are currently embarking on a large prospective birth cohort study known as the CHILD study. This study will provide valuable information on long term immunologic consequences associated with the late preterm population and will take into account familial history of atopy.

In our initial studies our goal was to assess innate immune responses in neonates of various gestational ages. With the large cohort of neonates analyzed we also wanted to compare immune responses at birth to those of older individuals in an attempt to better understand how innate immune responses change throughout life. Hence, the next portion of our studies focused on TLR responses to LPS, PGN and 3M011 in full term neonates, children, adolescents and adults. Yerkovich *et al* are the only group which has previously studied TLR4 responses at multiple time points throughout life (165). Here we examined a larger population of subjects and tested much larger panel of TLR ligands as well as broader range of cytokine responses. Our study demonstrated several important differences in neonates and other age groups. For instance, we found markedly

reduced neonatal capacity to produce the anti-inflammatory cytokine IL-10 compared to all other groups. Reduced capacity to generate IL-10 could contribute to the increased risk of sepsis (ie uncontrolled inflammation) in the neonatal period (229). In addition, we are the first to report an overall reduced capacity of neonates to respond to viral agonists at birth. Responses to viral agonists generally appeared to increase with age, with the most robust cytokine responses observed in the adult population. In contrast to previous studies which suggest that neonates have an augmented capacity to produce IL-6 (64,113,118,124), we demonstrate here that production of IL-6 is equivalent or reduced in neonates compared to adults depending on the TLR pathway tested. In addition, children and teens produce markedly higher levels than both neonates and adults. Our results are supported by reports which have found an increased capacity of children to respond to LPS compared to adults (110). This increased capacity to produce IL-6 and CCL2 following bacterial stimulation was also observed in the adolescent population, suggesting that these TLRs may be particularly useful as therapeutic targets in these populations.

In the current literature examining TLR responses in neonates compared to adults there is a generally accepted belief in an increased propensity of neonates to generate Th2 skewing immune responses (64,118,124). However, this conclusion is perhaps misleading as it is based on the observation that neonates have a reduced TNF:IL-6 ratio compared to adults (64). In contrast, we demonstrate here that neonates have a reduced ability to produce both Th1 (IP-10)

and Th2 (MDC) skewing cytokines compared to other age groups. Our results also showed that adults had higher levels of Th-1 skewing IP-10 than children.

Further studies are necessary to develop a better understanding of how TLR responses are modified at different developmental time points. One possibility is that there are different numbers of responsive cells in each group. To determine if this is the case, the ELISPOT assay, which tests the frequency of responsive cells, would be a useful assay. Another approach would be to examine the TLR mRNA levels and absolute number of TLR2,4,7 and 8 positive cells in the CBMCs and PBMCs of individuals at various time points. It is possible that the proportion of cells expressing the various TLRs varies over time. It would also be interesting to further examine anti-viral immune responses throughout life through; this could be achieved through the use of whole virus (ie RSV) and examining type 1 interferon production.

In recent years the complex roles of epigenetic modifications in linking environmental and developmental cues with the expression of genes have become better understood (194). The focus of the next portion of our study was to further examine how neonatal innate immune responses are regulated by studying endogenous HDAC and HAT activity in cord blood. Currently there are no studies published which have examined endogenous HDAC and HAT activity at birth; therefore our first objective was to measure the activity of these important transcriptional modifiers. Interestingly, we found significantly reduced HDAC activity in neonates compared to children. In contrast, HAT activity was augmented in full term neonates compared with children. In addition, we found

significantly reduced HDAC and HAT activity in preterm neonates compared to those born at term. Impaired gene regulation could contribute to increased susceptibility to infections and sepsis in the neonatal period, especially in neonates born preterm.

Following the measurement of endogenous HDAC and HAT activity in neonates we next wanted to determine what effect modifying the endogenous balance would have on neonatal innate immune responses. To accomplish this we used both a specific HDAC inhibitor, TSA and an environmental compound, cigarette smoke condensate, which also inhibits HDAC activity *ex vivo*. We found that inhibition of endogenous HDAC activity by either TSA or CSC had an inhibitory effect on TLR-induced cytokine production. In addition, HDAC inhibition lead to a reduction in TLR4 mRNA expression following LPS exposure, further supporting the conclusion that endogenous HDAC activity is essential in maintaining adequate TLR-related gene expression. Our results provide a potential mechanism for the results reported by Noakes *et al* who found that infants which were exposed to environmental tobacco smoke throughout development had reduced TLR responses at birth (203).

In the final phase of our studies we sought to develop a broader understanding of neonatal innate immune responses by utilizing proteomic techniques to study global protein profiles in CBMC following TLR stimulation. Proteomics has become a valuable tool in the identification of novel markers involved in human disease (208). Our initial goal was to analyze the protein profiles of several preterm and full term neonates following TLR activation in an

attempt to identify important differences in proteins involved in the innate immune response between these groups. Our study is the first to attempt to define global protein profiles of cultured primary cells from neonates. Although we were able to identify several secreted proteins present in the supernatants tested we had technical challenges which prevented the analysis of the relatively low abundance cytokines and chemokines which were our primary focus. One of our major problems was the high level of contamination of our samples with proteins of intracellular origin. In order to minimize this problem one potential solution is to increase to amount of our proteins of interest by culturing a larger number of cells. In addition, we could centrifuge the supernatants for a longer time period in order to deposit as much of the cellular debris as possible. Based on the large number of proteins identified which are typically associated with the intracellular compartment it is likely that there was some contaminated with cellular material in the samples we tested. If we can increase the sensitivity of our techniques, proteomic analyses have to potential to provide a vast amount of information regarding the important mediators regulating innate immune responses in neonates as well as older individuals.

