MATERNAL ATOPY, HEMATOPOIETIC PROGENITORS AND IMMUNE RESPONSES IN EARLY LIFE

INFLUENCE OF MATERNAL ATOPY AND INNATE AND ADAPTIVE IMMUNE STIMULI ON CORD BLOOD HEMATOPOIETIC PROGENITOR CELLS

By

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Abstract

The recent and dramatic rise in allergic disease, coupled with the manifestation of the disease within the first years of life, suggests that *in utero* events are likely critically important to the inception of allergy. Epidemiological and experimental evidence suggest that both genetic predisposition and prenatal environmental exposures (e.g., *in utero* microbial exposures) play a role in modulating neonatal immunity and subsequent development of allergy. Of relevance to the work in this thesis, reports suggest that bacterial agents can directly alter myelopoiesis and, in connection to allergy, we have previously shown that cord blood (CB) progenitors from high-atopic risk infants demonstrate altered hematopoietic responses. However, whether CB progenitor cell hematopoietic responses are directly altered by microbial stimulation, and what effect maternal atopy has on these responses are unclear. Therefore, this thesis examines the influences of bacterial lipopolysaccharide (LPS) stimulation (innate immunity), maternal atopy, and adaptive immune stimuli (representative of an atopic milieu) on CB progenitor cell eosinophilopoiesis. We show that CB progenitors from healthy, pregnant women respond to LPS through increased eosinophil-basophil (Eo/B) colony forming units (CFU) via the mitogen-activated protein kinase (MAPK) signalling pathway (Chapter 2), whereas the presence of maternal atopy (as defined by skin prick test positivity) is associated with reduced CB $CD34^+$ cell LPS-induced Eo/B CFU formation (Chapter 3). To investigate the potential mechanism of reduced eosinophilopoiesis in high-atopic risk infants, CB progenitors stimulated with IL-4 (a surrogate *ex vivo* for maternal atopy), but

not IL-13, demonstrate reduced LPS-induced MAPK activation and Eo/B CFU formation (Chapter 4). This novel work provides insight into mechanisms relating to the influence of maternal atopy and/or potential intrauterine exposures (e.g., prenatal cytokines) on the responsiveness of CB progenitor cells to LPS, which may be of key importance for the development of atopic illnesses. These observations may help in the generation of novel biomarkers and therapeutic targets for childhood atopy.

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List of Abbreviations

AHR	Airway hyperresponsiveness
ARI	Acute respiratory infection
AP-1	Activated protein- 1
BAL	Bronchoavelolar lavage
βc	Beta-chain
BM	Bone marrow
BcL	B cell lymphoma
BHR	Bronchial airway hyperresponsiveness
BSA	Bovine serum albumin
CB	Cord blood
CEBPα	CCAAT/ enhancer binding protein alpha
CFU	Colony forming unit
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CpG	Cytosine phosphate guanine
DC	Dendritic cell

DMSO	Dimethyl sulfoxide
ECP	Eosinophil cationic protein
Eo/B	Eosinophil/basophil
EDN	Eosinophil-derived neurotoxin
EDTA	Ethylenediaminetetraacetic acid
EPO	Eosinophil peroxidase
ERK	Extracellular signal regulated kinase
FACS	Fluorescence activated cell sorter
FCS	Fetal calf serum
FBS	Fetal bovine serum
FcεRI	Fc epsilon Receptor I
FEV1	Forced expiratory volume in 1 second
FOXP3	Forkhead box P3
GM-CSF	Granulocyte-macrophage colony stimulating factor
GMP	Granulocyte and macrophage progenitor
HSC	Hematopoietic stem cell

Ig	Immunoglobulin
IL	Interleukin
IFN	Interferon
IRAK-M	Interleukin receptor associated kinase M
IRAK-4	Interleukin receptor associated kinase 4
JAK	Janus Kinase
JNK	c-Jun N terminal kinase
LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
LRR	Leucine rich repeats
LTC4	Cysteinyl leukotriene C4
LTD4	Cysteinyl leukotriene D4
LTE4	Cysteinyl leukotriene E4
LTB4	Leukotriene B4
MAPK	Mitogen activated protein kinase
MBP	Major basic protein

MD-2	Myeloid differentiation factor-2
MEP	Megakaryocyte and erythrocyte progenitor
MKK	MAPK kinase kinase
MMP	Multipotent progenitor
MNC	Mononuclear cell
mRNA	messenger RNA
MyD88	Myeloid differentiation primary response protein 88
NAMNC	Nonadherent mononuclear cell
NF-κB	Nuclear factor kappa beta
NK	Natural killer
NOD	Nucleotide-binding oligomerization domain
NLR	NOD-like receptor
OVA	Ovalbumin
PAMP	Pathogen associated molecular pattern
PB	Peripheral blood
PBS	Phosphate buffered saline

PGD2	Prostaglandin D2
PI3-K	Phosphoinositide 3-kinase
PPV	Positive predictive value
PRR	Pathogen recognition receptor
PUFA	Polyunsaturated fatty acid
Rα	Receptor alpha
RLR	RIG-I-Like Receptor
RPMI	Roswell Park Memorial Institute
RSV	Respiratory syncytial virus
sCD14	soluble CD14
SCF	Stem cell factor
SDF-1	Stromal derived factor-1
sMFI	Specific median fluorescence intensity
SNP	Single nucleotide polymorphism
SOCS	Suppressor of cytokine signalling
SPT	Skin prick test

STAT	Signal transducer and activator of transcription
TARC	Thymus and activation-regulated chemokine
TGFβ	Transforming growth factor beta
T_H	T helper
TIR	Toll/Interleukin-1 receptor
TNF	Tumour necrosis factor
TLR	Toll-like receptor
T_{Reg}	T regulatory cell
TRIF	TIR-domain containing adaptor inducing interferon β
TSLP	Thymic stromal lymphopoietin

CHAPTER 1

Introduction

The prevalence of atopic diseases such as allergic asthma, eczema and rhinoconjunctivitis has increased dramatically in recent decades, resulting in a substantial increase in morbidity, mortality and socio-economic burden [1]. Epidemiological studies repeatedly show that allergy is a complex disease involving interactions of genes and environmental factors. The early onset of allergic disease in childhood [2,3] suggests that *in utero* events could play a key role. In support of this, pre-symptomatic differences in neonatal toll-like receptor (TLR) immunity have been identified in infants destined to become allergic [4,5]; however these studies have focused mainly on cord blood (CB) monocytes and T cells. In this connection, we have previously shown that maternal atopy [6] and gestational environmental exposures [7] influence other hematopoietic cells in the CB, such as eosinophil/basophil (Eo/B) progenitor cells. With increased interest in the role of prenatal TLRs/microbial exposure in the development of allergy [8,9], and the fact that TLRs alter bone marrow (BM) progenitor cell hematopoiesis [10-12], the purpose of this thesis was therefore to extend our understanding of how CB progenitor hematopoietic responses are influenced by both maternal atopy and innate and adaptive immune stimuli. The underlying hypothesis was that *maternal atopy influences neonatal progenitor cell phenotype and functional responses.* The rest of this chapter will be divided into sections which introduce the main themes of this thesis: allergic inflammation and eosinophils, mechanisms of hematopoiesis and progenitor cells, and finally microbes (TLRs) and infant allergy.

1.1 Allergy and allergic inflammation

Atopic diseases such as allergic asthma, allergic rhinitis and atopic dermatitis (AD) are common inflammatory disorders influenced by genetic and environmental factors. The term atopy describes a genetic predisposition to form IgE antibodies upon exposure to common airborne, food and other antigens in the environment, whereas allergy is the hypersensitivity reaction and ensuing inflammation mediated by these IgE antibodies, involving the cross-linking of high-affinity IgE receptors on mast cells after antibody binding to specific allergen. An atopic individual is “sensitized” when ‘primed’ to respond to the allergen, via circulating or mast cell-bound, allergen-specific IgE. Once a person is sensitized to a specific allergen, subsequent exposures lead to allergic inflammatory responses, which can be divided into two phases: the immediate or early-phase response (occurs within minutes of allergen exposure) and the late-phase response (occurs within hours of exposure) [13].

Continuous exposure to allergen results in increasing numbers of IgE-coated mast cells which transverse the epithelium. Cross-linking of IgE bound to high-affinity IgE receptor (FcεRI), as a result of antibody binding to specific allergen, initiates the immediate secretion of various granule-associated preformed mediators (such as tryptase, chymase and histamine) as well as newly-synthesized mediators such as arachidonic acid metabolites (including PGD2, LTB4, LTC4) [14]. These cells also produce many cytokines, chemokines and growth factors (such as IL-1, -3, -4, -5, -6, -8, GM-CSF, TNFα and TGFβ) [14,15], some of which, as preformed mediators, play a key role in the edema and vasodilation which predominately characterizes the early-phase reaction [16]

as well as the subsequent recruitment of leukocytes into the inflammatory site, thereby maintaining the allergic response [17].

The late-phase occurs approximately 4-8 hours after the early-phase [13] and is characterized by the recruitment and activation of effector cells such as eosinophils, basophils, neutrophils, T lymphocytes and macrophages [18]. Once these cells are recruited to the inflammatory site, $CD4^+$ T_H2 cells release IL-4, -5, -9 and -13, which both augment and sustain the inflammatory response [19] through the promotion of IgE production, eosinophil chemoattraction and survival, as well as mast cell recruitment [13]. Recruited eosinophils release pro-inflammatory and cytotoxic mediators such as cytoplasmic granular mediators (e.g., major basic protein (MBP) and eosinophil cationic protein (ECP)) and lipid mediators (e.g., leukotriene C4 (LTC4)) [20,21], which contribute to chest symptoms (wheeze, cough, tightness, dyspnea) characteristic of asthma through smooth muscle contraction and mucus production; these events lead, in the airways, to bronchoconstriction and airway hyperresponsiveness (AHR) [17]. Both T_H2 cytokines and eosinophils have prominent roles in this allergic inflammatory response in various tissues of the body, such as the airways.

1.1.1 T_H2 cytokines and allergic responses

Although the recruitment of inflammatory cells, such as eosinophils, basophils and lymphocytes found at the site of allergic inflammation, is important, the presence and production of T_H2-type cytokines, such as IL-4, -5, -9 and -13 [19] have been shown to be key mediators in both the early and late phases of allergic inflammation. Although these cytokines are predominately secreted by $CD4^+$ T_H2 cells, a number of different cells

involved in the allergic response, including eosinophils and mast cells [19], secrete them as well. Genes for these cytokines are found in a cluster on the human chromosome 5q31 [22], and play important roles in the development of atopy and AHR [23].

An essential biological activity of IL-4 in the development of allergic inflammation is to drive the differentiation of T_H2 cells and for optimal IgE class switching in B cells [16]. In relation to eosinophil development, studies of IL-4 have shown divergent results. While IL-4 has been shown to potentiate the development of eosinophils and basophils from CB cells stimulated with IL-3 *in vitro* [24] in one study, another study showed that IL-4 reduced IL-3- or IL-5-mediated eosinophil differentiation *in vitro* [25]. IL-5 has been recognized as the major maturation and differentiation factor for eosinophils [20], and is responsible for BM mobilization and recruitment of eosinophils to the site of inflammation [26]. Additionally, the expression of IL-5 mRNA in bronchial biopsies of asthmatic patients is increased, the predominate source of which is the $CD4^+$ T cell [16]. IL-13 plays a key role in the allergic response via its actions on epithelial and smooth muscle cells [16], thereby contributing significantly to mucus secretion, AHR and tissue remodelling [27]. Studies have shown that both IL-13 mRNA and protein are elevated in bronchial biopsy specimens and bronchoaveolar lavage (BAL) cells from allergic individuals [28,29]. IL-13 has also been shown to increase eosinophilopoiesis of CB progenitor cells stimulated with IL-5 or GM-CSF [30]. IL-9 has been described to have profound effects on both inflammatory and structural cells [19], much like IL-13. IL-9 increases the numbers of mast cells and T_H2 cells in the lung, and increases airway inflammation, mucus production and AHR [19,23,31]. The expression of

IL-9 and its receptor is increased in allergic asthma [32,33], coincident with increased airway eosinophilia and IgE production in murine models of asthma [34]. Also, *in vitro* evidence shows that IL-9 prolongs eosinophil survival, as well as IL-5-mediated differentiation and maturation [35], which suggests that IL-9 may potentiate eosinophil function *in vivo* [36].

Finally, it is important to note the recent and novel roles for epithelial-derived cytokines like IL-25, -33 and thymic stromal lymphopoietin (TSLP), which have emerged as key influences on allergic responses. These cytokines promote the development of T_H2 cell responses by acting on basophils and dendritic cells [37], as well as exacerbating eosinophilia in murine models of asthma [38]. Additionally, not only is the expression of IL-33, IL-25 and TSLP [16,39] increased in the airways of asthmatics, but these cytokines have been recently shown to activate gut-derived immune cell production of T_H2 (IL-5 and -13) responses [39]. The cells involved are referred to as 'nuocytes' or multipotent progenitor (MPP) cells [40], and respond to these epithelial-derived cytokines by augmenting mucus production and eosinophilia or the differentiation of T_H2 cells, respectively [39]. Table I provides a summary of T_H2 and T_H2-inducing cytokines in allergic responses.

Although the above T_H2 (and associated) cytokines are all implicated in allergic inflammation, IL-4 and IL-13 are critical since they play a key role in the development of allergic sensitization (atopy). With respect to neonatal studies-- and therefore of direct relevance to this thesis--these cytokines have been shown to influence CB-derived eosinophil differentiation. Interestingly, CB cells have been shown to respond to IL-4 and

IL-13 differently with respect to eosinophil differentiation. Whereas IL-4 added to CB mononuclear cells results in the inhibition of IL-3- and IL-5-mediated eosinophil differentiation *in vitro* [25], the addition of IL-13 enhances IL-5- and GM-CSF-mediated eosinophil differentiation in mice [30]. Therefore, the effects of the prototypical T_H2 cytokines IL-4 and IL-13 were chosen for studies described below investigating hematopoietic responses of CB progenitor cells to adaptive immune stimuli.

1.1.2 Eosinophils in allergy

Eosinophils are multifunctional leukocytes which are strongly implicated in inflammatory processes, including helminth infections and allergic diseases [20,21]. A role for eosinophils in the immunopathogenesis of asthma was shown in studies using eosinophil-deficient mice that develop markedly attenuated disease [41]. In fact, a pivotal role for IL-5 in eosinophilic inflammation was confirmed by using neutralizing antibodies to IL-5 which inhibited eosinophilic infiltration to the airways in allergen-challenged mice [16]. Furthermore, the overexpression both of IL-5 and eotaxin in the lungs of mice produces pulmonary pathology markedly similar to that observed in moderate to severe asthma, including epithelial desquamation and mucus production, leading to airway obstruction, airway smooth muscle hyperplasia and methacholine-induced AHR [41].

Eosinophils, when released from the BM, are in circulation for approximately (10-12 hours). Tissue eosinophils can survive up to 2 weeks [20]. In response to stimuli such as allergen exposure, circulating eosinophils are recruited to sites of inflammation. This trafficking process is coordinated by a wide array of T_H2- and endothelial-derived cytokines (e.g., IL-4, IL-5 and IL-13), chemokines (e.g., eotaxins and RANTES) and

adhesion molecules (e.g., β1, -2 and -7 integrins) [20,21]. Mobilization of eosinophils from the BM is regulated by IL-5, whereas egress from the circulation to the tissue is controlled by eotaxin. Additionally, the T_H2 cytokines IL-4 and IL-13 are important in the transmigration of eosinophils from the vascular bed into the tissue [42] through interactions between eosinophil expressed very late antigen-4 (β1 integrin) and endothelial expression of vascular cell adhesion molecule-1 [20].

Once inside the inflamed tissue, eosinophils can contribute to allergic manifestations through the release of granular proteins and pro-inflammatory mediators. For example, granular proteins increase vascular permeability and stimulate mucus production, resulting in tissue edema and airway obstruction [42]. Granular proteins produced by eosinophils such as MBP, ECP and eosinophil peroxidise (EPO) are toxic to the respiratory epithelium [18,43]. Furthermore, MBP-mediated mast cell degranulation causes the release of leukotrienes and histamine, which result in airway constriction and obstruction [44]. Additionally, pro-inflammatory lipid mediators such as leukotriene B4 (LTB4), the cysteinyl leukotrienes (LTC4, LTD4, and LTE4) and prostaglandin (PG) D2 regulate eosinophil accumulation and migration [45-48]. Eosinophils can also secrete an array of preformed and de novo synthesized cytokines, chemokines and growth factors (e.g., IL-4, -5, -8, -13, GM-CSF, SCF and TGF β, eotaxin and RANTES) [20,42] which in turn promote airway inflammation [49,50]. These factors not only support the recruitment of other eosinophils and leukocytes to the site of inflammation, but also provide cellular survival signals, which augment the inflammatory response.

Table I. T_H2 cytokines and T_H2-inducing cytokines in allergic responses.

Cytokine	Major Source	Major Target	Functions
IL-4	$CD4^+T_H2$ cells, Basophils, Mast cells	$CD4^+T_H2$ cells, B cells, Basophils	Differentiation of naïve $CD4^+$cells into T_H2 cells, IgE isotype switching, inducing T_H2 cytokine production
IL-5	$CD4^+T_H2$ cells, Eosinophils	Eosinophils, Basophils, $CD34^+$ cells	Eosinophil differentiation, survival, activation, migration (from BM)
IL-9	$CD4^+T_H2$ cells, T_H9 cells, Eosinophils, Mast cells	Mast cells, Epithelium, smooth muscle	Growth and differentiation of mast cells, enhancing IL-5-mediated eosinophilia, globet cell hyperplasia, AHR, mucus production
IL-13	$CD4^+T_H2$ cells	Smooth muscle, Epithelium	globet cell hyperplasia, AHR, mucus production, enhancing IL-5-mediated eosinophilia
IL-25	Epithelium $CD4^+T_H2$ cells, Mast cells, Eosinophils, Basophils	$CD4^+T_H2$ cells, Basophils, MMP, Nuoctyes, DCs	Basophil activation, enhancing eosinophilia and IgE secretion, induction of T_H2 responses
IL-33	Epithelium, airway smooth muscle, DCs	$CD4^+T_H2$ cells, Basophils, Nuoctyes, DCs	Basophil activation, enhancing eosinophilia and IgE secretion, induction of T_H2 responses
TSLP	Epithelium, Basophils and Mast cells	$CD4^+T_H2$ cells, Basophils, MMP, Nucotyes, DCs	Basophil and DC activation, induction of T_H2 responses (including $CD34^+$ cell IL-5/IL-13 production)

TSLP- thymic stromal lymphopoietin, DC- dendritic cell, T_H- T helper, AHR- airway hyperresponsivness, IgE- immunoglobulin E, IL- interleukin, BM- bone marrow

1.2 Hematopoiesis

The majority of hematopoietic activity takes place in the BM, under the control of resident stromal cells as well as T lymphocytes, monocytes and their products [42]. An important marker of hematopoietic progenitors is the CD34 antigen, an integral glycoprotein, whose density is greatest on primitive progenitor cells and decreases as the cell matures [51]. Hematopoietic progenitor cells are derived from the hematopoietic stem cell (HSC), which is defined by its capacity to self-renew as well as to differentiate into all the lineages (e.g., myeloid, lymphoid, erythroid etc) of the hematopoietic system. Figure 1 shows a diagram of the hematopoietic tree [52]. The generation of mature cells from pluripotent $CD34^{+}$ progenitor cells involves commitment to a specific cell lineage, followed by terminal differentiation [53]. Although it is proposed that cell fate decisions are the result of either stochastic events or induced by environmental cues mediated through receptor-ligand interactions, it is likely that a combination of both stochastic gene expression, and instructive soluble factors imposing lineage commitment [54], operates to accommodate host requirements for hematopoietic cells.

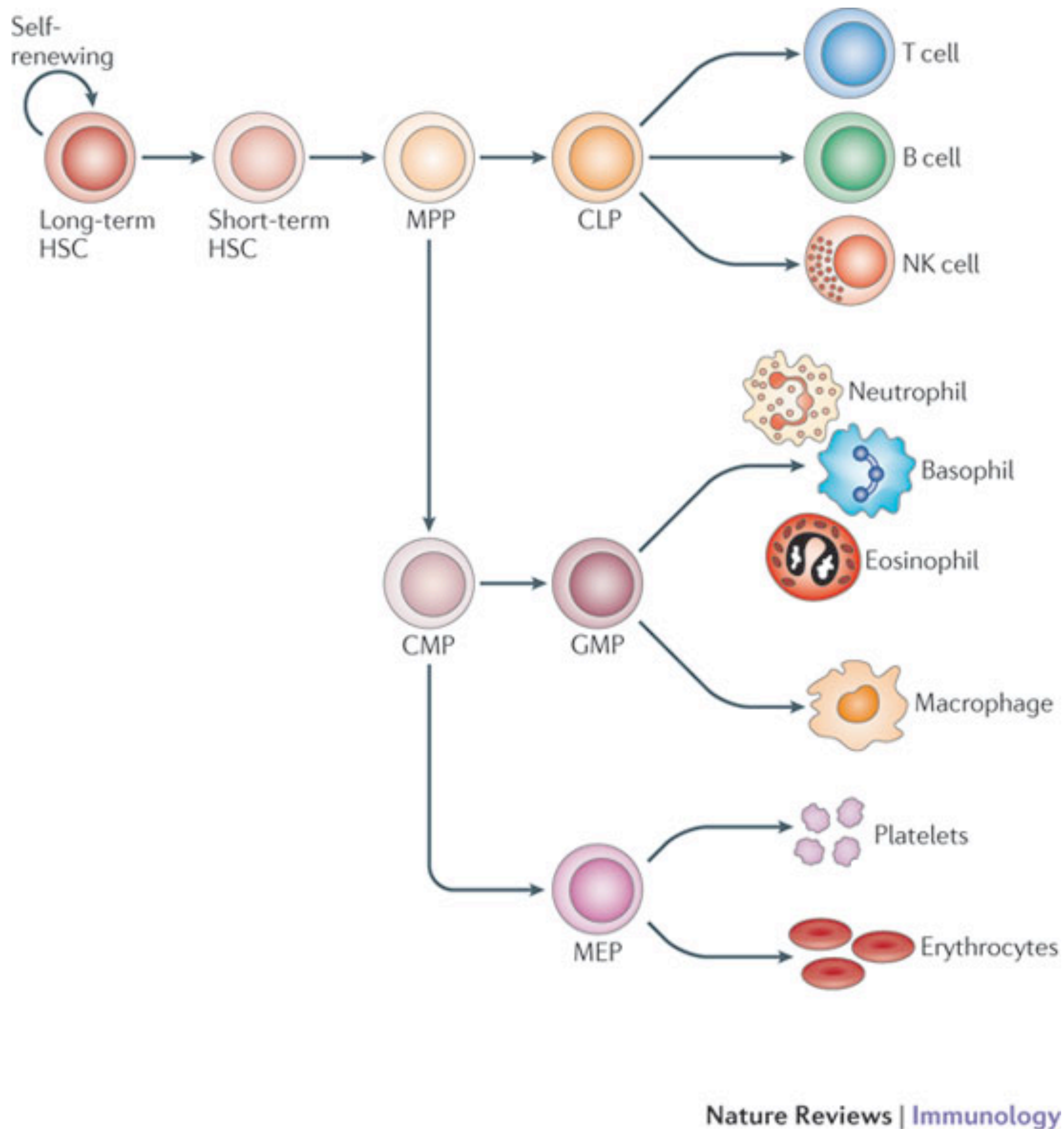

Figure 1. The hematopoietic tree.

Long term hematopoietic stem cells (HSCs) have limitless capacity to self-renew and also give rise to all the cell types of the bone marrow and peripheral blood. Other pluripotent progenitors, short-term HSCs and multipotent progenitors (MPPs) have less self-renewal capacity. MPPs are thought to differentiate into the two main branches of hematopoietic development that arise from the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP). Mature peripheral blood cells, shown on the right, are derived from these progenitors. GMP, granulocyte and macrophage progenitor; MEP, megakaryocyte and erythrocyte progenitor; NK, natural killer Adapted from [52].

1.2.1 Toll like receptors (TLRs)

Toll-like receptors (TLRs) have been recognised as prototypical innate microbial sensors and immune receptors which respond to pathogen-associated molecular patterns (PAMPs) such as endotoxin [55]. In humans, ten TLR family members have been identified [56] which recognize a wide array of pathogens (Table II [55]). TLRs are structurally similar transmembrane pathogen recognition receptors (PRRs) with large leucine rich (LRR) extracellular domains and cytoplasmic domains that are similar to the IL-1 receptor, referred to as the Toll/IL-1 receptor (TIR) domain [57]. TLR activation results in myeloid differentiation primary response protein 88 (MyD88)-dependent or -independent (in the case of TLR-3) signalling, which initiates the sequential activation of intracellular signals, ultimately involving nuclear factor kappa B (NF-κB)- and mitogen-activated protein kinase (MAPK)-dependent transcription of pro-inflammatory gene expression [58]. Alternatively, TLR-3 signalling activates the TIR-domain containing adaptor inducing interferon β (TRIF) to activate NF-κB [58].

Table II. Human Pathogen-recognition Receptors.

	Recognizes	Expressed by	Functions
Toll-like receptors			
TLR-1	Bacterial lipoproteins, lipotechoic acid	Many immune and epithelial cell types	Cell activation, dimerizes with TLR-2
TLR-2	Bacterial lipoproteins, peptidoglycan, lipotechoic acid, lipoarabinomannan, fungal zymosan	Many immune and epithelial cell types	Cell activation, dimerizes with TLR-1 or TLR-6
TLR-3	Viral double-stranded RNA	Many immune and epithelial cell types, cell activation	
TLR-4	Bacterial endotoxin, respiratory syncytial virus coat proteins	Many immune and epithelial cell types, cell activation	
TLR-5	Bacterial flagellin	Many immune and epithelial cell types, cell activation	
TLR-6	Bacterial lipoproteins, lipotechoic acid	Many immune and epithelial cell types, cell activation	Cell activation, dimerizes with TLR-2
TLR-7	Viral single-stranded RNA	Many immune and epithelial cell types, cell activation	
TLR-8	Viral single-stranded RNA	Many immune and epithelial cell types, cell activation	
TLR-9	Bacterial/viral unmethylated CpG DNA	Many immune and epithelial cell types, cell activation	
TLR-10	Unknown	Many immune and epithelial cell types, cell activation	
MD-2	Bacterial endotoxin	Many immune and epithelial cell types	TLR-4 coreceptor
CD14	Bacterial endotoxin	Many immune and epithelial cell types	TLR-4 enhancer
LPS binding protein	Bacterial endotoxin	Liver	TLR-4 enhancer

Adapted from [55] with permission.

1.2.2TLRs in hematopoiesis

With regard to factors that influence the decisions of stem cells to differentiate, recent studies have revealed that HSC can directly sense pathogens, which can "push" the HSC to differentiate into a lineage committed cell [52,59]. In fact, stimulation of HSCs by microbial ligands may affect the timing and manner in which HSCs differentiate, as well as the progeny they subsequently produce. Studies have shown that not only do HSCs express TLRs but receptor engagement can exert a direct effect on HSC activation and hematopoietic potential [10-12,60].

Infection poses a natural stress on the hematopoietic system, since immune cells are 'consumed' in the fight against invading pathogens. Homeostasis, therefore, requires

ongoing replenishment of immune effector cells through stimulation and differentiation of hematopoietic progenitors [52]. Studies demonstrating that sepsis is associated with both neutropenia [61], and peripheral and BM progenitor cell mobilization and differentiation into neutrophils [62], suggest that HSCs have the ability to respond to pathogens and/or inflammatory environments. Hematopoietic progenitors can be 'pushed' to differentiate into cells which both replenish the immune system and combat the invading pathogen.

Murine models were first utilised to investigate this phenomenon [60]. Nagai *et al*., [60] showed that murine HSCs express TLR-2 and -4 and respond to receptor ligation, since *in vitro* stimulation of HSCs resulted in their proliferation and the ability of granulocyte macrophage progenitor (GMPs) to differentiate into myeloid committed cells. Yanez *et al*., [63] similarly pointed out this phenomenon following *Candida albicans* exposure. Human studies by Souid *et al*.,[10,11] and De Luca *et al*., [12] have likewise shown that BM-derived HSCs express TLRs and are 'pushed' to myeloid differentiation upon stimulation *in vitro*; these findings have recently been recapitulated *in vivo* [64]. Together, these studies point to an important role for TLR signalling in hematopoietic progenitor cell lineage commitment; HSCs can directly 'sense' microbial danger signals and subsequently proliferate to produce a supply of effector cells to 'confront' the invading pathogen [52,59].

1.2.3 Eosinophil development

Eosinophils are produced in the BM from $CD34^+$ progenitor cells under the influence of granulocyte macrophage-colony stimulating factor (GM-CSF), IL-3 and IL-5 [65]; these cytokines are commonly found in allergic tissue [66]. IL-3 and GM-CSF are

early-acting cytokines in eosinophil lineage commitment; depending on the cytokine microenvironment, progenitor cells exposed to IL-3 or GM-CSF could also differentiate into other myeloid cells (e.g., neutrophils). Conversely, IL-5 effects appear mostly specific to the eosinophil lineage, leading to the terminal differentiation of eosinophil lineage committed progenitor cells [41,67]. The critical role for IL-5 in the production of eosinophils was best demonstrated by genetic manipulation of mice: overexpression of the gene for IL-5 results in profound eosinophilia [68], whereas deletion causes marked reduction of eosinophils in the blood and lungs after allergen challenge [69]. IL-5 is also involved in the growth, survival and functional activity of eosinophils [41], as well as in stimulating their release from the BM [70]. The three cytokines (IL-3, GM-CSF, and IL-5), which utilise a common β-subunit [71], are responsible for providing both proliferative and differentiation signals through the activation of signal transduction pathways and lineage-specific transcription factors (e.g., GATA-1).

1.2.4 Signalling pathways in eosinophil differentiation

The receptors for IL-3, IL-5 and GM-CSF signalling are a part of the gp140 family. These receptors have a unique ligand-binding α chains and share the common β (gp140) signal transducing subunit [71]. Important signalling pathways that these cytokines activate include the Janus kinase (JAK)/ Signal transducer and activator of transcription (STAT) and MAPK, which have been shown to be crucially involved in eosinophil differentiation from human CB progenitor cells *in vitro* [72-74].

There are four members of the JAK family, JAK 1, JAK2, JAK 3 and TYK2 [71], of which JAK2 is involved in β receptor signalling [75]. STAT proteins are a family of cytoplasmic transcription factors, activated by a variety of stimuli, including growth factors and cytokines, and participate in diverse processes such as differentiation [75]. There are 7 members of the STAT family (STAT1-4, STAT5A and 5B and STAT6) [75]. When the α and β subunits of IL-3, IL-5 or GM-CSF are not engaged, the JAK protein is complexed with the β receptor in a catalytically inactive latent state [71]. Receptor dimerization, due to ligand binding, results in the activation (phosphorylation) of receptor associated JAKs. Activation of this kinase leads to direct tyrosine phosphorylation of STAT proteins followed by STAT dimerization. Homo- or hetero-dimerized STAT proteins then translocate to the nucleus where they bind to specific promoter regions and induce gene transcription [75]. Although the precise role in the differentiation process remains to be clarified, STAT5 may be responsible for promoting the survival of myeloid progenitors via transcriptional upregulation of anti-apoptotic proteins Bcl-X_L, thereby allowing myeloid differentiation to occur [71,75].

The MAPKs extracellular regulated signal kinase (ERK) 1/2 and p38 MAPK have also been shown to be activated by eosinophilopoietic cytokines IL-3, GM-CSF and IL-5 [76]. Upon cytokine binding, the adaptor molecule Shc is rapidly phosphorylated and associated with the β chain. Recruitment of Shc leads to interaction with the adaptor protein Grb2, which then associates with mSos, a nucleotide exchange factor for Ras. The activation of Ras leads to the activation of upstream kinases such as MAPK kinase (MKK) 3/MKK6 which activates p38 MAPK [77] or MEK1 which activates ERK 1/2

[78]. These cumulative signals result in the activation of a number of transcription factors, including AP-1 [77] and c-fos [78].

1.2.5 Transcription factors involved in eosinophil differentiation

Eosinophil lineage commitment is controlled by at least three classes of transcription factors, including GATA-1 (a zinc finger family member), PU.1 (an Ets family member) and c/EBP members (CCAAT/enhancer-binding protein family) [79]. GATA-1 is clearly the most important factor for eosinophil lineage specification, since targeted deletion of high-affinity GATA-binding sites in the GATA-1 promoter results in complete loss of eosinophil development [80] and expression of GATA-1 in myeloid progenitor cells exclusively promotes development and terminal maturation of eosinophils [81]. Additionally, GATA sites are found in the regulatory regions of eosinophil-specific genes, including the eotaxin receptor CCR3, MBP and the IL-5Rα genes [20], all of which are important to the development and functions of eosinophils in allergic responses [42]. Although our laboratory has previously evaluated the kinetics of GATA-1 mRNA expression in CB progenitor cells during eosinophilopoiesis [82], an examination of this transcription factor was outside the scope of study of this thesis.

1.2.6 Tissue in situ hematopoiesis

It was previously thought that the differentiation and maturation of hematopoietic progenitors was restricted to the BM. With particular importance to this thesis, accumulating evidence suggests that eosinophil progenitors present in allergic tissue may

contribute to the process of allergic inflammation by giving rise to mature eosinophils *in situ*, having trafficked as lineage-committed, but not fully differentiated, cells through the peripheral circulation to the allergic tissue. Due to rich combination of cytokines present at the site of inflammation, these newly recruited committed progenitor cells can differentiate into potent effector cells (e.g., eosinophils, basophils), thereby producing an ongoing supply of effectors and mediators which can perpetuate the inflammatory response. This process is referred to as "in situ hematopoiesis" [83,84].