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APPENDIX A

Table 5. Characteristics of Full Term Neonates

Log Number	Date	Gender	Gestation	Weight	Comments
20	05-Nov-05	F	39	2711	
22	08-Nov-05	M	37	2905	
25	22-Nov-05	F	38	3240	
26	07-Dec-05	F	39	3522	
32	24-Jan-06	M	41	3879	
39	25-Jan-06	F	38	2753	
42	26-Jan-06	M	39	4179	LGA
43	07-Feb-06	F	40	2989	
45	14-Feb-06	M	39	3237	
48	14-Feb-06	F	41	3545	
49	22-Feb-06	F	40	3079	
50	27-Feb-06	F	40	3643	
52	27-Feb-06	M	40	3230	
53	27-Feb-06	F	40	2690	
58	20-Mar-06	M	40	4340	LGA
59	03-Apr-06	Unknown			
60	03-Apr-06	Unknown			
61	03-Apr-06	Unknown			
62	04-Apr-06	F	40	3780	
63	03-Apr-06	F	40	3780	
65	03-Apr-06	F	40	3760	
66	10-Apr-06	F	39	3102	
67	09-Apr-06	F	39	3894	
68	03-May-06	F	39	2810	
69	13-Jun-06	M	40	4065	LGA
71	12-Jun-06	F	40	2670	
72	27-Jun-06	F	40	3925	LGA
74	10-Jul-06	Unknown			
76	18-Jul-06	F	37	2960	
77	18-Jul-06	Unknown			
80	01-Aug-06	M	41	4057	LGA
81	09-Aug-06	F	39	2845	
84	27-Aug-06	Unknown			
85	30-Aug-06	M	39	4330	LGA
86	05-Sep-06	Unknown			
90	05-Sep-06	F	41	3763	
92	06-Sep-06	Unknown			
93	27-Sep-06	F	38	3405	
102	28-Sep-06	F	38	4014	LGA
107	13-Oct-06	F	39	3504	
108	21-Oct-06	F	41	3593	

111	27-Oct-06	F	39	4026	LGA
113	29-Oct-06	M	37	3503	
115	30-Oct-06	M	40	2993	
116	29-Oct-06	F	40	3686	
118	04-Dec-06	M	38	3751	
119	04-Dec-06	M	41	3400	
120	05-Dec-06	M	39	3803	LGA
122	07-Dec-06	M	38	3199	
128	18-Dec-06	F	39	3627	
129	18-Dec-06	M	40	3361	
130	06-Mar-07	F	40	3658	
133	27-Mar-07	M	40	3704	
134	02-May-07	M	40	3313	
135	17-May-07	M	39	3109	
136	17-May-07	F	39	4377	
137	21-May-07	M	39	3664	
138	22-May-07	M	37	2956	
139	20-Jun-07	F	40	3269	
140	26-Jun-07	F	41	3784	
141	28-Jun-07	Unknown			
142	29-Jun-07	Unknown			
146	22-Jul-07	F	38	2760	
147	25-Jul-07	Unknown			
148	14-Aug-07	F	39	3721	
149	15-Aug-07	M	39	3280	
150	28-Aug-07	F	39	4109	LGA
151	28-Aug-07	Unknown			
152	03-Sep-07	F	41	3879	
154	12-Sep-07	Unknown			
155	17-Sep-07	F	39	3722	
156	19-Sep-07	M		3384	
160	27-Sep-07	F		3657	
161	03-Oct-07	M		3793	
162	03-Oct-07	F		3346	
163	17-Oct-07	Unknown			
164	22-Oct-07	F		4507	LGA
165	23-Oct-07	F		3378	
166	25-Oct-07	M		3441	
167	30-Oct-07	F	40	3179	
168	31-Oct-07	F	38	3207	
169	31-Oct-07	M	41	3884	
170	01-Nov-07	M	41	3625	
171	07-Nov-07	Unknown			
172	06-Nov-07	F	40	3911	
173	06-Nov-07	M	38	3544	
176	15-Nov-07	F	41	3652	

177	15-Nov-07	F	38	3197	
178	18-Nov-07	M	41	3709	
179	19-Nov-07	M	40	3213	
180	22-Nov-07	Unknown			
181	22-Nov-07	F	40	3404	
182	26-Nov-07	F	40	3963	LGA
183	26-Nov-07	M	40	3995	LGA
184	27-Nov-07	M	38	3234	
185	27-Nov-07	M	38	3887	
186	09-Dec-07	M	38	3270	
187	11-Dec-07	F	37	3469	
188	11-Dec-07	F	41	3665	
189	17-Dec-07	F	37	2687	
191	06-Jan-08	M	40	3625	
192	11-Jan-08	Unknown			
193	15-Jan-08	F	39	2997	
194	15-Jan-08	F	41	3700	
196	23-Jan-08	M	37	2761	
197	27-Jan-08	F	39	3646	
198	28-Jan-08	M	40	3783	
199	29-Jan-08	F	40	3365	
200	05-Feb-08	F	40	3741	
201	12-Feb-08	F	39	3909	LGA
202	12-Feb-08	M	39	3344	