Evidence for the importance of this process came originally from the observation that allergic rhinitics during the pollen season had reduced progenitor cells in circulation compared to before the pollen season, and that through the season these progenitors kinetically decreased, then rebounded back to baseline (high) levels following the season [85,86]; this suggested that progenitor cells had trafficked into tissues in response to allergen. In support of this, it has been demonstrated that not only does nasal polyp tissue contain $CD34^+$ cells that can differentiate into Eo/B CFU *ex vivo* [87], but that there are increased $CD34^+$ cells and eosinophils bearing the CD34 antigen in the nasal mucosa during the pollen season [88]. Additionally, Robinson *et al*., [89] demonstrated that atopic asthmatics have increased $CD34^+$/IL-$5Ra^+$ cells within the bronchial mucosa compared to normals. Local differentiation was demonstrated by the finding that *ex-vivo* incubation of nasal biopsy tissue from allergic rhinitics with allergen or IL-5 resulted in a decrease in $CD34^{+/}$IL-$5R\alpha^+$ cells and an increase in mature eosinophil MBP^+ cells [90]. Relevant to this thesis, it has been recently demonstrated that freshly isolated CB $CD34^+$ progenitor cells respond to IL-33 and TSLP by releasing copious amounts of inflammatory T_H2

cytokines such as IL-5, IL-13 and GM-CSF, as well as the chemokines IL-8 and CCL17 [91,92], providing further support that the allergic inflammatory microenvironment can activate $CD34^+$ cells to directly contribute to the ongoing inflammation. Therefore, in addition to differentiating into effector cells of allergic inflammation (i.e., *in situ* production of Eo/B), $CD34^+$ cells may thus themselves exert potent inflammatory activity, which is enhanced by IL-33 in synergy with other locally produced cytokines from the epithelium (such as TSLP) [93]. Indeed, $CD34^+$ cells producing IL-5 and IL-13 were detected in the sputum of allergic asthmatics and these numbers increased 24 and 48 hours following allergen challenge [91]. Additional support for progenitor cell effector function was provided by the fact that allergen itself can directly activate BM $CD34^+$ progenitors cells to secrete IL-5, thus contributing to their own differentiation via autocrine regulation [94] in a murine model of asthma. These observations and others, which will be presented in later sections (1.3-1.3.1), provide the basis for this thesis project investigating hematopoietic progenitor mechanisms relevant to the development of infant allergic disease.

1.3 Systemic processes of allergy: circulating and BM-derived progenitor cells

Accumulation of eosinophils and basophils in the tissues of allergic patients is a characteristic of the inflammatory response observed in allergic rhinitis, nasal polyposis and asthma [95]. In fact, allergen inhalation by sensitized individuals results in increased airway eosinophils and basophils [96,97], which are derived from progenitor cells from the BM and blood. These tissue inflammatory events are coincident with relevant changes and fluctuations of circulating and BM populations of Eo/B progenitors [98].

The clinical relevance of Eo/B progenitor cells in allergic responses has been evaluated using methylcellulose cultures to grow Eo/B colony forming units (CFU), as well as through enumeration of $CD34^+$ populations using flow cytometry. The earliest studies demonstrated that $CD34^+$ cells (Eo/B progenitors) are indeed increased in the PB of patients with allergic rhinitis, nasal polyposis or asthma, compared to nonallergic controls [85,99,100]. Allergen-induced late asthmatic responses and airway inflammation is associated with increased Eo/B CFU in circulation [97,98,101] and BM [102,103]. Similar increases in Eo/B CFU are observed in the BM of ovalbumin (OVA) sensitized and challenged mice [104], which is coincident with increased numbers of airway eosinophils. These results are consistent with the hypothesis that eosinophils and basophils accumulate at the sites of allergic inflammation, at least in part by the direct recruitment of circulating and BM progenitors [95].

Through the use of flow cytometry, $CD34^+$ cells as well as $CD34^+$/IL-5Rα and $CD34^+$/$CCR3^+$ cells are present and inducible in the BM, circulation and in induced sputum samples from allergic asthmatics; especially those that go on to develop the late-phase response to allergen [100,102,103,105-107]. These findings have been confirmed and extended in murine models of asthma; 24 hours after OVA challenge showed increased BAL eosinophils and BM-derived $CD34^+$/IL-5R $mRNA^+$ cells [108]. Furthermore, in terms of progenitor cell mobilization, $CD34^+$/$CCR3^+$ cells have increased migration towards eotaxin *in vitro* [107,109], as well as a reciprocal decrease in expression of stromal derived factor-1 (SDF-1) receptor CXCR4 on BM $CD34^+$ cells [109]; this latter receptor is responsible for hematopoietic progenitor cell retention within

the BM compartment [42]. Therefore, data from human and murine studies suggest that an increase in both IL-5Rα and CCR3 on $CD34^{+}$ cells may favour their mobilization to the site of allergic inflammation [107,109], and their differentiation into eosinophils, which subsequently contribute to blood and tissue eosinophilia [105] in response to allergen.

The involvement of progenitor cells in the systemic process of allergy is further demonstrated by influence of therapies targeted to reduce the symptoms of allergic disease. Inhaled corticosteroids are considered the gold standard for treating allergic diseases. Withdrawal of inhaled corticosteroids results in increased Eo/B progenitor cells in the blood of mild asthmatics [110], with restoration of progenitor numbers back to baseline. Additionally, Gauvreau *et al.*, [111] demonstrated that inhaled corticosteroids suppress PB Eo/B CFU in patients with asthma. These results suggest, at least in part, that inhaled corticosteroids act systemically to control asthma responses through the suppression of hematopoietic signals [42]. Contrastingly, Wood *et al.*, [106] showed that inhaled low-dose corticosteroids attenuated both allergen-induced responses, as well as blood and airway eosinophilia, with little effect on BM Eo/B progenitors [106]. However, since the baseline numbers of BM Eo/B progenitors were reduced after the low-dose treatment, it may be that the dose and duration used in the study was insufficient to reduce allergen-induced progenitor cells numbers [42]. Additional studies with nasal polyp tissue have demonstrated that intranasal corticosteroid therapy results in the local increase in $CD34^{+}$ mononuclear cells, that is associated with a reduction of eosinophils

[95], suggesting that corticosteroids cause the accumulation of CD34$^+$ cells by blocking eosinophil differentiation [42].

Although not a corticosteroid, anti-IL-5 therapy (mepolizumab) was found to have similar inhibitory effects in the BM. Mepolizumab treatment significantly reduced terminal differentiation of eosinophils within the BM, without an overall effect on the numbers of early eosinophil progenitors (CD34$^+$/IL-5Rα or Eo/B CFUs), suggesting it is involved in the maturational arrest of BM eosinophil differentiation [112]. These studies clearly demonstrate that events in the airways are related to events occurring the BM. Taken together, allergic inflammation, may result in part, from airway signalling to the BM, release of progenitor cells which traffic to the site of inflammation and their ability to differentiate in situ thus providing an ongoing supply of effector cells that augment the inflammatory response [42].

1.3.1 Cord blood (CB) progenitor cells

The above mechanisms, delineated in adults with allergic disease, led our group to postulate that umbilical CB progenitor cells may likewise be involved in the development of allergic disease in infancy and early childhood. The working hypothesis is that the phenotype or functional responsiveness of these eosinophil progenitors is altered in infants at-risk for atopy and/or those destined to ultimately develop atopic disorders; subtle changes in cord blood hematopoietic progenitors may provide biomarkers of disease development in later life [95]. We have demonstrated both phenotypic and functional differences in CB CD34$^+$ progenitors between children at low- and high-risk

for atopy, as determined by parental history and/or maternal allergy skin prick test (SPT) positivity. The first observations on this by Upham *et al*., [6] compared the expression of hematopoietic cytokine receptors on CD34$^+$ progenitor cells in CB in at-risk infants (by history only). Interestingly, the expression of GM-CSF receptor on CD34$^+$ cells was reduced in the at-risk infants, showing an association between infant risk for atopy and phenotypic changes on progenitor cells, which may not only be related to *in utero* events, but also supports the notion that the allergic phenotype may begin to evolve in the prenatal period [65].

Diet during pregnancy and in early life may have profound effects on the developing immune system [95]. Maternal consumption of omega-3 fatty acids, such as polyunsaturated fatty acid (PUFA), has been associated with reduced development of allergy early life [113]. In a randomized, placebo-controlled trial, n-3 PUFA supplementation at 20 weeks gestation influenced the characteristics of CB progenitor populations in infants at high-risk for allergy [7]. Allergy was defined as doctor-diagnosed maternal allergic rhinitis and/or asthma and one or more positive SPT to common allergens (including house dust mite, grass, mould and cat) [7]. This study showed that PUFA supplementation significantly affected CD34$^+$ progenitor populations in CB, leading to both increased numbers of undifferentiated CD34$^+$ cells and IL-5-responsive Eo/B CFU *ex vivo*. Not only were functional differences in the CD34$^+$ populations observed, but IL-5-responsive Eo/B CFU also positively predicted AD and wheeze at the first year of life [7]. Additional studies demonstrated PUFA supplementation was associated with decreased CB T cell cytokine responses to allergen

(compared to placebo), and that these children were less likely to have positive SPT to egg [113]. Thus, environmental exposures during pregnancy can have immunomodulatory effects at birth and influence clinical outcomes into infancy as reflected by changes in CB progenitor cell phenotype and function [95].

Post-natal environmental responses to infections in early life may be reflected by alterations in CB of at-risk infants [114]. A study by Fernandes *et al*., [114] has shown that at-risk infants (defined by 1 parent having medical diagnosed eczema, hayfever or asthma and a positive SPT) develop fever and wheeze after respiratory syncytial virus (RSV) infections, related to increased $CD34^+$ cells expressing GM-CSF and IL-3 receptors and increased GM-CSF and IL-3 responsive Eo/B CFU [114]. Therefore, altered CB progenitor cells may serve as harbingers of severity of acute respiratory infections (ARI) in at-risk infants [114]. Collectively, these studies suggest that maternal atopy, dietary exposures as well as early postnatal environmental exposures to infectious agents can be associated with altered CB progenitor responses, which may ultimately influence the development of allergic disease.

1.4 Hygiene Hypothesis and the "farm effect"

The prevalence of allergic diseases such as asthma, allergic rhinitis and AD is higher in the developed world. It has been proposed that the variance in development of allergies is dependent on environmental exposure. One of the earliest observations of increased prevalence of allergy in affluent communities was found by studying white and Metis communities in Saskatchewan [115]. Gerrard *et al*., found that the Metis

communities had lower levels of asthma and eczema, which was associated with untreated viral and bacterial diseases, as compared to their white counterparts [115]. A study in 1989 by Strachan [116] demonstrated that family size and the position of birth were related to the expression of atopic disease [116]. This study lead to the postulation of the "Hygiene Hypothesis" suggesting a correlation between early life microbial exposure and the development of allergy. The initial interpretation was that exposure to infections during early life or prenatally transmitted through unhygienic contact with older siblings, or prenatally through maternal interactions with these siblings, drives the maturation of the neonatal immune system towards T_H1 phenotype and away from the T_H2 phenotype (which is associated with allergic disease) at birth [116]. Although this interpretation presents a very attractive hypothesis that is well supported by farming studies [117-119] (see below), there are a few caveats [120]. One of these is the fact that Bach *et al*., [121] showed that although there is a decreased amount of infectious disease in the developed world (due primarily to increased vaccination), there is increased prevalence of both T_H1- (e.g., Crohn's disease) and T_H2-mediated diseases (e.g., asthma). If an increase in T_H2-mediated diseases was thought to be the reason for increased incidence of allergy, there would be expected to be a reciprocal decrease in T_H1-mediated diseases. Another shortcoming of the Hygiene Hypothesis is the fact that individuals with helminth infections, in endemic areas, typically develop increased T_H2 responses (e.g., T_H2 cytokines, IgE and eosinophilia), but do not have increased rates of allergic sensitization or allergic manifestations [122]. These observations suggest therefore that other mechanisms, possibly involving innate immune activation (and not only adaptive

T_H immunity) may be operative in the development of allergies early life, warranting further investigation.

Numerous epidemiological studies have shown that children who are raised on farms are protected from asthma, allergic rhinitis and allergic sensitization [118,119,123,124]. Comparison of children from different rural Bavarian regions has shown that the most pronounced allergy protective effects of the farming environment are present at sites having livestock in the stables adjacent to living quarters [118,124]. In fact, early life contact (e.g., pregnancy) with livestock [8,125] and their fodder (grass, corn and grain), and the consumption of unpasteurized milk [125] have been identified as the most effective protective exposures [117,118,126]. Studies of the immunobiology of this phenomenon point to activation and modulation of innate and adaptive immune responses by intense microbial exposures before or soon after birth. This has been termed the “farm effect”, with the strongest effect observed for microbial exposures that occur *in utero* and during the first years of life. Therefore, maternal exposure to farm animals might represent a model of natural immunotherapy [119]. Taken together, all these observations suggest that the farming environment may influence the developing immune system leading to reduced risk of the development of allergies in the offspring. A “window of opportunity” may thus exist which involves exposure to protective environmental factors early in life, presumably *in utero*. Figure 2 illustrates the potential influences of farming environments on neonatal development in relation to allergy [119].

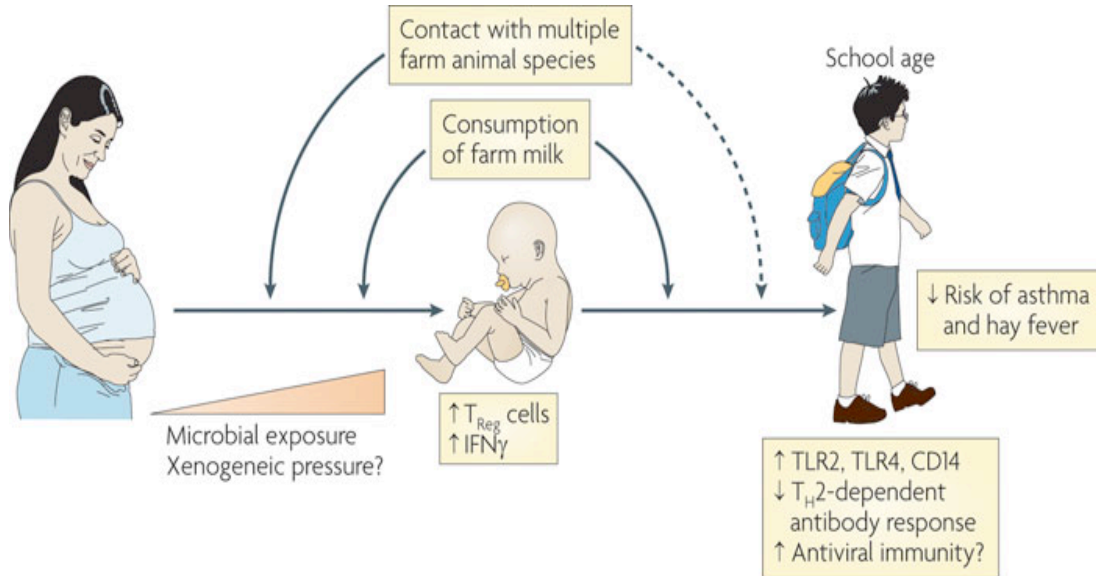

Figure 2. A working model for immunobiology of farm exposure.

Contact with multiple animal species, combined with consumption of farm milk, results in strong microbial exposure of women who carry out farming duties during pregnancy. These combined exposures occurring in early life result in increased T regulatory (T_{Reg}) cell function and interferon- γ (IFNγ) production at birth, which in turn enhance innate immune responses (through increased expression of pattern-recognition receptors), and dampen T helper 2 (T_H2) cell-dependent allergic inflammation in early childhood. Exposure to animals and farm milk in early life reinforces the protective effects of prenatal exposures. The ability to produce high levels of IFNγ at birth may also ensure effective responses to respiratory viral infections in early life, thereby counteracting the contribution of these infections to increased asthma susceptibility. TLR, Toll-like receptor. Figure adapted from [119].

1.4.1 Maternal influences on neonatal immunity

As allergic diseases start to develop early in life, it has been proposed that genetic predisposition as well as multiple prenatal factors (e.g., *in utero* microenvironment, microbial exposures, smoking) influence *in utero* programming of the fetal immune system and, therefore, susceptibility to allergy. Since the mother provides the first environment for the developing fetus, maternal responses to environmental stimuli may exert direct immunologic effects. This is likely to occur through the variations in cytokine milieu at the materno-fetal interface and trafficking of maternal antibodies, cytokines or cells across the placenta [127]. Although animal models may not adequately reflect human responses, it should be noted that transfer of maternal allergen-specific IgG through the placenta or breast milk mediates tolerance and protection for the development of allergy in murine offspring [128,129]. In connection to the above, it has been shown that that a substantial number of maternal cells can cross the placenta and reside in fetal lymph nodes, inducing the development of T regulatory cells (T_{Reg}) that suppress fetal reactivity of maternal antigens. This antigen specific tolerance, which is induced *in utero,* can persist until early adulthood and may be active in regulating immune responses after birth [130].

Maternal allergy is a well recognized risk factor for the development of infant allergic disease, and also has significant effects on neonatal immune function [131,132]. In fact, a number of maternal environmental factors, including diet [113], smoking [133], and microbial exposure [8], in early life can modify neonatal immune responses; notably, these are the same culprits identified as potential immune modifiers in epidemiological

studies of allergic disease [132]. These findings have fuelled extensive investigations into the differences in neonatal immune function in CB of infants deemed at high- or low-risk of allergic development. Longitudinal studies have been completed which have repeatedly shown that immaturity of T_H1 function is associated with the development of infant allergy [134,135]. However, there is also emerging evidence to show that allergic disease may also be associated with differences in neonatal innate immunity (e.g., TLR function in CB) [4,131,136]. Therefore, since differences in immune function are in evidence at birth in newborns that develop allergic disease [132], there is increased interest in the prenatal factors that may alter neonatal immunity, with particular interest in the role of early life microbial exposures [119,132,137] and the development of allergy.

1.4.2 Early life exposure to microbes and allergic development

With direct relevance to the Hygiene Hypothesis, investigations into the role of maternal microbial exposures on neonatal development of allergy have been conducted. Indeed, a number of animal studies demonstrate the importance of *in utero* (maternal) exposure to microbial products that prevent allergic outcomes in the progeny [138-141]. In humans, a study from New Zealand provides further evidence that *in utero* exposure to farming environment (a surrogate for high microbial exposure) protects against the development of childhood eczema, asthma and allergic rhinitis; this was found to be independent of postnatal exposure [126]. This is consistent with previous studies in German farming environments where prenatal exposure to high microbial burden has been associated with altered expression of innate immune genes and reduced risk of allergic disease in the progeny [8]. Mechanistically, prenatal exposure to farming

environments has been associated with production of IFN γ and TNF α [125] and increased production and function of T_{Reg} cells [137] in CB. Additionally, maternal farm exposure during pregnancy is associated with decreased IgE in CB [8], presumably through T_H1 polarization of the neonate [137,142]. Although murine models have quite elegantly shown that prenatal microbial exposure results in protection of allergic development in the offspring [138-141] through epigenetic modification of the IFNγ promoter [141], human intervention studies using microbial products are mainly limited to probiotics. Although they have been shown to reduce the risk of eczema, it is not clear whether this effect is the result of prenatal or postnatal supplementation (or both) [127]. Collectively, these studies suggest that not only is the timing of microbial exposure crucial to the protection against the development of allergy, but that pregnancy and early life may represent a biological ‘window of opportunity’ for shaping subsequent immune responses [119].

Epidemiologic studies of microbial exposure and allergy have generally revealed a picture that is consistent with a preventive influence of microbial PAMP exposure in early life. Endotoxin concentrations are highest in stables from farmer families, but also farmer households contain higher levels of endotoxin than non-farmer households [118]. Interestingly, the levels of endotoxin [123] and muramic acid (peptidoglycan) [143] in children’s mattresses are inversely related to the occurrence of allergic rhinitis, atopic asthma and atopic sensitization. Higher house dust levels of endotoxin are associated with lack of allergic sensitization in infants and higher proportions of T_H1 cells in the PB

[144], and also with less AD [145], allergic sensitization and asthma [123,146] in infancy and childhood.

1.5. Endotoxin (Lipopolysaccharide) signalling

TLR-4 was the first human TLR identified; this receptor binds to bacterial endotoxin, a lipopolysaccharide (LPS) PAMP that makes up most of the outer layer of the cell wall of gram-negative bacteria [55]. TLR-4 specifically recognizes the lipid A moiety (LPS) of endotoxin, which is highly conserved and imparts most of endotoxin's biological activities [147,148]. Responsiveness to LPS can be attributed as well to TLR-4 related enhancer proteins, LPS binding protein (LBP), CD14, and the TLR-4 recognition co-factor, myeloid differentiation factor (MD-2). LBP is a soluble protein which binds LPS and shuttles it to TLR-4 [149]. CD14 is also a soluble protein as well as cell surface receptor that binds LPS and improves TLR-4 sensitivity and detection of endotoxin [150]. MD-2 is an extracellular adaptor protein that is constitutively associated with TLR-4 and is required for TLR-4 recognition of endotoxin [151]. TLR-4 signalling has been shown to activate a number of signalling pathways in human monocytes [152], including p38 MAPK, ERK 1/2 and c-jun N terminal kinase (JNK). The activation of these protein kinases results in the activation of number of different transcription factors which contribute to cytokine gene transcription [152]. Figure 3 is a schematic of the signalling pathways activated by LPS [152]. In connection to allergy, TLR-2, -4 and -9 have been widely investigated [153]; given its particular involvement in myeloid differentiation [60], and impaired signalling associated with infant allergic-risk [154,155], TLR-4 has been the focus of the thesis.

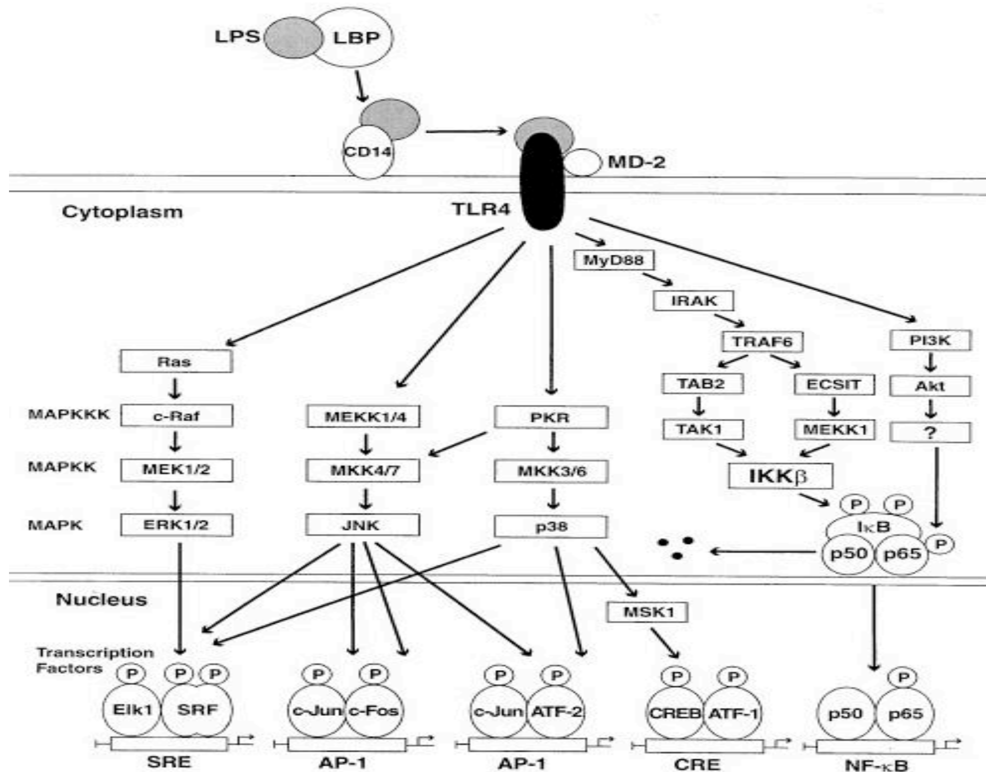

Figure 3. LPS stimulation of monocytes activates signalling pathways and transcription factors.

LPS binds to the serum protein LBP and is transferred to the CD14 at the cell surface. LPS then interacts with the signalling receptor TLR4 and the accessory protein MD-2. LPS stimulates the activation of various MAPK pathways, including the ERK, JNK, and p38 pathways. These pathways directly or indirectly phosphorylate and activate various transcription factors, including Elk-1, c-Jun, c-Fos, ATF-1, ATF-2, SRF, and CREB. In addition, LPS activates the IKK pathway via MyD88, IRAK, and TRAF6. TAK1–TAB2 and MEKK1–ECSIT complexes phosphorylate IKKβ, which in turn phosphorylates IκBs. Subsequent degradation of IκBs permits nuclear translocation of NF-κB/Rel complexes, such as p50/p65. The PI3K–Akt pathway phosphorylates and activates p65 via an unknown kinase. Figure adapted from [152].

1.5.1 Prenatal and early life TLRs and allergy

Early analysis of school-age children showed that PB cells from farm children expressed higher levels of CD14 and TLR-2 mRNA than cells from non-farm children [156]. Since these children have lower rates of allergic sensitization and asthma [119], these findings suggested that the immune system of farmers' children responds to the microbes in their environment to protect against allergic disease. Since epidemiological evidence also supports the protective effects of farm living, the relationship between TLR expression and prenatal farm exposures was evaluated: this demonstrated that exposure of pregnant mothers to stables, rather than exposure of their infants during childhood, was associated with enhanced TLR-2, -4 and CD14 expression in PB of school-age children [8]. This expression was also related, in a dose-dependent manner, to the number of farm animals the mother encountered [8]. This study not only agrees with the former [156], but extends their findings by demonstrating the importance of prenatal exposures on PRR expression in childhood. Another study extended the results of Ege *et al*., [8] to include increased TLR-7 and -8 expression in CB [157] with maternal farming during pregnancy, as well to noting increases in TLR-4, -5 and -6 expression at 1 year of age with consumption of raw milk in early life [157]. Likewise, a study by Ruduit *et al*., [9] demonstrated that not only did maternal contact with animals during pregnancy increase TLR-5 and -9 gene expression in offspring CB, but further illustrated that the expression of these TLRs was inversely related to the development of AD. Collectively, these studies indicate that prenatal and/or early life exposure to microbial environments induces an

upregulation of innate immune receptors that is robust and long lasting [119], and may be correlated to the development of allergic disease [9].

The level of sCD14 has also been implicated in allergy: low levels in serum [158], amniotic fluid and breast milk [159] have been associated with the development of AD in early life. In this connection, mRNA expression of CD14, TLR-2 and TLR-4 in newborns of allergic mothers has been shown to be reduced [160], along with reduced TLR-2 responses in CB [131] and PB [154,161]. Prescott *et al*., found no difference in TLR-2 mediated responses in CB of infants born to allergic mothers [4]; however, these studies varied in their use of TLR-2 agonists and sample size. Likewise, children with atopic histories exhibit impaired responses to TLR-4 signalling [155] in PB monocytes. These observations indicate that maternal allergy could potentially influence allergic predisposition of the child through effects on neonatal TLR expression and immune responses [131,154,161].

1.6 Studies completed for this thesis

The purpose of this thesis was to investigate the responsiveness of CB progenitors to LPS stimulation in relation to maternal atopy and the presence of T_H2 cytokines. The general hypothesis for these studies is that *CB progenitor cell responsiveness to LPS is modulated by maternal atopy.* Each study completed has a specific hypothesis. The studies were completed in a sequential manner, with each one building on the findings of the previous chapter. The goal of the studies was to collectively broaden our understanding of the role that maternal atopy and associated adaptive immunity (T_H2 cytokines) play on the responses of neonatal $CD34^+$ cells to innate immune stimuli (LPS). *Modulation of Eo/B differentiation may ultimately influence the development of allergic responses in infancy.*

In chapter 2 of the thesis, we investigated the hypothesis that *LPS stimulation of CB progenitor cells alters Eo/B CFU formation through LPS-induced hematopoietic cytokine secretion,* based on a number of reports demonstrating that TLR ligands, like LPS, can induce myeloid cell differentiation of BM-derived progenitor cells [10-12,60]. In order to do this, CB was collected from healthy pregnant women to delineate the potential mechanisms of LPS-induced eosinophilopoiesis. We show that CB progenitor cells respond to LPS through p38 MAPK-dependent GM-CSF secretion which supports Eo/B CFU *ex vivo*.

Given the results of chapter 2, we utilized CB samples collected from women participating in an Australian birth cohort study in order to ascertain the influence of maternal atopy on CB $CD34^+$ cell LPS-induced Eo/B CFU. We tested the specific

hypothesis that *the presence of maternal atopy alters CB progenitor TLR phenotype and Eo/B differentiative response to LPS stimulation*. To accomplish this, progenitor cells were isolated from CB and assessed for baseline TLR expression as well as Eo/B CFU formation after stimulation with LPS. The central observation of this study was that $CD34^+$ cells from infants at high-risk for atopy (as defined by maternal allergic sensitization) had reduced TLR expression as well as reduced Eo/B CFU formation after stimulation with LPS. The results of this research are presented in chapter 3.

The observation that the presence of maternal atopy was associated with decreased numbers of LPS-induced Eo/B CFU, sparked the studies pursued in chapter 4. We sought to gain a greater understanding of the role of T_H2 cytokines (representing an atopic milieu) in LPS-induced Eo/B CFU formation *ex vivo*. We hypothesized that *CB progenitor cell TLR-induced Eo/B CFU is altered by* T_H2 *cytokines*. This study therefore examined the mechanisms leading to reduced Eo/B CFU formation observed in high-atopic risk infants, by stimulating CB $CD34^+$ cells with IL-4 and IL-13 *ex vivo*. Interestingly, we demonstrate that the there is a differential effect of T_H2 cytokines on LPS-induced eosinophilopoiesis: IL-4:IL-4Rα signalling inhibits LPS-induced Eo/B CFU by regulating ERK 1/2 expression in CB $CD34^+$ cells, whereas IL-13 does not. The studies comprising this thesis are either published or submitted for publication.

CHAPTER 2

Toll-like Receptor mediated Eosinophil-Basophil Differentiation: autocrine signalling by GM-CSF in cord blood hematopoietic progenitors

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Recent studies have demonstrated that both murine [60] and human [10-12] BM-derived progenitor cells respond to TLR ligands through increased myeloid cell differentiation. However these studies focus on the production of DCs and macrophages, whereas the production of eosinophils, a known contributor to the immunopathogenesis of allergy [42], is unknown. Since Eo/B development is dependent of hematopoietic cytokine (e.g., IL-5, GM-CSF, IL-3) signalling [162], and TLR stimulated CB [163] or BM [10-12] $CD34^+$ cells have been shown to secrete detectable levels of these cytokines, we questioned whether LPS stimulation of CB $CD34^+$ cells resulted in hematopoietic cytokine secretion which could facilitate Eo/B differentiation. We show that CB $CD34^+$ progenitor cells isolated from healthy pregnant women respond to LPS stimulation through p38 MAPK-dependent GM-CSF secretion thereby facilitating Eo/B CFU formation *ex vivo*.

Drs. Judah Denburg, Roma Sehmi, and I were responsible for the experimental design and interpretation of the results obtained. Dr. Michael Cyr was also involved in the initial experimental design and provided methodological support in LPS stimulation assays as well as helpful discussion for data interpretation. Adrian Baatjes provided considerable expertise on flow cytometric analysis of $CD34^+$ cells in initial data

collection. I was responsible for generating the data and for writing the article. Both Drs. Denburg and Sehmi were involved in the editing process.

Toll-like Receptor mediated Eosinophil-Basophil Differentiation: autocrine signalling by GM-CSF in cord blood hematopoietic progenitors

Short title: LPS influences cord blood eosinophil-basophil differentiation

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Summary

Eosinophils are multi-functional leukocytes that play a role in inflammatory processes including allergy and infection. Although bone marrow (BM) inflammatory cells are the main source of eosinophil/basophil (Eo/B) differentiation-inducing cytokines, a recent role has been demonstrated for cytokine induction through Toll-like receptor (TLR)-mediated signalling in BM progenitors. Since we have previously demonstrated that cord blood (CB) progenitors induce Eo/B CFU after lipopolysaccharide (LPS) stimulation, we sought to investigate the intracellular mechanisms by which LPS induces Eo/B differentiation. Freshly isolated CD34-enriched human CB cells were stimulated with LPS (and/or pharmacological inhibitors) and assessed for alterations in hematopoietic cytokine receptor expression and signalling pathways by flow cytometry, Eo/B colony forming units (CFU) in methylcellulose cultures, and cytokine secretion using Luminex assays. LPS stimulation resulted in a significant increase in GM-CSF-responsive, as opposed to IL-5-responsive, Eo/B CFU which also correlated with significant increases in $CD34^+$ cell GM-CSFRα expression. Functionally, CB $CD34^+$ cells secrete abundant amounts of GM-CSF following LPS stimulation, via a p38 MAPK-dependent mechanism; this secretion was responsible for Eo/B CFU formation *ex vivo,* as evidenced by antibody blockade. We show for the first time that LPS stimulation of CB progenitor cells results in autocrine activation of p38 MAPK-dependent GM-CSF secretion facilitating Eo/B differentiation *ex vivo*. This work provides evidence that early life exposure to products of bacterial agents can modulate Eo/B differentiation, representing a

novel mechanism by which progenitor cells can respond to microbial stimuli and thus affect immune and inflammatory responses.