Table 6. Characteristics of Preterm Neonates

Log #	DOB	Sex	Gestation (weeks)	Weight (g)	Comments*
51	24-Feb-06				
54	28-Feb-06	M	34	2607	
55	07-Mar-06	F	36	3933	LGA
56	08-Mar-06	F	36	2550	
57	18-Mar-06	F	36	2050	SGA
64	04-Jun-06	M	35	2941	
70	06-Apr-06	M	34	1983	
73	07-May-06	F	36	2119	SGA
75	11-Jul-06	M	30	1575	
78	18-Jul-06	F	36	2247	
79	18-Jul-06	F	36	1950	
82	21-Jul-06	M	33	2330	
83	22-Aug-06	F	34	2124	
87	06-Sep-06	M	34	2340	
88	05-Sep-06	M	36	3349	
89	11-Sep-06	M	32	1918	
91	20-Sep-06	F	36	3125	

94	04-Oct-06	M	35	2920	
95	25-Oct-06	M	32	1509	
96	07-Nov-06	M	36	2920	
97	08-Nov-06	F	33	1804	
98	09-Nov-06	M	35	2600	
99	12-Nov-06	F	36	2711	Emerg CS
100	12-Nov-06	M	28	950	Emerg CS
101	13-Nov-06	F	36	3162	
103	14-Nov-06	M	36	2188	Emerg CS/SGA
104	14-Nov-06	M	35	2241	
105	15-Nov-06	M	36	2610	
106	16-Nov-06	M	36	3053	
109	21-Nov-06	M	33	2152	
110	27-Nov-06	M	36	2560	
112	27-Nov-06	F	36	2935	
114	29-Nov-06	M	35	2960	
117	12-Mar-06	F	35	3410	Emerg CS/LGA
121	12-Jun-06	F	36	2905	
123	12-Nov-06	F	36	2640	
124	12-Nov-06	F	35	2418	
125	12-Oct-06	F	35	1984	
126	12-Nov-06	M	29	2024	
127	17-Dec-06	F	34	2485	
131	10-Apr-07	M	35	2855	
132	17-Apr-07	M	34	2425	NICU
143	03-Jul-07	F	35	2665	
144	05-Jul-07	F	34	1345	Emerg CS/SGA
145	10-Jul-07	M	34	2475	
153	13-Sep-07	F	35	3020	
157	23-Sep-07	F	35	2835	
158	23-Sep-07	F	35	2620	
159	23-Sep-07	M	32	1704	
174	07-Nov-07	F	36	2805	
175	15-Nov-07	M	36	2880	
190	12-Dec-07	M	35	2915	
195	16-Jan-08	M	35	2185	
203	14-Mar-08	M	32	3344	

(*Note SGA denotes small for gesational age, $<10^{th}$ percentile and LGA denotes large for gestational age, $>90^{th}$ percentile)

APPENDIX B

Table 7. Proteins identified in supernatants from LPS stimulated CBMC via 2D RP HPLC-ESI MS/MS

log(e)	pI	Mr (kDa)	Description	Location
-322.7	5.58	80.6	Gelsolin precursor, plasma (Actin-depolymerizing factor) (ADF)	Cytoplasm
-223.4	8.72	15.2	ALPHA 2 GLOBIN.	Serum
-162.8	5.07	98.1	HEAT SHOCK PROTEIN HSP 90-ALPHA (HSP 86).	Secreted/Cytoplasm
-146.1	5.47	70	Heat shock 70 kDa protein 1 (HSP70.1) (HSP70-1/HSP70-2).	Secreted/cytoplasm
-73	6.31	102.7	Hexokinase, type I (EC 2.7.1.1) (HK I) (Brain form hexokinase).	Cytoplasm
-61.4	5.27	88.4	INTEGRIN BETA-1 PRECURSOR (FIBRONECTIN RECEPTOR BETA SUBUNIT) (CD29 ANTIGEN)	Cell membrane
-59.8	4.72	32.9	Tropomyosin 1 alpha chain (Alpha-tropomyosin). Source: Uniprot/SWISSPROT P09493	Cytoplasm
-56.6	5.91	41.2	HLA class I histocompatibility antigen, alpha chain H precursor (HLA- AR) (HLA-12.4).	Cell Membrane
-56.3	4.98	101.3	Adapter-related protein complex 1 beta 1 subunit (Beta-adaptin 1)	Cell Membrane
-53.6	5.13	58.8	KERATIN, TYPE I CYTOSKELETAL 10 (CYTOKERATIN 10) (K10) (CK 10).	Cytoplasm
-53.6	5.13	59.5	Keratin, type I cytoskeletal 10	Cytoplasm
-50.5	7.58	46.9	Beta-enolase (EC 4.2.1.11) (2-phospho-D-glycerate hydro-lyase) (Muscle-specific enolase)	Cytoplasm
-41	7.52	35.9	Immunoglobulin heavy chain C gene segment Source: IMGT/GENE_DB IGHG2	Cell membrane
-39.6	9.13	65.8	Tyrosine-protein kinase LYN (EC 2.7.1.112).	Cytoplasm
-38.1	10.4	11.3		
-36.2	5.89	49.2	Heterogeneous nuclear ribonucleoprotein H (hnRNP H).	Nucleus
-33.2	4.95	33.6	HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEINS C1/C2 (HNRNP C1 / HNRNP C2).	Nucleus
-32.9	9.15	23.7	Ras-related protein Rab-8 (Rab-8A) (Oncogene c-mel).	Cell Membrane