Keywords

LPS, p38 MAPK, cord blood, eosinophil-basophil, GM-CSF

Abbreviations

CB	cord blood
CFU	colony forming unit
Eo/B	eosinophil-basophil
ERK	extracellular signal regulated kinase
BM	bone marrow
sMFI	specific median fluorescence intensity
MAPK	mitogen activated protein kinase
STAT	signal transducer and activator of transcription
JAK	janus kinase
TLR	Toll-like receptor
LPS	lipopolysaccharide
DMSO	dimethyl sulfoxide
PBS	phosphate buffered saline
FBS	fetal bovine serum
NF- κB	nuclear factor kappa B

Introduction

Eosinophils are multi-functional leukocytes involved in a number of infectious and inflammatory processes, including allergic diseases. [1] Eosinophil-basophil (Eo/B) lineage commitment is a highly regulated process that involves the common βc- subunit binding cytokines, in particular GM-CSF and IL-5, [2] which when co-linked to specific, high affinity α chains, stimulate $CD34^+$ progenitor cells in the bone marrow (BM) via activation of several signal transduction pathways.[3] Both the janus kinase (JAK)/ signal transducer and activator of transcription (STAT) and mitogen activated protein kinase (MAPK) pathways drive eosinophil differentiation of cord blood (CB) derived progenitor cells.[4,5] Although the production of GM-CSF and IL-5 is generally derived from inflammatory cells within the BM, it has recently been shown that BM-derived $CD34^+$ cells secrete these cytokines after stimulation with Toll-like receptor (TLR) agonists.[6-8]

TLRs recognize microbial pathogens to activate intracellular signalling pathways during innate immune responses. TLR-4 signalling is initiated by the binding of lipopolysaccharide (LPS) to the TLR-4/MD-2 receptor complex on cellular membranes leading to activation of multiple signalling pathways including NF-κB and MAPK, and resulting in inflammatory cytokine gene transcription.[9] There are recent reports that hematopoiesis can be induced via direct TLR activation, independent of hematopoietic cytokines.[6,7,10] Specifically, extrinsic microbial stimuli are able to 'push' progenitor cells toward a myeloid-committed cell fate. [11] In relation to this, we have previously shown

that TLRs are expressed by human CB progenitor cells and that stimulation with LPS, a prototypical TLR-4 ligand, can induce Eo/B colony forming units (CFU).[12] Although the relationship to atopic predisposition was assessed previously,[12] the primary focus of this work was to investigate the biological effects of LPS stimulation on CB progenitors; specifically, we aimed to delineate intracellular mechanisms by which TLR-4 signalling may regulate Eo/B differentiation. Since LPS signalling can influence BM progenitor cell differentiation both *in vitro* [13] and *in vivo* [14] with clinical implications related to survival from sepsis [15] and risk of allergic disease,[12] we evaluated LPS-activated intracellular mechanisms involved in Eo/B CFU formation[12] of CB $CD34^+$ cells. Our results provide novel insights into the molecular mechanisms associated with LPS-induced hematopoietic progenitor cell Eo/B differentiation.

Materials and Methods

Cord blood collection

Pregnant mothers admitted to the Labour and Delivery ward at McMaster University Medical Centre, Hamilton, ON, Canada provided informed consent for CB donation prior to delivery. The CB samples were collected from otherwise healthy pregnant women as we were interested in investigating the mechanisms in CB $CD34^+$ cells. Upon delivery, each CB sample was collected in a 60mL syringe containing 2mL of heparin (1000 units/mL; Sigma, Mississauga, ON) and stored at 4°C until processing. This study was approved by the Hamilton Health Sciences/McMaster Faculty of Health Sciences Research Ethics Board.

Cord blood processing and $CD34^+$ cell enrichment

CB samples were depleted of erythrocytes using gravity sedimentation as previously described.[12] To enrich the sample for $CD34^+$ cells, the pellet was resuspended at a concentration of 5×10^7 cells/mL in RoboSep Buffer (PBS containing 2% FBS and 1mM EDTA; Stem Cell Technologies, Vancouver, BC). The cells were transferred to a 5mL falcon polystyrene round-bottom tube (Becton Dickenson 2058, Franklin Lakes, NJ) and EasySep Negative Selection Human Progenitor Cell Enrichment Cocktail with CD41 depletion (Stem Cell Technologies) at a concentration of 50µL/mL cells was added. The solution was mixed and incubated for 15 min at room temperature. The magnetic nanoparticles (Stem Cell Technologies) were added at a concentration of 50µL/mL cells and incubated for 15 min at room temperature. The cell suspension was then brought to a

total volume of 2.5 mL by adding RoboSep Buffer and the tube was placed inside the RoboSep Magnet (Stem Cell Technologies) for 10 min at room temperature. This sample was further enriched by placing the liquid portion in a new 5mL tube and re-incubating the sample in the magnet for 10 min. The purity of $CD34^+$ cells was between 85-90%.

LPS stimulation of CB $CD34^+$ cells

LPS from Escherichia coli 0111:B4 was purchased from Sigma and used at the optimal concentration of 10μg/ml as previously reported.[12] For stimulation studies, $CD34^+$ enriched cells were stimulated with LPS overnight (37°C, 5% CO_2) in tissue culture plates (Falcon) supplemented with RPMI complete (RPMI plain, HEPES, Pen/ Strep and FBS). After overnight incubation, cells were centrifuged and resuspended in FACS buffer for flow cytometry staining. Immunofluorescent staining for GM-CSFRα and IL-5Rα expression were performed as previously described.[12]

Phospho-flow to detect intracellular activation of signalling pathway molecules

Analysis of intracellular proteins followed a protocol that was described previously.[16] Briefly, following incubation (37°C 5% CO_2) of enriched CB $CD34^+$ cells with LPS for 5, 15, 30, 45 or 60 min, cells were fixed using PhosFlow CytoFix Buffer (BD Biosciences, Ontario, Canada), and then centrifuged for 10 min at 1500 RPM. After washing, cells were permeabilized (PhosFlow Perm Buffer III; BD Biosciences) for 30 min on ice, washed with FACS Buffer (PBS, 0.1% sodium azide) then stained with a PE-conjugated phospho-specific monoclonal antibody against p38 MAPK (pT180/pY182), ERK 1/2 (pT202/pY204) or STAT5 (pY694), FITC conjugated CD45 and PerCP conjugated CD34

or isotype control, all purchased from BD Biosciences. The amount of phosphorylated p38, ERK 1/2 or STAT5 was calculated as stimulation index equal to the median fluorescence intensity (MFI) $_{\text{stimulated cells}}$/MFI $_{\text{unstimulated cells}}$.[16]

Acquisition and analysis

Acquisition was performed using a LSR II flow cytometer (BD Bioscience); 5 x 10^3 events were collected for analysis. To enumerate $CD34^+$ cells, we used an established multiparameter gating strategy as previously described.[12]

Methylcellulose cultures

Methylcellulose colony assays were completed as previously described [12] using enriched CB $CD34^+$ cells at a plating concentration of 2 x 10^4 cells/ 35mm x 10 mm culture dish (Falcon Plastics, CA) in duplicate.

Duplicate cultures were also grown in the presence of supernatant (1/10 final dilution in culture) for 14 days (5% CO_2, 37ºC). The role of GM-CSF and IL-5 in supernatant stimulated Eo/B CFU formation was confirmed by adding 5μg/mL of anti-GM-CSF or anti–IL-5 (Peprotech, Rocky Hill, NJ) monoclonal antibodies to the supernatant stimulated methylcellulose cultures. Eo/B colonies were defined as tight, round refractile cell aggregates of 40 cells or more, staining pink with eosin using Wright-Giemsa (Diff-Quik; Seimens, Newark, USA) and visualized by inverted light microscopy (Olympus CK 40, Olympus Co. Ltd, Tokyo Japan).[17]

CD34⁺ cell cytokine assays

Freshly isolated $CD34^+$ progenitor cells were cultured in RPMI complete medium in the absence or presence of LPS overnight. After overnight incubation (37°C, 5% CO_2), the cell-free supernatant was harvested and stored at −80°C for subsequent analysis. Multi-analyte profiling was performed and acquired using a Perkin Elmer CS 1000 Autoplex Analyzer (Luminex XMAP Technology). A bioplex cytokine assay was used that simultaneously measured the concentrations of GM-CSF and IL-5 in culture supernatant using a human cytokine/chemokine MILLIPLEX MAP kit (Millipore, Mississauga, ON, CA). The assay sensitivities of these cytokines were 2.3 and 0.1 pg/mL respectively. All analyses were performed according to the manufacturer's instructions. In order to determine the mechanism of GM- CSF secretion, $CD34^+$ cells were stimulated with 50μM STAT5 inhibitor [18] or 50μM PD98059 [19] (ERK 1/2 inhibitor), or 20μM SB203580 [5] (p38 MAPK inhibitor) (Calbiochem, Cambridge, MA) or dimethylsulfoxide (DMSO) vehicle control for 45 min before LPS was added for overnight stimulation to induce GM-CSF secretion. These concentrations were found to be non-toxic to cells and of optimal dosage as determined by preliminary experiments.

Statistical analysis

Data were analyzed using IBM SPSS Statistics version 20.0 (Chicago, IL) and presented in figures as mean ± SEM. Data that were not normally distributed were log transformed and subsequently analyzed with the t- test to compare differences between two groups, or the ANOVA with a Dunnett *post hoc* analysis for many groups. We further applied the

Mann-Whitney U test to assess the sensitivity or robustness of the results, and the results were consistent. We set the criterion for statistical significance a priori at $\alpha = 0.05$. All p values were reported to two decimal places.

Results

LPS stimulation of CB progenitor cells enhances GM-CSF-induced Eo/B CFU

We have previously shown that CB CD34$^+$ progenitor cells express functional TLR-4 and respond to LPS stimulation through Eo/B CFU formation.[12] In order to confirm and extend those findings, freshly isolated CD34$^+$ cells were stimulated with LPS and hematopoietic cytokines for 14 days in methylcellulose cultures. Although LPS alone could not induce Eo/B CFU formation, the combination of GM-CSF ($p= 0.02$) and LPS resulted in a significant increase in the number of enumerable Eo/B colonies (Fig. 1A). Although the mean value was increased, IL-5-responsive Eo/B CFU formation in the presence of LPS did not reach significance (Fig 1B).

LPS stimulated CB progenitors secrete significant amounts of GM-CSF

We next assessed whether CD34$^+$ cells stimulated with LPS secrete the Eo/B differentiation inducing cytokines, GM-CSF and IL-5, using a bioplex cytokine assay. While none of these cytokines was found in the culture medium, CD34$^+$ cells alone do secrete ambiently low levels of cytokines. As shown in Fig. 2A, LPS induces significant levels of GM-CSF ($p=0.02$) from CB progenitors. The mean level of IL-5 was increased in LPS stimulated supernatant but this did not reach significance (Fig 2B).

LPS stimulated CD34$^+$ cells activate p38 MAPK signalling

Phospho-flow cytometry is an especially valuable tool for investigating signalling pathways in rare cell populations,[20] like CD34$^+$ progenitor cells. Since it has been

previously used to detect MAPK and STAT5 signalling pathways,[16] which may be involved in cytokine secretion from TLR stimulated CB progenitor cells,[21] we investigated whether these pathways were activated by LPS stimulation of CB CD34$^+$ cells. As shown in Fig 3, detectable levels of phosphorylated p38 MAPK were seen 5 min after LPS stimulation (p=0.046) followed by a steady decline thereafter. Additionally, there was a trend to increased ERK 1/2 between 5 to 30 min (p=0.06) with LPS stimulation. No significant differences in STAT5 expression, as evaluated over time, were detected in LPS stimulated CB progenitor cells.

LPS-induced p38 MAPK is involved in GM-CSF secretion by CD34$^+$ cells

Since we show that LPS induces a significant increase in GM-CSF secretion from CB CD34$^+$ cells (Fig 2), and that LPS can induce the rapid activation of p38 MAPK (Fig 3), we next assessed whether these pathways were involved in GM-CSF secretion by CB CD34$^+$ cells. To do this, CD34$^+$ cells were pre-incubated with MAPK inhibitors SB203580 (p38 MAPK inhibitor) or PD98059 (ERK 1/2 inhibitor) or a STAT5 inhibitor and GM-CSF secretion was assessed by Luminex. Although ERK 1/2 and STAT5 inhibition were found to reduce the mean percentage of CD34$^+$ cells secreting GM-CSF after LPS stimulation, it was only the addition of SB203580, a specific p38 MAPK inhibitor,[5] that significantly suppressed GM-CSF secretion (p=0.002) compared to LPS stimulated CD34$^+$ cells alone (Fig 4).

LPS-induced GM-CSF secretion by CD34$^+$ cells facilitates Eo/B CFU formation

We were next interested in whether LPS-induced GM-CSF could support Eo/B CFU formation. Indeed, as shown in Fig 5A, the supernatant of LPS stimulated CD34$^+$ cells induced Eo/B CFU formation which could be blocked by the addition of GM-CSF cytokine specific monoclonal antibodies ($p=0.02$); the reduction in Eo/B CFU formation by anti- IL-5 monoclonal antibodies was not significant. Morphology of the cells in the colonies indicated characteristic bi-lobed nuclei and eosinophilic granulation (Fig. 5B).

LPS stimulation of CB CD34$^+$ progenitor cells increases GM-CSFRα expression

Since alterations in Eo/B CFU production could be due to modulation of hematopoietic cytokine receptors, CD34$^+$ cells were stimulated with LPS overnight and then analyzed for receptor expression using flow cytometry. As shown in Fig. 6, LPS stimulation of CB progenitors increased the sMFI of GM-CSFRα ($p=0.04$). Although the mean level density of IL-5Rα was also increased, this value did not reach significance.

Discussion

TLRs are sentinels of the innate immune system,[22] and have recently been ascribed a new role in the regulation of myeloid lineage commitment.[7] Since hematopoietic processes are central to allergic inflammation [2] and systemic bacteraemia,[15] and given that LPS modulates CB progenitor cell [12] and BM progenitor cell differentiation both *in vitro* [13] and *in vivo*,[14] we further investigated the potential intracellular mechanisms regulating LPS-induced Eo/B CFU formation [12] in human CB CD34$^+$ cells.

We show that LPS enhancement of Eo/B CFU is specific to GM-CSF-responsive CD34$^+$ progenitor cells, as opposed to IL-5-responsive progenitor cells, and is also associated with preferential upregulated expression of GM-CSFRα (Fig 6). Additionally, we show that CB CD34$^+$ cells stimulated with LPS activate p38 MAPK signalling pathways which are involved in the autocrine secretion of GM-CSF; this cytokine plays an important role in facilitating Eo/B CFU formation *ex vivo,* as evidenced by antibody blockade. We had previously observed that *in vitro* Eo/B maturation of CD34$^+$ progenitors is accompanied by an increase in GM-CSF mRNA and protein in maturing colony cells;[23] our current finding of increased expression of GM-CSFRα after LPS stimulation, and its association with increased functional responsiveness of these cells to GM-CSF in colony assays, provides an additional explanation for this autocrine effect, as others have also noted.[24] In support of this, blocking signal transduction via GM-CSFRα through GM-CSF inhibition reduced Eo/B CFU formation. Whether or not secreted GM-CSF auto-regulates GM-CSFRα expression is unknown to us; however, we cannot refute this possibility since GM-CSF has been shown to alter the expression of its cognate receptor in peripheral

blood eosinophils.[25] Collectively, these findings highlight a role for GM-CSF/GM-CSFRα in LPS enhancement of Eo/B differentiation, which is consistent with previous observations on the development of myeloid cells [26] and eosinophil progenitors.[27]

TLR stimulation has been shown to involve the activation of MAPK signalling pathways in human monocytes, [9,28] macrophages, [29] eosinophils [30] and CB progenitor cells.[21] In relation to progenitor cells, we have previously shown that IL-5- or GM-CSF-stimulated peripheral blood progenitor cells undergo rapid phosphorylation of p38 MAPK within 1 to 5 min using phosopho-ELISA.[17] Although not in a kinetic study, *Kim et al.,* also showed that in CB progenitors stimulated with TLR-9 agonists there is upregulation of both p38 MAPK and ERK 1/2.[21] Our findings therefore complement and extend the latter study, showing that significant phosphorylation of p38 MAPK is also detected in CB $CD34^+$ cells stimulated with other TLR (LPS) agonists.

While others have reported that BM derived $CD34^+$ cells respond to TLR stimulation with the production of cytokines including GM- CSF,[6-8] the potential mechanism(s) of this secretion were not investigated. Our demonstration that blocking p38 MAPK signalling in CB $CD34^+$ cells suppresses LPS-induced GM-CSF secretion is thus novel. Relatedly, *Kim et al.,* have demonstrated that TLR-9 stimulation of CB $CD34^+$ cells [21] activates p38 MAPK and ERK 1/2 pathways involved in IL-8 secretion. Our data show for the first time that LPS-induced GM-CSF production, which facilitates Eo/B CFU, directly involves TLR4/p38 MAPK signal transduction in CB $CD34^+$ cells. In this way, LPS is only one component of this autocrine effect, a co-factor in Eo/B CFU formation, which

utilizes the production of GM-CSF through MAPK signalling pathways to induce Eo/B differentiation from CB CD34^{+} cells. This is in line with studies which have shown that p38 MAPK is an integral part of the TLR4 axis of signal transduction.[31]

We have previously shown that CB progenitor cells from high-atopic risk infants have reduced capacity for Eo/B CFU formation after LPS stimulation.[12] It has recently been shown that children of atopic mothers have reduced TLR-dependent p38 MAPK signalling in their blood monocytes up to the age of 2 years.[32,33] In light of our current results, we hypothesise that reduced CB Eo/B differentiation after LPS stimulation in high-atopic risk infants [12] may be due to reduced p38 MAPK-induced GM-CSF production by CD34^{+} cells, possibly related to epigenetic effects on p38 MAPK expression *in utero*. Along these lines, prenatal exposure to bacterial microflora (*A. lwoffii F78*) has been shown to prevent the development of allergy in offspring [34] through microbial-induced epigenetic regulation of the IFN-γ promoter.[35] Although the assessment of atopy was not the objective of this study since we were interested solely in the biological implications of LPS stimulation on human CB CD34^{+} cells, we are now in position to examine this hypothesis in prospective birth cohorts.

Multi-potent progenitor cells have been previously shown to express TLRs and respond to receptor ligation by production of myeloid committed cells;[6,7,13] indeed, alterations in hematopoiesis may represent another mechanism for targeted redirection of the immune response to confront invading pathogens. For example, it has been shown that sepsis is sometimes associated with neutropenia,[36] accompanied by peripheral blood and BM

myeloid progenitor cell mobilization and differentiation.[37] In the case of eosinophils, there is a documented case of cryptococcal infection combined with sepsis, resulting in eosinophilia in a healthy individual.[38] Likewise, LPS has been shown to influence hematopoietic dynamics through direct effects on progenitor cells, including rapid myeloid differentiation.[13] Increased Eo/B CFU production after LPS stimulation of CB $CD34^+$ cells may represent a mechanism through which hematopoietic progenitor cells [15,37] or their resulting mature progeny [39] can help respond to invading bacterial species during acute infections. These mechanisms may also be operative in allergic (eosinophilic/basophilic) inflammation.

Our data are interesting in the context of the type of immune response that can be generated in response to bacterial agents. Of note, IL-5 is an eosinophil-specific inducing cytokine,[40] whereas GM-CSF-responsive progenitors represent earlier stages of lineage commitment and therefore contribute to the development of several myeloid cells [37] including of Eo/B cells, macrophages and neutrophils. Therefore, the apparent skewing of the Eo/B progenitor population towards GM-CSF- (Fig 1A), as opposed to IL-5-responsive lineages (Fig 1B), with noted increases in GM CFU (data not shown), suggests that the progenitor response to LPS involves production of multi-cellular (Eo/B [39] and GM [37]) inflammatory responses to pathogens or allergens.

Although relatively high doses of LPS were used in the *ex vivo* culture system, this must be tempered by knowledge of the bio-availability of LPS *in vivo*. Physiologically, the foetus is exposed *in vivo* to LPS, since gram-negative bacteria and associated LPS can be

isolated from amniotic fluid in median concentrations of 0.05μg/mL.[41] Though the minimal concentration of biologically active LPS present within the intrauterine environment is unknown, soluble factors (e.g., sCD14) can modulate immune cell responses to LPS at 1000 fold lower concentrations than those observed in amniotic fluid.[42] The LPS concentration which we have utilized in the current studies is in line with other *in vitro* progenitor cell studies [12,13] which have found minimal progenitor cell responses to LPS below 10μg/mL. In addition, Roy et al., have demonstrated that endotoxin levels range between 1 – 6 μg/g house dust in rural and urban homes.[43] Thus, our utilized dose of LPS appears to be in the physiological range of *natural* LPS exposure.

We cannot conclude without addressing a couple of limitations of this study. Firstly, the sample size used in this study appears to be small but we have completed the necessary sample size calculations which show that as few as 4 CB samples are necessary to see differences in hematopoietic cytokine-induced CFUs. With this in mind, we are reassured of the significance of the findings and our interpretation that GM-CSF-mediated Eo/B CFU formation is an important pathway induced by LPS-stimulated $CD34^+$ cells. Finally, there was a slight limitation with the type of LPS used for the study. We understand that this was not an ultrapure version of LPS, and therefore could be activating TLRs other than TLR-4. However, this study was not designed to investigate which TLR LPS signals through, but instead was designed to determine the biological effect (e.g., activation of signalling pathways involved in Eo/B CFU formation) of LPS stimulation of $CD34^+$ cells.

In conclusion, the novel autocrine mechanism of LPS-mediated Eo/B differentiative capacity shown herein points to the potential importance of TLR-mediated hematopoiesis *in utero* in relation to the development of allergic inflammation or immune responses to microbial stimulation. With interest increasing in p38 MAPK as a therapeutic target in inflammatory disorders,[44] an understanding of the biology of TLR-mediated Eo/B differentiation may aid in the development of therapeutic interventions for high-atopic risk infants [12] or neonatal responses to infection.

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Figure Legends

Figure 1A,B. LPS stimulation of CB CD34$^+$ cells enhances Eo/B CFU formation. CB CD34$^+$ cells were stimulated with 10 μg/mL LPS and optimal concentrations of A) GM-CSF (10 ng/mL) or B) IL-5 (1 ng/mL). Cultures were incubated for 14 days at 37°C and 5% CO_2. Eo/B cultures are tight, granular clusters of 40 cells or more. Data are presented as mean ± SEM of 4 experiments per condition. Significant findings were $p< 0.05$ and indicated on the graph.

Figure 2A,B. Stimulation with LPS induces the production of hematopoietic cytokines by CD34$^+$ cells. CB CD34$^+$ cells were stimulated with 10 μg/mL LPS overnight and culture supernatants were collected. Baseline and stimulated supernatants were assessed for GM-CSF (A) or IL-5 (B) using Luminex technology. Data are presented as mean ± SEM of 6 experiments per condition. Significant findings were $p< 0.05$ and indicated on the graph. N.D. not detected

Figure 3A-C. Kinetics of LPS-induced phosphorylation of signalling molecules in CB CD34$^+$ cells. CB CD34$^+$ cells were stimulated with 10 μg/mL LPS for 5 to 60 min (different shades of grey), with a time 0 control (light gray) and assessed for A) p-p38 MAPK, B) p-ERK 1/2 and C) p-STAT5 expression using intracellular flow cytometry. A representative histogram of each time point is shown. A total of 4 experiments were conducted for each time point.

Figure 4. Inhibition of p38 MAPK suppresses GM-CSF secretion by LPS-stimulated CD34$^+$ cells. CB CD34$^+$ cells were stimulated with 50μM PD98059 (ERK 1/2 inhibitor),

20μM SB203580 (p38 MAPK inhibitor) or 50μM anti-STAT5 inhibitor for 45 min before the addition of 10 μg/mL LPS overnight. Culture supernatants were collected and baseline and stimulated supernatants (with/or without inhibitors) were assessed for GM-CSF using Luminex. Data are presented as mean ± SEM of 6 experiments per condition. Significant findings were $p < 0.05$ and indicated on the graph.

Figure 5A, B. LPS induced cytokines from CD34$^+$ cells induce Eo/B CFU.

(A)The supernatants of previously stimulated CB CD34$^+$ cells were incubated alone or with anti-IL-5 or anti-GM-CSF antibodies in methylcellulose culture for 14 days (37°C and 5% CO_2). Eo/B cultures are tight, granular clusters of 40 cells or more. Data are presented as mean ± SEM of 6 supernatant stimulated cultures. Significant findings were $p < 0.05$ and indicated on the graph. B) Picographs were developed from cytospins of Eo/B colonies. Eo/B cells were stained pink with eosin using DiffQuik solution. Images of Eo/B colonies and Eo/B cells are shown at 40X and 400 X magnifications respectively. N.D. not detected

Figure 6A-C. Stimulation of CB CD34$^+$ cells with LPS increases GM-CSFRα expression. CB CD34$^+$ cells were stimulated with 10 μg/mL LPS overnight before being stained with antibodies to GM-CSFR α (A) or IL-5Rα (B). Histograms shown are representative of one experiment, light tinted histograms are isotype controls for the unstimulated and stimulated samples, and filled histograms are of the receptor of interest in the unstimulated (black) and stimulated samples (dark gray). Data were also presented as the mean ± SEM of 6 experiments measured by specific median fluorescent intensity

(sMFI [12]), which represents the number of receptors per cell (C). Significant findings were $p < 0.05$ and indicated on the graph.

Figure 7. p38 MAPK-dependent GM-CSF secretion by LPS stimulated CD34$^+$ cells induces Eo/B CFU formation. LPS signalling in CB CD34$^+$ cells induces rapid p38 MAPK phosphorylation (1). This activation results in the intracellular production of GM-CSF, which is secreted (2), and serves to induce Eo/B CFU *ex vivo* (3).

Figure 1

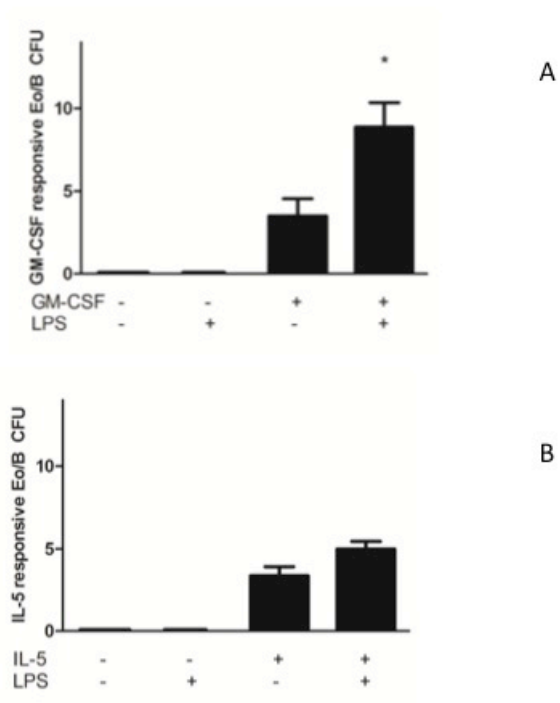
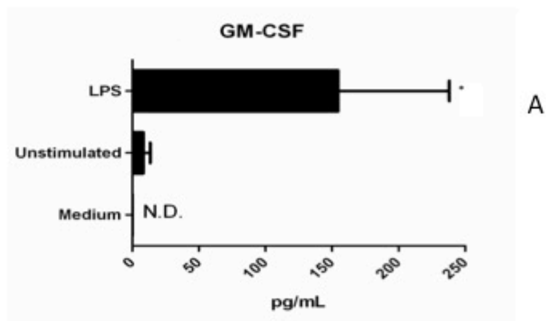


Figure 2



A

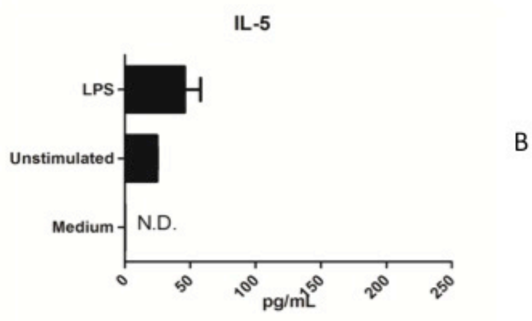


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Figure 3

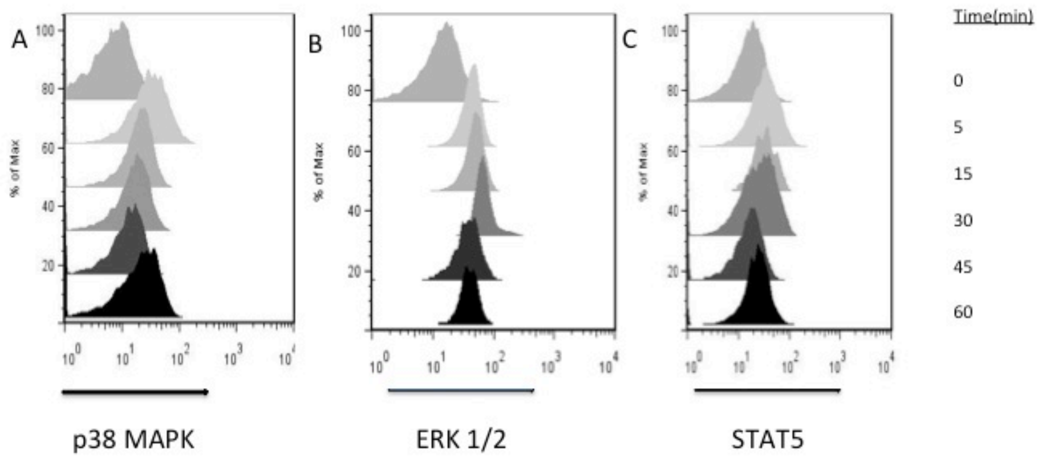


Figure 4

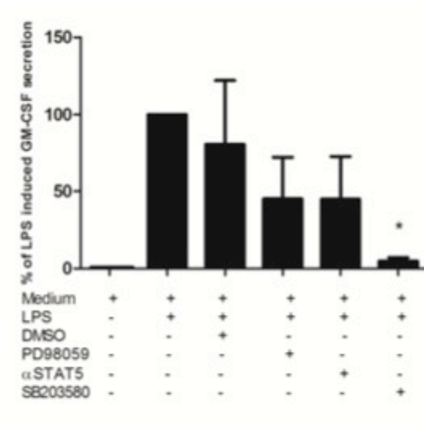


Figure 5

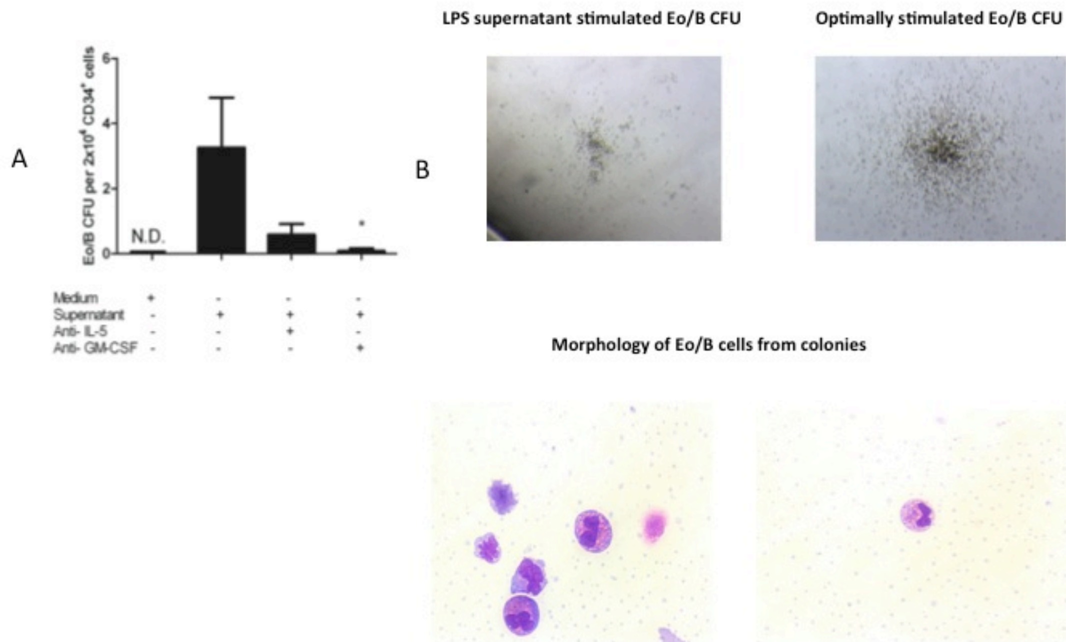


Figure 6

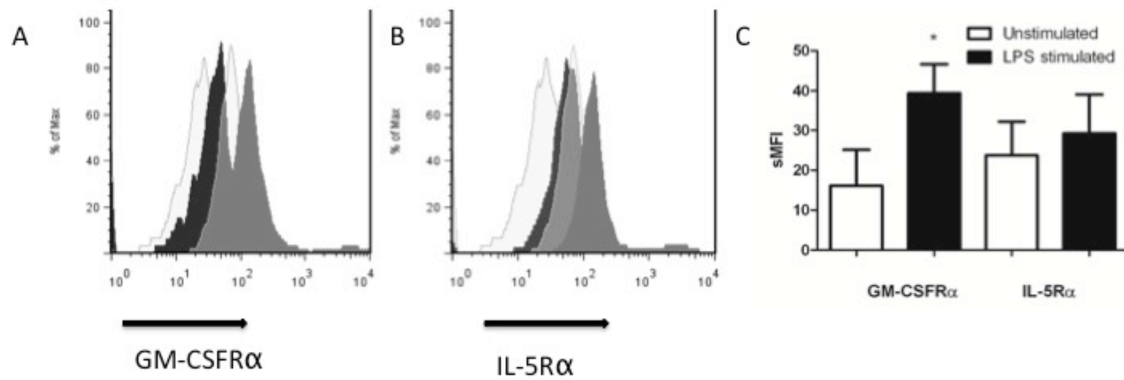
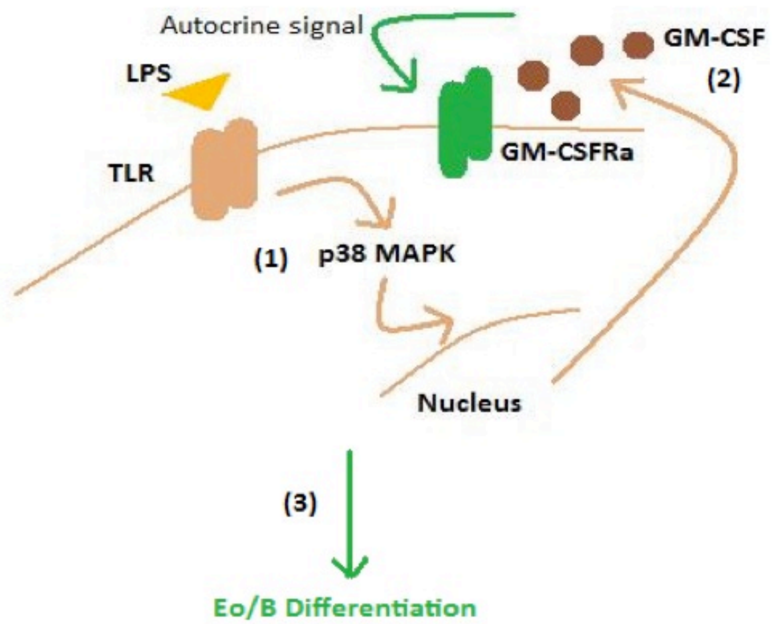


Figure 7



CHAPTER 3

Maternal allergy modulates cord blood progenitor $CD34^+$ cell toll like receptor expression and function

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Our group has repeatedly shown that both the phenotype and differentative function of CB progenitor cells are altered in infants at risk for allergy [6,7,114]. Additionally, there is ample evidence that TLR expression [9,160] and function [131] are altered in CB of at-risk infants, or infants that subsequently develop AD [9]. Despite this, whether a genetic predisposition to allergy alters $CD34^+$ TLR expression or responsiveness to LPS stimulation in relation to Eo/B CFU formation is unknown. We show that CB $CD34^+$ cells from high-atopic risk infants have reduced TLR expression and LPS-induced Eo/B CFU formation. Modulation of neonatal innate immune responses may be a novel approach to prevent allergic responses in early life.