-31.4	4.82	10.4	SH3 DOMAIN-BINDING GLUTAMIC ACID-RICH-LIKE PROTEIN 3	Cytoplasm
-29.8	5.19	51	Heterogeneous nuclear ribonucleoprotein K (hnRNP K)	Nucleus
-29.7	6.03	68.5	Radixin.	Nucleus
-27.1	6.74	101.9	Staphylococcal nuclease domain containing 1; EBNA-2 co-activator (100kD)	Cytoplasm
-26.1	6.4	96.6	Glycogen phosphorylase, brain form (EC 2.4.1.1).	Cytoplasm
-25.2	6.17	23.7	Interleukin-6 precursor (IL-6) (B-cell stimulatory factor 2) (BSF-2) (Interferon beta-2)	Secreted
-24.9	8.09	60	KERATIN, TYPE II CYTOSKELETAL 6F (CYTOKERATIN 6F) (CK 6F) (K6F KERATIN).	Cytoplasm
-24.8	5.82	75.6	Arachidonate 12-lipoxygenase, 12S-type (EC 1.13.11.31) (12-LOX)	Serum
-24.7	4.73	86.9	AMYLOID BETA A4 PROTEIN PRECURSOR (APP) (ABPP) (ALZHEIMER-S DISEASE AMYLOID PROTEIN)	Cytoplasm
-24.3	6.44	2207.5	titin isoform novex-3; connectin; CMH9, included; cardiomyopathy, dilated 1G	Cytoplasm
-24	5.49	103.2	Puromycin-sensitive aminopeptidase (EC 3.4.11.-) (PSA).	Cytoplasm
-23.9	8.09	60	KERATIN, TYPE II CYTOSKELETAL 6E (CYTOKERATIN 6E) (CK 6E) (K6E KERATIN).	Cytoplasm
-22.3	5.64	36.1	Apolipoprotein E precursor (Apo-E).	Cell Membrane
-22.2	4.75	10.4	Protein S100-P (S100 calcium-binding protein P).	Cytoplasm
-20	9.32	61.1	heparanase; heparanase-1.	Cytoplasm
-18.9	6.94	33.7	Calponin H2, smooth muscle (Neutral calponin).	Cytoplasm
-18	5.17	126.9	DNA DAMAGE BINDING PROTEIN 1 (DAMAGE-SPECIFIC DNA BINDING PROTEIN 1)	Cytoplasm
-17.9	6.02	71.4	ELONGATION FACTOR 1-DELTA (EF-1-DELTA) (ANTIGEN NY-CO-4).	Nucleus
-17.7	4.91	47.2	Gamma enolase (EC 4.2.1.11) (2-phospho-D-	Cytoplasm

			glycerate hydro-lyase) (Neural enolase) (NSE)	
-17.2	6.11	44.5	Cathepsin D precursor (EC 3.4.23.5)	Cytoplasm
-15.8	5.13	856.8	Bullous pemphigoid antigen 1 isoforms 1/2/3/4/5/8 (230 kDa bullous pemphigoid antigen)	Cytoplasm
-15.7	10.46	11.3	(CXCL1) (Neutrophil-activating protein 3) (NAP-3) (GRO-alpha(1-73))	Secreted
-15.4	9.17	38.7	Heterogeneous nuclear ribonucleoprotein A1	Nucleus
-14.6	7.66	20.5	Dual specificity protein phosphatase 3 (EC 3.1.3.48) (EC 3.1.3.16)	Cytoplasm
-14.1	5.9	42.7	Leukocyte elastase inhibitor (LEI) (Serpin B1) (Monocyte/neutrophil elastase inhibitor)	Cytoplasm
-14.1	6.23	139	Complement factor H precursor (H factor 1).	Serum
-13.4	8.3	80.1	HSP 75 KDA, (TNF Receptor- Associated Protein 1).	Secreted/cytoplasm
-13.1	5.88	25.9	Ribosyldihydronicotinamide dehydrogenase 2) (Quinone reductase 2) (QR2)	Cytoplasm
-12.7	8.74	48.4	UBIQUINOL-CYTOCHROME C REDUCTASE COMPLEX CORE PROTEIN 2	Cytoplasm
-12.5	6.11	33.2	Syntaxin-11. Source: Uniprot/SWISSPROT O75558	Cytoplasm
-12.4	5.36	49.5	nicotinate phosphoribosyltransferase domain containing 1	Cytoplasm
-11.7	6.33	71.9	KININOGEN PRECURSOR (ALPHA-2-THIOL PROTEINASE INHIBITOR)	Cytoplasm
-11.5	7.72	16.4	ubiquitin-conjugating enzyme E2 variant 2	Cytoplasm
-11.5	6.18	43.4	Dual specificity (MAP kinase kinase 1) (MAPKK 1) (ERK activator kinase 1) (MEK1)	Cytoplasm
-11.5	7.95	225.4	CH-TOG protein (Colonic and hepatic tumor over-expressed protein).	Nucleus
-11.2	5.74	99.9	cGMP-specific 3-,5--cyclic phosphodiesterase (EC 3.1.4.17) (CGB-PDE)	Nucleus
-10.8	7.5	31.5	Outer mitochondrial membrane protein porin 2	Cytoplasm
-10.8	5.23	33.2	ALPHA-SOLUBLE NSF ATTACHMENT PROTEIN (SNAP-ALPHA)	Cytoplasm