Dr. Judah Denburg and myself were responsible for the experimental design(s) and interpretation. Lynn Crawford, Amudhinie Thanderan and I were responsible for the generation of data, whereas Dr. Denburg and I were involved in writing the article. Dr. Sehmi provided considerable expertise on flow cytometric analysis. Dr. Susan Prescott medically assessed and recruited the pregnant women approved for the study, as well as provided the CB samples from the cohort for analysis. Drs. Tulic and Prescott were vital

in the editing process. Dr. Thabane was instrumental in providing statistical advice and approval.

Maternal Allergy Modulates Cord Blood Hematopoietic Progenitor Toll-Like Receptor Expression and Function

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Abstract

Background: Little is known regarding the prenatal determinants of innate immune responses in relation to infant allergic risk. Environmental exposures, including microbial stimuli, may predispose susceptible individuals to atopy and asthma in early infancy or even *in utero*.

Objective: Since Toll-like receptors (TLRs) recognize microbial products, and since cord blood (CB) progenitor alterations have been observed in neonates at risk for atopy, we investigated the expression and function of TLRs on CB hematopoietic progenitors, in relation to atopic risk as defined by maternal allergic sensitization.

Methods: Thirty-two (15 low- and 17 high-atopic risk) infant CB samples were assessed for phenotypic and functional alterations in $CD34^+$ cells by flow cytometry and methylcellulose cultures, respectively. $CD34^+$ hematopoietic progenitors were stained for TLR-2, -4, -9, GM-CSFRα, IL-5Rα and IL-3Rα, or cultured in methylcellulose assays for hematopoietic cytokine-stimulated eosinophil-basophil (Eo/B) colony forming units (CFU), with or without lipopolysaccharide.

Results: High-atopic risk infants had significantly lower CB $CD34^+$ cell TLR-2, TLR-4 and TLR-9 expression ($p=0.009$). High risk infant progenitors gave rise to significantly more Eo/B CFUs ($p=0.002$) upon hematopoietic cytokine (IL-3, IL-5 or GM-CSF) stimulation *ex vivo*. While LPS co-stimulation induced Eo/B CFUs from both low- and high-risk infant CB $CD34^+$ cells, this response was significantly ($p=0.020$) muted in the high-risk CB progenitors.

Conclusions: Neonatal CB CD34$^+$ hematopoietic progenitor cell TLR expression and functional responsiveness are altered in CB from atopic at-risk infants. Maternal allergic sensitization may modulate hematopoietic progenitor TLR expression and function *in utero*; specifically, Eo/B 'lineage priming' at birth may be circumvented through engagement of TLR pathways in early life.

Clinical Implications

Since hematopoietic processes at birth may play an important role in the development of atopy, investigating progenitor cell phenotypic and functional alterations may reveal novel pathways and predictors of early childhood atopic outcomes.

Capsule Summary: This work provides evidence of early maternal influences on cord blood hematopoietic progenitor cell TLR expression and function, which may provide key diagnostic and therapeutic information in the management of allergic disease in early childhood.

Keywords: atopy, Toll-like receptors, cytokine receptors, cord blood, CD34, eosinophil-basophil

Abbreviations

TLR	Toll-like receptor
LPS	lipopolysaccharide
BM	bone marrow
CB	cord blood

Eo/B	eosinophil-basophil
sMFI	specific median fluorescence intensity
CFU	colony forming units
GM-CSF	granulocyte-macrophage colony-stimulating factor

Introduction

The developmental immune mechanisms in the fetal and neonatal period that influence infant atopic risk are not clearly understood. Immunological responses to environmental stimuli in early life may influence patterns of immune development [1,2]. Since studies have provided evidence that antenatal differences in immune function are associated with subsequent allergic disease [3] and that childhood allergies manifest in the first years of life [4,5], it is possible that the earliest manifestation of allergy results from intrauterine regulation of prenatal responses to environmental stimuli; however, little is known about the prenatal determinants of the innate immune response related to atopic development.

It has been proposed that gestational environmental exposures in the appropriate genetic context may influence the risk of pediatric allergy [6], and specifically that hematopoietic and/or immune cord blood (CB) profiles might be useful in predicting atopy in early life. For example, peri-natal cytokine[7] and cytokine receptors[8] have been associated with increased risk of postnatal allergic disease such as atopic dermatitis. Likewise, we have previously shown that hematopoietic cytokine receptor expression on CB myeloid ($CD34^{+}/45^{+}$) progenitor cells is associated with atopic risk – as determined by parental history and/or maternal allergic sensitization [9] – and first year atopic and respiratory outcomes[10,11]. Since maternal allergy is associated with alterations in TLR expression[12], CB progenitor TLR expression may also be a potential biomarker identifying children at risk for allergy.

Rural farming environments with high microbial loads have been repeatedly associated with lower childhood allergy[13], presumably through immunomodulatory effects on the

neonatal innate immune system[2,14]. Furthermore, there is evidence that microbial stimulation modulates hematopoiesis[15-18]. In fact, we have found that CB progenitors respond to TLR ligation through the induction of Eo/B lineage primed cells– activation of CB $CD34^{+}/45^{+}$/IL-(3) $5R\alpha^{hi}$/GM-$CSFR\alpha^{hi}$ cells that are highly responsive to hematopoietic cytokine stimulation ex vivo (Reece et al., manuscript submitted). Neonates at risk for allergy also have increased Eo/B “lineage priming”[10]; however, whether allergic risk modulates TLR-mediated effects on these progenitors and their lineage commitment has not yet been studied.

Therefore, we investigated whether maternal allergic sensitization (conferring atopic ‘high-risk’ to their infants, by definition) impacts CB progenitor TLR-2, TLR-4 and TLR-9 expression and function. We hypothesized that progenitor cell TLR expression and function are altered in atopic ‘high-risk’ infants. To test this, we quantified the level of TLR expression and the role of TLR ligation in CB hematopoietic progenitor differentiation, in a population of women participating in an Australian birth cohort study.

Methods

Study Design & Population

Pregnant females, booked for antenatal appointments in local metropolitan hospitals (including St John of God Hospital, King Edward Memorial Hospital and Osborne Park Hospital) between August 2002 and October 2004 were screened by a researcher using a telephone or interview questionnaire. Healthy pregnant volunteers (n=32) were recruited into the study, including similar numbers of non-atopic (n=15) and atopic (n=17) participants. Subjects were excluded from analysis if they delivered pre-term (<36 weeks gestation), or if there was any clinically important neonatal morbidity or disease. Maternal allergy status was assessed by skin prick testing (SPT) to common allergens (house dust mite, grasses, moulds, cat, dog, feathers and cockroach). These extracts were purchased from Hollister-Steir Laboratories (Spokane, WA, USA). A wheal size of ≥ 3 mm above the negative control was considered positive. Mothers were defined as allergic if they had a history of disease and one or more positive SPT. All volunteers recruited into the study received verbal and written information about the study and signed written consent. For laboratory assessments, research personnel were unaware of maternal allergy status. Infant cord blood samples were considered "high risk" if the mothers had at least one positive SPT, and low risk if the mothers were SPT negative. Allergic symptoms were reported by the mothers as doctor diagnosed but this was not confirmed by the investigators. As such, these symptoms were not considered in the inclusion criteria. The study was approved by the Princess Margaret Hospital Ethics Committee, Perth, Australia

and by the Hamilton Health Sciences/McMaster Faculty of Health Sciences Research Ethics Board in Hamilton.

Cord Blood Collection and Sample Cryopreservation

Cord blood was collected at delivery into an equivolume of RPMI (Gibco, ON, Canada) plus heparin (Sigma Aldrich Ltd, ON, Canada). Mononuclear cells were isolated using Lymphoprep (Nycomed Pharma AS, Oslo, Norway) gradient centrifugation at 500g for 30 minutes and washed twice in RPMI and then once in RPMI containing 2% FCS (Gibco, ON, Canada). Cells were enumerated using a hemocytometer (Neubauer Chamber, Hausser Scientific, Blue Bell, PA) and diluted to 40 x 10^6 cells/ml RPMI/2% FCS and placed on ice.

Cryopreservation and shipment of CB samples

Cells were diluted to 40 x 10^6/ml RPMI/2% FCS (MultiSer, Biosciences Pty, Ltd, Australia) and placed on ice. An equal amount of cooled 15% dimethyl sulphoxide (BDH, Victoria, Australia) in FCS was added drop wise. Samples were frozen in 1ml aliquots each containing 20 x 10^6 cells and were cooled to -80°C at a rate of 1°C per minute in a freezing container (Nalgene cryocontainer) containing isopropyl alcohol (BDH), and transferred to a liquid nitrogen tank for storage and shipment. We and others have shown reliably and reproducibly that cryopreservation and thawing in this way do not distort cellular immune responses, including functional and phenotypic characteristics of $CD34^+$ progenitor cells[10] or alter the pattern of TLR cellular function[19]. Mononuclear cells stored in liquid nitrogen (2 x 10^7 CB mononuclear cells per patient) were transported from Australia to Hamilton by courier.

Cells were thawed by drop wise application of RPMI complete, resuspended in McCoy's 3+ media (Gibco), and incubated (37°C, 5% CO_2) for 2 hours in a plastic flask to remove adherent mononuclear cells. Nonadherent mononuclear cells (NAMNC) were then resuspended in 1ml Iscoves 2+ culture medium (for methylcellulose cultures) or 1ml FACS buffer (for flow cytometry) and a cell count was performed. Average viability was greater than 98% and average recovery was 81.5% of the total number of cells frozen.

TLR ligand used for methylcellulose cultures

LPS from Escherichia coli 0111:B4 was purchased from Sigma (Mississauga ON). Optimal culture conditions for LPS were determined including optimal cell numbers and ligand doses. Preliminary experiments using the maximal number of Eo/B CFU as an endpoint measure revealed that 10μg/ml LPS was an optimal concentration for all assays performed.

Methylcellulose colony assay

Methylcellulose colony assays were performed as previously described[11]. Briefly, CB NAMNC were cultured in duplicate with 2 x 10^5 cells/ 35 mm x 10 mm culture disk (Falcon Plastics, CA) in 0.9% methylcellulose (Sigma) with Iscove's 2+ [modified Dulbecco's medium (Gibco) and 20% FCS supplemented with 1% penicillin-streptomycin and 5 x 10^{-5} M 2-mercaptoethanol] in the presence of recombinant human cytokines at optimal doses, IL-3 (1 ng/ml), IL-5 (1 ng/ml) or GM-CSF (10 ng/ml) (BD Biosciences, ON), or equal amounts of Iscoves 2+ as a control. Cultures were incubated for 14 days (37˚C, 5% CO_2) in the presence of IL-3, IL-5 or GM-CSF. For TLR stimulation, 10 μg/ml LPS was added to the hematopoietic cytokine stimulated cultures.

Eo/B-CFU responsive to each cytokine were enumerated from duplicate cultures using inverted light microscopy (colonies were defined as > 40 cells). The researcher counting colonies was blinded to any information about the subjects.

Antibodies

Receptors of study include Phycoerythrin (PE)-anti human TLR-2 (CD282), PE-anti human TLR-4 (CD284), PE-anti human TLR-9 (CD289), PE-anti human IL-3Rα (CD123), PE-anti human IL-5Rα (CDw125) and PE-anti human GM-CSFRα (CD116). TLR antibodies were purchased from eBioscience (San Jose CA), and hematopoietic cytokine receptor antibodies were purchased from BD Pharmigen (San Jose CA). Isotype control for TLR-2 and TLR-4 was PE-mouse immunoglobulin G2a (IgG2a κ). Isotype control for TLR-9 was PE-rat IgG2a κ. Isotype control for hematopoietic cytokine receptors was PE-mouse IgG1a κ. Peridinin chlorophyll protein (PerCP)-conjugated CD34 antibody was used to label progenitor cells. The antibodies Fluorescein isothiocyanate (FITC), Allophycocyanin (APC), PerCP and PE-conjugated CD45 antibody were used for leukocyte labelling and compensation.

Cell Staining

Four colour immunofluorescent staining was performed by using an adapted procedure[9]. The CD34 enriched cells were resuspended in 1ml of FACS buffer (PBS, 0.1% sodium azide) and aliquoted into individual tubes at a concentration of 1 x 10^5 cells/tube. Cells were resuspended in mouse block (PBS containing 0.2% sodium azide, 2.5% mouse serum and 2.5% human serum) and incubated in the refrigerator for 30 minutes. Optimal amounts of PE labeled anti-IL-3Rα, IL-5Rα, GM-CSFRα, TLR-2, TLR-4 or their

respective isotype control were added to the tubes and they were incubated for 30 minutes in the refrigerator. Cells were then washed with FACS buffer and fixed in 250μL of 1% paraformaldahyde. Compensation tubes were likewise prepared, staining cells individually with anti-CD45-FITC, anti-CD45-PE, anti-CD45-APC and anti-CD45-PerCP. For intracellular analysis of TLR-9, cells were resuspended in a fixing solution (Caltag Laboratories, Carlsbad, California) for 20 minutes at RT, then washed with FACS buffer and resuspended in permabilization buffer (Catlag Laboratories). Antibodies for PE-anti-human TLR-9 and rat isotype control were added to the permabilization solution and incubated for 30 minutes at RT. Cells were then washed and resuspended in 250μL 1% PFA. All samples were covered with aluminum foil and refrigerated until ready for acquisition.

Acquisition and Analysis

Acquisition was performed using a LSR II flow cytometer (BD Bioscience). Five thousand progenitors were collected for analysis. To enumerate $CD34^+$ cells by flow cytometry, we used an established multi-parameter gating strategy[9] and a four colour staining protocol to assess the expression of TLR and hematopoietic cytokine receptors. Flow Jo software (Tree Star) was used for the analysis of data. $CD34^+$ cells were identified as having high CD34 expression, low- intermediate CD45 and low forward and side scatter (consistent with lymphoblastoid cells). For gating analysis, quadrant markers were set so that 2% of cells were stained with isotype control (negative staining). Cytokine receptor expression was calculated as the percentage of $CD34^+$ cells staining positively for the receptor. This was obtained by subtracting the staining achieved by the

designated isotype control from the staining of the $CD34^+$ cells with the receptor of interest. Absolute numbers of CD34 cells were not determined due to the use of an enrichment protocol. Specific median fluorescence intensity (sMFI), which represents the numbers of receptors per cell, was calculated by subtracting the MFI of the isotype from the MFI of the receptor of interest.

Statistical Analysis

The sample size for this study (n = 32) was based on feasibility consideration as the overall goal of the study was exploratory or hypothesis-generation. The demographic and prognostic characteristics of the patients were analyzed using descriptive statistics reported as mean (standard deviation [SD]) or median (minimum [min], maximum [max] for continuous variables and counts for categorical variables. We used the t-test to compare the two groups (low- and high-atopic risk defined by maternal sensitization) on the basis of $CD34^+$ cell phenotypic bio- markers or functional outcomes. We further applied the Mann Whitney U test to assess the sensitivity or robustness of the results, and the results remained the same. The criterion for statistical significance was set a priori at $\alpha = 0.050$. We did not adjust α for multiple testing because the analyses were primarily exploratory (i.e. for hypothesis generation)[20] . The results are reported as estimates of the difference (95% confidence interval) and associated p values. All p values are reported to three decimal places with p values less than 0.001 reported as $p<0.001$. All analyses were performed using PASW Statistics (formerly named SPSS) version 18 (Chicago, IL).