-10.7	9.23	33	ATP synthase gamma chain, mitochondrial precursor (EC 3.6.3.14).	Cytoplasm
-10.7	9.59	46.4	HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN D-LIKE	Nucleus
-10.1	5.88	23.7	Tumor protein D54 (hD54) (Tumor protein D52-like 2).	cytoplasm
-9.9	8.3	46.3	Acyl-coenzyme A thioesterase 4 (EC 3.1.2.2) (Acyl-CoA thioesterase 4)	Cytoplasm
-9.8	8.25	27.8	Electron transfer flavoprotein beta-subunit (Beta-ETF).	Cytoplasm
-9	5.27	39.4	Glycogenin-1 (EC 2.4.1.186). Source: Uniprot/SWISSPROT P46976	Cytoplasm
-8.9	5.14	158.4	Eukaryotic translation initiation factor 4 gamma 1 (eIF-4-gamma 1) (eIF-4G1) (eIF-4G) (p220).	Cytoplasm
-8.7	5.46	33.4	3-(2-), 5--BISPHOSPHATE NUCLEOTIDASE 1; BISPHOSPHATE 3--NUCLEOTIDASE	Cytoplasm
-8.3	6.19	277.3	Fatty acid synthase (EC 2.3.1.85)	Cytoplasm
-7.5	10.11	10.7	11 kDa protein	Cytoplasm
-6.7	8.8	17.1	Tubulin--tyrosine ligase-like protein 3 (HOTTL)	Cytoplasm
-6.5	11.07	47.7	60S RIBOSOMAL PROTEIN L4 (L1).	Nucleus
-6.3	5.81	37.4	N-acetylglucosamine kinase (EC 2.7.1.59) (GlcNAc kinase).	Cytoplasm
-6	6.97	25	Transmembrane emp24 domain-containing protein 10 precursor	Cell Membrane
-5.7	5.6	88.9	Heterogenous nuclear ribionucleoprotein U(HNRNP U) (SCAFFOLD ATTACHMENT FACTOR A)	Nucleus
-5.6	6.13	96	Programmed cell death 6-interacting protein (Hp95).	Cytoplasm
-5.5	5.32	91.6	Vacuolar protein sorting 35 (Vesicle protein sorting 35) (hVPS35) (Maternal-embryonic 3).	Cytoplasm
-5.2	5.34	17.5	ATP synthase delta chain, mitochondrial precursor (EC 3.6.3.14).	Cytoplasm
-5.1	5.4	88.1	Protein-tyrosine phosphatase, non-receptor type 12 (	Cytoplasm

-4.8	8.34	38.3	BETA-2-GLYCOPROTEIN I PRECURSOR (APOLIPOPROTEIN H)	Cell Membrane
-4.7	8.79	26.6	C-type lectin domain family 1, member B	Cell Membrane
-4.6	4.73	20.5	Ras-related protein Rap-2b.	Cell Membrane
-4.6	10.48	26.2	Transmembrane protein 109 precursor (Mitsugumin-23) (Mg23).	Cell Membrane
-4.6	5.76	19	Translocon-associated protein delta subunit precursor (TRAP-delta)	Cytoplasm
-4.4	5.41	59.7	T-COMPLEX PROTEIN 1, THETA SUBUNIT (TCP-1-THETA) (CCT-THETA).	Cytoplasm
-4.3	9.61	71.7	Splicing factor 1 (Zinc finger protein 162) (Transcription factor ZFM1) (	Cytoplasm
-4.1	5.38	318.9	Lipopolysaccharide-responsive and beige-like anchor protein (CDC4-like protein)	Cytoplasm
-4.1	5.47	102.4	Rho guanine nucleotide exchange factor 1 (p115-RhoGEF) (p115RhoGEF) (	Nucleus
-4	7.02	43.9	VACUOLAR ATP SYNTHASE SUBUNIT C (EC 3.6.3.14) (V-ATPASE C SUBUNIT)	Cytoplasm
-4	5.82	106.8	alpha glucosidase II alpha subunit.	Cytoplasm
-3.9	6.4	110.6	platelet endothelial aggregation receptor 1	Cell Membrane
-3.9	7.66	26.9	3-HYDROXYACYL-COA DEHYDROGENASE TYPE II (EC 1.1.1.35) (TYPE II HADH)	Cytoplasm
-3.8	5.3	35.6	Serine/threonine protein phosphatase 2A, catalytic subunit, alpha isoform (EC 3.1.3.16)	Cytoplasm
-3.8	9.03	29.2	Antithrombin-III precursor (ATIII).	Serum
-3.7	4.91	23	RAS-RELATED PROTEIN RAB-30.	Cell Membrane
-3.5	9.07	13.7	CYTOCHROME C OXIDASE POLYPEPTIDE VB, MITOCHONDRIAL	Cytoplasm
-3.4	6.44	59	Nonspecific lipid-transfer protein (EC 2.3.1.176)	Cell membrane
-3.4	9.13	35.4	Retinol dehydrogenase 11 (EC 1.1.1.-)	Cytoplasm
-3.4	7.33	74.6	Acyl-coenzyme A oxidase 1, peroxisomal (EC 1.3.3.6) (Palmitoyl-CoA oxidase) (AOX).	Cytoplasm
-3.4	5.71	102.3	Hexokinase, type II (EC 2.7.1.1) (HK II) (Muscle form hexokinase).	Cytoplasm
-3.3	5.18	41.3	Hsc70-interacting protein (Hip) (Putative	Cytoplasm

			tumor suppressor ST13)	
-3.3	5.86	47.7	N-acylglucosamine 2-epimerase (EC 5.1.3.8) (GlcNAc 2-epimerase)	Cytoplasm
-3.3	7.48	71.1	Thioredoxin reductase 1, cytoplasmic precursor (EC 1.8.1.9) (TR) (TR1).	Cytoplasm
-3.3	7.58	80.8	GLYCEROL-3-PHOSPHATE DEHYDROGENASE, MITOCHONDRIAL PRECURSOR (EC 1.1.99.5)	Cytoplasm
-3.2	7.08	47.3	Flotillin-1. Source: Uniprot/SWISSPROT O75955	Cell Membrane
-3.2	5.84	37.2	protein phosphatase 1, catalytic subunit, beta isoform 1; protein phosphatase 1,	Cytoplasm
-3.2	5.76	124.5	MYELOBLAST KIAA0223 (FRAGMENT).	Cytoplasm
-3.2	5.77	22.9	Plasma retinol-binding protein precursor (PRBP) (RBP) .	Serum
-3	5.94	17.3	Prefoldin subunit 5 (C-myc binding protein Mm-1) (Myc modulator 1).	Cytoplasm
-3	5.44	69.2	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 63 kDa subunit precursor	Cytoplasm
-2.9	4.69	20.2	Coatomer zeta-1 subunit (Zeta-1 coat protein) (Zeta-1 COP) (CGI-120) (HSPC181).	Cytoplasm
-2.9	4.97	34.5	COATOMER EPSILON SUBUNIT (EPSILON-COAT PROTEIN) (EPSILON-COP).	Cytoplasm
-2.9	6.6	37.4	GMP reductase 1 (EC 1.7.1.7) (Guanosine 5--monophosphate oxidoreductase 1)	Cytoplasm
-2.9	6.37	39.5	PTK9L PROTEIN TYROSINE KINASE 9-LIKE (A6-RELATED PROTEIN); PROTEIN TYROSINE KINASE 9-LIKE	Cytoplasm
-2.8	4.89	23.3	Synaptosomal-associated protein 23 (SNAP-23) (Vesicle-membrane fusion protein SNAP-23).	Cell Membrane
-2.8	6.96	107.5	Adapter-related protein complex 2 alpha 1 subunit (Alpha-adaptin A)	Cell Membrane
-2.8	5.64	111.6	Ubiquitin-activating enzyme E1 homolog (D8).	Cytoplasm
-2.8	5.77	40.8	Uroporphyrinogen decarboxylase (EC 4.1.1.37) (URO-D) (UPD).	RBC
-2.7	4.51	108.4	Reticulon 4 (Neurite outgrowth inhibitor)	Cell Membrane

			(Nogo protein) (Foocen)	
-2.7	8.55	29.6	Ubiquinol-cytochrome C reductase iron-sulfur subunit, mitochondrial precursor	Cytoplasm
-2.7	6.49	42.9	Cytoplasmic protein NCK2 (NCK adaptor protein 2) (SH2/SH3 adaptor protein NCK-beta)	Cytoplasm
-2.7	7.09	59.5	Histidine-rich glycoprotein precursor (Histidine-proline rich glycoprotein) (HPRG).	Secreted
-2.6	6.08	83.8	MITOCHONDRIAL INNER MEMBRANE PROTEIN (MITOFILIN) (P87/89).	cytoplasm
-2.5	4.97	49.5	cytokine receptor-like factor 3; cytokine receptor-like molecule 9	cell membrane
-2.5	6.08	47.4	26S proteasome non-ATPase regulatory subunit 11 (26S proteasome regulatory subunit S9)	Cytoplasm
-2.5	5.18	42.9	Interleukin enhancer-binding factor 2 (Nuclear factor of activated T- cells 45 kDa).	Nucleus
-2.5	5.51	18.8	Neudesin precursor (Neuron-derived neurotrophic factor)	Secreted
-2.5	8.3	47.4	Inhibin beta A chain precursor (Activin beta-A chain) (Erythroid differentiation protein) (EDF).	Secreted
-2.5	6.34	19.3	Uncharacterized protein C20orf27.	Unknown
-2.4	4.93	94.2	epidermal growth factor receptor pathway substrate 15-like 1	Cell Membrane
-2.4	4.87	24.3	ADP-sugar pyrophosphatase (EC 3.6.1.13) (EC 3.6.1.-)	Cytoplasm
-2.4	6.01	126.5	FH1/FH2 domains-containing protein	Cytoplasm
-2.3	5.93	123	Ankyrin repeat and SAM domain-containing protein 1 (Odin).	Cell Membrane
-2.3	11.64	19.3	Splicing factor, arginine/serine-rich 3 (Pre-mRNA-splicing factor SRP20).	Cytoplasm
-2.3	5.38	27	Glycoprotein NB1 precursor.	Cytoplasm
-2.3	4.89	92.4	Eukaryotic translation initiation factor 3 subunit 9 (eIF-3 eta)	Cytoplasm
-2.2	9.64	46.1	Gamma-interferon-inducible protein Ifi-16 (IFI 16).	Nucleus
-2.1	4.98	38.4	UNR-interacting protein (WD-40 repeat protein PT-WD) (MAP activator with WD	Cell Membrane

			repeats).	
-2.1	4.81	25	Eukaryotic translation initiation factor 3 subunit 11 (eIF-3 p25) (eIF3k)	Cytoplasm
-2.1	5.89	79.4	NADH-UBIQUINONE OXIDOREDUCTASE 75 KDA SUBUNIT, MITOCHONDRIAL PRECURSOR	Cytoplasm
-2.1	5.49	15.9	TRANSTHYRETIN PRECURSOR (PREALBUMIN) (TBPA) (TTR) (ATTR).	Serum
-2	6.34	83.9	endo-beta-N-acetylglucosaminidase	Cytoplasm
-2	5.92	133.9	MOKA isoform a.	Nucleus
-1.9	9.7	10.9	ATP synthase f chain, mitochondrial (EC 3.6.3.14).	Cytoplasm
-1.8	5.01	40.3	Leukosialin precursor (Leucocyte sialoglycoprotein) (Sialophorin) (CD43 antigen)	Cell Membrane
-1.8	9.24	47.1	Probable saccharopine dehydrogenase (EC 1.5.1.9). Source: Uniprot/SWISSPROT Q8NBX0	Cytoplasm
-1.7	9.65	37.6	stearoyl-CoA desaturase 4	Cytoplasm
-1.7	6.48	39.6	inorganic pyrophosphatase 2 isoform 2	Cytoplasm
-1.7	6.13	43.8	PROLIFERATION-ASSOCIATED PROTEIN 2G4 (CELL CYCLE PROTEIN P38-2G4 HOMOLOG) (HG4-1).	Cytoplasm
-1.7	9.74	58.6	regulator of G-protein signalling like 2	Cytoplasm
-1.7	6.46	123.7	Potassium voltage-gated channel subfamily H member 8	Cytoplasm
-1.7	9.62	65.5	Nuclear receptor coactivator 5 (NCoA-5) (Coactivator independent of AF-2) (CIA).	Nucleus
-1.6	8.8	16.9	OTTHUMP00000030191. Source: Uniprot/SPTREMBL Q9NTT1	Cytoplasm
-1.6	8.68	28.3	GLYCOSYLTRANSFERASE-LIKE 1B; ORTHOLOG OF MOUSE GLYCOSYLTRANSFERASE-LIKE 1B.	Cytoplasm
-1.6	4.79	34	Small glutamine-rich tetratricopeptide repeat-containing protein A (Vpu-binding protein) .	Cytoplasm
-1.6	9.07	53.2	CGI-04 protein	cytoplasm
-1.6	5.18	127.9	voltage-gated calcium channel alpha(2)delta-4 subunit.	Cytoplasm

-1.6	6.64	315.2	Polycystic kidney disease 1-like 1 protein (Polycystin 1L1).	Cytoplasm
-1.6	11.42	39.6	Splicing factor, arginine/serine-rich 6 (Pre-mRNA splicing factor SRP55).	Nucleus
-1.6	4.96	48.2	Transcobalamin I precursor (TCI) (TC I).	Secreted (neutrophil granules)
-1.5	5.28	38.1	CD5 antigen-like precursor (SP-alpha) (CT-2) (IgM-associated peptide).	Cell Membrane
-1.5	8.54	53.5	TNF receptor associated factor 4	Cell membrane
-1.5	10.32	24.2	40S ribosomal protein S8.	Cytoplasm
-1.5	9.53	32.6	Endonuclease G, mitochondrial precursor (EC 3.1.30.-) (Endo G)	Cytoplasm
-1.5	7.78	33.8	SULFATASE MODIFYING FACTOR 2 PRECURSOR (C-ALPHA-FORMYGLYCINE- GENERATING ENZYME 2)	Cytoplasm
-1.5	8.84	40.6	CAMP-DEPENDENT PROTEIN KINASE, ALPHA-CATALYTIC SUBUNIT (EC 2.7.1.37) (PKA C-ALPHA).	Cytoplasm
-1.5	5.92	47.7	ADENOSYLHOMOCYSTEINASE (EC 3.3.1.1) (S-ADENOSYL-L-HOMOCYSTEINE HYDROLASE)	Cytoplasm
-1.5	4.63	9.7	Small nuclear ribonucleoprotein F (snRNP-F) (Sm protein F) (Sm-F) (SmF).	Nucleus
-1.5	8.71	43.1	42 kDa protein	Nucleus
-1.5	6.31	56.1	Transcription factor SOX-9.	Nucleus
-1.5	6.08	151.8	APOPTOTIC CHROMATIN CONDENSATION INDUCER IN THE NUCLEUS (ACINUS).	Nucleus
-1.4	8.83	131.5	Insulin receptor substrate-1 (IRS-1).	Cell Membrane
-1.4	6.18	37.7	Aldose 1-epimerase (EC 5.1.3.3) (Galactose mutarotase) (BLOCK25 protein).	Cytoplasm
-1.4	8.24	70.5	Potassium voltage-gated channel subfamily D member 2 (Potassium channel Kv4.2).	Cytoplasm
-1.4	5.73	128.2	Ankyrin repeats- and FYVE domain-containing protein 1	Cytoplasm
-1.4	6.03	50.2	RUVB-LIKE 1 (EC 3.6.1.-) (49-KDA TATA BOX-BINDING PROTEIN-INTERACTING PROTEIN)	Nucleus

-1.4	5.6	23.7	Neuroendocrine protein 7B2 precursor (Secretory granule endocrine protein I)	Secreted
-1.3	5.09	24.9	Ras-related protein Rab-27A (Rab-27) (GTP-binding protein Ram).	Cell Membrane
-1.3	7.81	27.3	Transmembrane emp24 domain-containing protein 9 precursor (Glycoprotein 25L2).	Cell Membrane
-1.3	4.79	346	Protocadherin 16 precursor (Cadherin-19) (Fibroblast cadherin 1).	Cell Membrane
-1.3	10	28.1	mitochondrial ribosomal protein S7; 30S ribosomal protein S7 homolog.	Cytoplasm
-1.3	6.41	31.5	Phosphatidylinositol transfer protein beta isoform (PtdIns transfer protein beta)	Cytoplasm
-1.3	5.15	34.5	Nucleotide-binding protein 1 (NBP 1).	Cytoplasm
-1.3	9.29	45.5	Synaptotagmin-7 (Synaptotagmin VII) (SytVII).	Cytoplasm
-1.3	8.26	73.8	KH-TYPE SPLICING REGULATORY PROTEIN (FUSE BINDING PROTEIN 2)	Cytoplasm
-1.3	6.45	138.5	Ubiquitin ligase protein DZIP3 (EC 6.3.2.-) (DAZ-interacting protein 3)	Cytoplasm
-1.3	5.75	144.7	Flightless-I protein homolog.	Cytoplasm
-1.3	8.05	88.7	PREDICTED: KIAA0953 protein	
-1.2	5.1	129.2	Integrin alpha-2 precursor (Platelet membrane glycoprotein Ia) (GPIa) , (CD49b).	Cell Membrane
-1.2	5.18	227.4		Cell Membrane
-1.2	8.69	9.1	40S RIBOSOMAL PROTEIN S21.	Cytoplasm
-1.2	6.43	39.8	MO25 protein (CGI-66).	Cytoplasm
-1.2	5.04	40.8	NSFL1 cofactor p47 (p97 cofactor p47). Source: Uniprot/SWISSPROT Q9UNZ2	Cytoplasm
-1.2	9.78	43.6	HIV-1 rev binding protein 2; Rev interacting protein.	Cytoplasm
-1.2	7.09	44.1	26S protease regulatory subunit S10B (Proteasome subunit p42)	Cytoplasm
-1.2	4.99	49.8	Ca(2+)/calmodulin-dependent protein kinase phosphatase	Cytoplasm
-1.2	8.71	97.4	Dystroglycan precursor (Dystrophin-associated glycoprotein 1)	Cytoplasm
-1.2	4.64	156.8	G protein-coupled receptor associated sorting	Cytoplasm

			protein 1	
-1.2	8.91	243.2	Myosin IXb (Unconventional myosin-9b).	Cytoplasm
-1.2	5.18	465.8	Cyclic AMP-dependent transcription factor ATF-6 beta	Nucleus
-1.2	4.8	20.4	MSFL2541	Secreted
-1.1	5.67	86.8	Integrin beta-7 precursor.	Cell membrane
-1.1	5.37	26.5	Glutathione S-transferase Mu 3 (EC 2.5.1.18) (GSTM3-3) (GST class-mu 3) (hGSTM3-3).	Cytoplasm
-1.1	6.1	30	Bisphosphoglycerate mutase (EC 5.4.2.4) (2,3-bisphosphoglycerate mutase, erythrocyte)	Cytoplasm
-1.1	5.21	40.9	Deoxyhypusine synthase (EC 2.5.1.46) (DHS).	Cytoplasm
-1.1	5.83	48.2	Farnesyl pyrophosphate synthetase (FPP synthetase)	Cytoplasm
-1.1	8.51	123	ubiquitin protein ligase E3B isoform a.	Cytoplasm
-1.1	8.68	25.2	cardiotrophin-like cytokine; neurotrophin-1/B-cell stimulating factor-3.	Secreted