Results

Characteristics of maternal and neonatal populations

The study participants included 15 non-atopic mothers and 17 atopic mothers (Table I). There was no statistical difference in maternal age or parity between the mothers in the 'low-atopic risk' and 'high-atopic risk' groups. In terms of neonatal characteristics, there were no statistically significant differences in the methods of delivery, gestational age, birth length or weight, head circumference or total IgE between the two groups (see Table I).

Presence of maternal allergy is associated with reduced CB progenitor $CD34^+$ cell TLR expression

We first sought to determine whether the expression profiles of three TLRs that have been associated with allergy (TLR-2, TLR-4 and TLR-9) were altered on human CB progenitor cells in relation to maternal allergic sensitization. As shown in Table II and Figure 1, in "high-risk" infants born to allergic mothers, CB $CD34^+$ cells had significantly reduced TLR-2 (mean difference: 8.18: 95% CI: (2.15,14.22), p= 0.012), TLR-4 (13.38 (2.83,23.94), p= 0.017), and TLR-9 (56.89 (16.77,97.02), p= 0.009) expression, when compared to "low-risk" (i.e., no maternal allergy) infant CB. Hematopoietic cytokine receptor percentage expression on CB $CD34^+$ cells was also significantly decreased in the high-atopic risk group or IL-3Rα (6.24 (1.85,10.64), p=0.009) and IL-5Rα (23.15 (13.32,32.97), p<0.001) (see Table II and Figure 2). This high risk group also had higher specific median fluorescence intensities for the respective cytokine receptors (data not

shown): i.e., the selective presence of subpopulations of $CD34^{+}/45^{+}/IL\text{-}5R\alpha^{hi}$ cells (and similarly for cells expressing IL-3Rα and GM-CSFRα).

Differences between low and high risk infants in eosinophil-basophil differentiation capacity of TLR-and hematopoietic cytokine co-stimulated CB CD34⁺ cells

Since we (Reece et al., manuscript submitted) and others[15-18] had previously demonstrated that TLR ligation of hematopoietic progenitor cells modulates their differentiation fates, we sought to determine what impact maternal allergic status had on Eo/B lineage commitment before and after TLR stimulation. First, we assessed Eo/B lineage commitment of CB $CD34^{+}$ progenitor cells in low- and high-risk infants to hematopoietic cytokine stimulation alone. Stimulation of CB $CD34^{+}$ cells from high-risk infants with the hematopoietic cytokines resulted in significantly *higher* IL-3- (-22.09 (-34.87,-9.31), p=0.002) and GM-CSF- (-18.53 (-30.35,-6.70), p=0.004) responsive Eo/B CFU, compared to Eo/B CFU from hematopoietic cytokine stimulated low-atopic risk CB $CD34^{+}$ cells (see Table III and Figures 3-5).

Since maternal allergy is associated with antenatal alterations in TLR expression [5] and function[3,21] on monocytes and CB progenitors (Figure 1), we next investigated whether *TLR-mediated* $CD34^{+}$ cell Eo/B differentiation was also influenced by maternal allergic status. Interestingly, when high risk infant CB $CD34^{+}$ cells were assessed for their functional responsiveness to LPS and hematopoietic cytokine co-stimulation, these progenitors responded with significantly *decreased* Eo/B CFU production to LPS and IL-

5 (4.88 (0.96,8.81), p=0.020) and GM-CSF (12.92 (1.42,24.41), p=0.032) when compared to low risk infant progenitors (see Table III and Figures 3-5).

Discussion

There is an increasing body of evidence supporting antenatal differences in TLR-related immune function and subsequent infant allergic disease. For example, it has recently been shown that infant allergic disease is associated with pre-symptomatic differences in TLR function at birth [3]. Maternal environmental exposures to farm animals [5] and cigarette smoke[19] have likewise been shown to modulate neonatal TLR expression and function. We have previously shown that neonatal CB progenitor cells display altered phenotype and function with respect to parental atopic history and maternal atopic status[9-10]; however, it is unknown whether the intrauterine environment of an allergic mother influences neonatal progenitor TLR expression and function. Our current study demonstrates that maternal allergic sensitization is associated with alterations in TLR expression and function on CB progenitor cells. We found that infant CB progenitor cell TLR-2, -4 and -9 expression was significantly reduced in high risk infants, and that ex vivo ligation of TLR-4 with LPS significantly decreased the functional responsiveness, and thus Eo/B lineage commitment, of CB progenitor cells.

That “high risk” infants’ (i.e., born to allergic mothers) CB progenitor cells have significantly decreased TLR-2, -4 and -9 expression suggests that factors affecting CB $CD34^+$ cell TLR expression may be operative in utero. Maternal allergic sensitization is typified by an ‘atopic milieu’, consisting of elevated ambient levels of the cytokines IL-4, IL-5 and IL-13, as well as higher IgE levels. It has been shown recently that peripheral blood dendritic cell TLR-9 suppression can be induced by IgE activation[22]. Whether[23] or not[24] maternal IgE can cross the placenta has been debated, but IgE produced in the

neonate, through cross reactivity with maternally derived allergens[24,25], has been demonstrated, and thus could explain the observed decreased TLR-9 expression in our high risk infants. However, this may be unlikely since there were no differences between circulating total CB IgE levels in the two groups (Table I). Likewise, T_H2 cytokines such as IL-4 and IL-13, both of which have been detected in placental tissue[26,27], have been shown to down-regulate TLR-4 expression and signalling[28], and could explain our finding of decreased CB $CD34^+$ cell expression of that TLR. Alternatively, genetic factors may also be involved in the modulated expression of these TLRs on CB progenitors. In fact, it has recently been shown that maternal genotype, in particular the ability to signal through TLR-2, -3, -4, -7 and -9, was of vital importance in the subsequent protection of offspring from the development of asthma[29].

Although the prenatal determinants predisposing an infant to allergy are relatively unknown, elucidating the mechanisms regarding poorly acquired immune deviation in children at risk for allergy may prove helpful[30]. Although innate immune responses to TLR ligation depend on a number of variables (e.g., age[31]), it is generally accepted that such stimulation results in the secretion of cytokines that favour Th1 immunity[32]; stimulation with TLR-2[33] or TLR-4[34] agonists directly activate Th1-mediated responses. Interestingly, a study by Pfefferle et al., 2010[14] demonstrated that maternal farming activities modulate CB cytokine profiles in the same fashion. Specifically, the authors showed that children born to mothers with regular farming contact, and presumably more microbial exposure, had increased IFNγ and TNFα levels at birth; both of these are cytokines associated with Th1 immunity. Reduced TLR expression on immunocompetent

cells, possibly in combination with reduced Th1 cytokine production at birth[14], may therefore result in a lower degree of immune deviation and a T_H2-skewed immune system, increasing the risk of developing allergies in later life. Our observation of reduced TLR expression on “high risk” infant CB hematopoietic progenitors, not only agrees with other studies on CB monocytes [5], but may reflect a more general phenotypic alteration across several immune compartments and cells (e.g., monocytes) – which are crucially involved in immune deviation.

Our finding that co-stimulation of CB hematopoietic progenitors with LPS and hematopoietic cytokines in high-risk children resulted in *decreased* Eo/B CFU production, is consistent with previous observations that TLR ligands can influence cell differentiation fates [15-18]. Moreover, the finding of reduced CB Eo/B CFU production in high risk infants after TLR stimulation suggests that TLR engagement on CB $CD34^+$ cells has the capacity to *suppress Eo/B ‘lineage priming’* of myeloid progenitors, decreasing the likelihood of development of allergic eosinophilic/basophilic inflammation, as well as disease maintenance or progression. Our results suggest that *there may exist a ‘window of opportunity’ to revert Eo/B ‘lineage priming’ in children at risk for allergy through engagement of progenitor cell TLR pathways in early life.* Although the operative mechanisms underlying these observations remain elusive, reduced TLR expression on CB progenitors has implications for the signalling cascade triggered by TLR ligation[35-36]; specifically those pathways involved in eosinophilopoiesis[37]. Likewise, early life programming of the developing fetal immune system through epigenetic modulation is

also possible, since bacterial products may alter methylation processes[38] or acetylation of histone proteins[39].

We thus propose *a novel role for TLR-mediated microbial (environmental) regulation of the allergic response: modulation of CB CD34$^+$ myeloid progenitor cell Eo-B differentiative function*, and thus of the capacity to mount an allergic inflammatory (e.g., eosinophilic) tissue response, independent of T-cells. LPS stimulation appears to have a unique suppressor effect on hematopoietic cytokine-mediated Eo/B differentiation *ex vivo*, thereby providing evidence for a novel TLR-mediated mechanism of immune modulation, i.e., down- regulation of eosinophilopoiesis in early life in children at risk for allergy. Although it has previously been reported that LPS stimulation of the nasal mucosa of atopic children suppresses allergen- induced T_H2 responses[34], and that LPS exposure in early life prevents the rate of wheeze in children[40,41], this is the first report of such suppressive effects on neonatal hematopoietic progenitor cell differentiative function in infants at risk for atopy.

It is also important to discuss the statistical considerations that were undertaken in this study. Of note, the exploratory nature of this study which, is generally hypothesis generating, justifies the use of a smaller sample size and no adjustment of the type I error for multiple testing (e.g., using the Bonferroni adjustment) [20]. There are strong statistical arguments against the use of stringent correction factors in such studies[20]. The fact that we observed some signals of significant differences between these two groups supports and strengthens the biological plausibility of the hypotheses. With this consideration, the

findings presented here warrant further investigation and validation in large studies (i.e., using larger sample sizes) where these correction factors would be more accurately applied to avoid Type I (α) errors. It is also noteworthy statistically, that despite the small sample size, the sensitivity analyses using non parametric methods resulted in similar significant findings, which adds to the robustness of the reported findings.

In conclusion, this report demonstrates that high-risk infant CB hematopoietic progenitors have not only significantly reduced TLR-2, -4 and -9 expression, but that this expression is negatively correlated with maternal allergic sensitisation. Moreover, co-stimulation with hematopoietic cytokines and TLR agonists results in *reduced* eosinophil-basophil differentiation in vitro, identifying a novel immunomodulatory effect of TLR stimulation on CB progenitors in relation to allergic risk. Our results suggest that not only may CB progenitor TLR expression potentially identify children at risk for allergy, but that the maternal atopic milieu could be regulating TLR expression and function on CB progenitor cells in utero, a finding which may shed light on how environmental and genetic factors influence the development of allergies even before birth.

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TABLE I. Characteristics of the study population

	Low Risk Infants (n= 15)	High Risk Infants (n=17)	P value
MATERNAL CHARACTERISTICS			
Maternal Age (years)	32.3 [5.2]	33.5 [3.9]	0.433
Parity	1.4 [0.8]	1.0 [0.0, 4.0]†	0.248
Method of Delivery			
Vaginal	12	12	
Caesarean	3	5	0.437
Allergic Phenotype			
Asthma	0	5 (29.4%)	<0.001
Allergic Rhinitis	0	16 (94.1%)	<0.001
Any SPT+	0	17 (100%)	<0.001
HDM SPT+	0	14 (82.3%)	<0.001
Grass pollen SPT+	0	12 (70.6%)	<0.001
Animal dander SPT+	0	3 (17.6%)	<0.001
NEONATAL CHARACTERISTICS			
Gestational Age (days)	278.1 [6.3]	276.5 [9.2]	0.590
Birth Weight (kg)	3.45 [0.4]	3.50 [0.4]	0.708
Birth Length (cm)	49.6 [1.8]	49.5 [1.6]	0.819
Total IgE (kU/L)	0.17 [0.00]	0.17 [0.18, 2.48] †	0.183
Female	11	10	0.405
Male	4	7	

Data are mean [SD]; † Represents median [min, max]; SPT: skin prick test; HDM: house dust mite

TABLE II. Differences between low and high atopic risk infant $CD34^+$ cell phenotype

Phenotype	**Groups: Mean [SD]**		**Mean Difference (95%CI)**	**P- value**
	LR (n=6)	*HR (n=9)*		
TLR-2	8.36 [8.54]	0.17 [0.34]	8.18 (2.15, 14.22)	**0.012**
TLR-4	14.4 [14.8]	1.02 [1.80]	13.38 (2.83, 23.94)	**0.017**
TLR-9	86.57 [6.04]	29.67 [44.66]	56.89 (16.77, 97.02)	**0.009**
GM-CSFRα	18.38 [5.61]	11.29 [6.70]	7.09 (-0.09, 14.27)	0.053
IL-3Rα	7.89 [6.00]	1.64 [1.30]	6.24 (1.85, 10.64)	**0.009**
IL-5Rα	32.46 [8.86]	9.31 [8.48]	23.15 (13.32, 32.97)	**<0.001**

Significant results are shown in Boldface, $p < 0.050$

LR: low-atopic risk, HR: high-atopic risk

SD: Standard deviation

TABLE III. Differences between low and high- atopic risk infant CD34+ cell differentiative function

Eo/B CFU Production	**Groups: Mean [SD]**		**Mean Difference (95%CI)**	**P-value**
	LR (n=9)	*HR (n=8)*		
Hematopoietic cytokine:				
IL-5-responsive	4.22 [4.03]	5.50 [5.93]	-1.28 (-6.47, 3.91)	0.607
IL-3-responsive	9.72 [6.04]	31.81 [16.87]	-22.09 (-34.87, -9.31)	**0.002**
GM-CSF-responsive	4.72 [4.16]	23.25 [16.11]	-18.53 (-30.35, -6.70)	**0.004**
	LR (n=6)	*HR (n=5)*		
Addition of LPS:				
IL-5-responsive	6.08 [3.71]	1.20 [1.15]	4.88 (0.96, 8.81)	**0.020**
IL-3-responsive	13.83 [12.61]	4.60 [3.79]	9.23 (-4.10, 22.57)	0.152
GM-CSF-responsive	14.92 [11.00]	2.00 [2.67]	12.92 (1.42, 24.41)	**0.032**

Significant results are shown in Boldface, $p < 0.050$

LR: low-atopic risk, HR: high-atopic risk, Eo/B CFU: eosinophil- basophil colony forming unit

SD: Standard deviation

Figure Legends

Table I. Characteristics of the Study Population. Data are shown as mean [SD] or median [min, max]. Data illustrating maternal atopic variables are represented as the number of individuals (percentage of group).

Table II. Differences between low and high-atopic risk infant $CD34^+$ cell phenotype. Data are presented as mean [SD] and mean differences 95% [CI]. Data were analyzed using t- test.

Table III. Differences between low and high-atopic risk infant $CD34^+$ cell differentiative function. Data are represented as mean [SD] and mean differences 95% [CI]. Data were analyzed using t- test.

Figure 1. Differential CB $CD34^+$ progenitor cell expression of TLR-2, -4 and -9 between high and low risk infants. CB MNC from high and low risk infants were stained with antibodies to surface TLR-2 and -4 and intracellular TLR-9. $CD34^+$ progenitor cells were gated on and analysed for differences in TLR expression. Data are presented as geometric mean (95%CI) and significant findings are indicated on the graph.

Figure 2. Differential CB $CD34^+$ progenitor cell expression of IL-3Rα, IL-5Rα and GM-CSFRα between high and low risk infants. CB MNC from high and low risk infants were stained with antibodies to surface IL-3Rα, IL-5Rα and GM-CSFRα. $CD34^+$ progenitor cells were gated on and analysed for differences in hematopoietic cytokine receptor expression. Data are presented as geometric mean (95%CI) and significant findings indicated on the graph.

Figure 3. Responses to hematopoietic cytokine (IL-3) with or without LPS co-stimulation of CB CD34$^+$ progenitor cells in high and low risk children. CB MNC from low- and high-risk infants were enriched for CD34$^+$ cells and stimulated with the hematopoietic cytokine IL-3 (1 ng/ml) or IL-3 and LPS (10μg/ml) for 14 days (5% CO_2, 37°C). Data presented as mean ± SEM and significant findings are indicated on the graph.

Figure 4. Responses to hematopoietic cytokine (IL-5) with or without LPS co-stimulation of CB CD34$^+$ progenitor cells in high and low risk children. CB MNC from low- and high-risk infants were enriched for CD34$^+$ cells and stimulated with the hematopoietic cytokine IL-5 (1 ng/ml) or IL-5 and LPS (10μg/ml) for 14 days (5% CO_2, 37°C). Data presented as mean ± SEM and significant findings are indicated on the graph.

Figure 5. Responses to hematopoietic cytokine (GM-CSF) with or without LPS co-stimulation of CB CD34$^+$ progenitor cells in high and low risk children. CBMNC from low- and high-risk infants were enriched for CD34$^+$ cells and stimulated with the hematopoietic cytokine GM-CSF (10 ng/ml) or GM-CSF and LPS (10μg/ml) for 14 days (5% CO_2, 37°C). Data presented as mean ± SEM and significant findings are indicated on the graph.

Figure 1

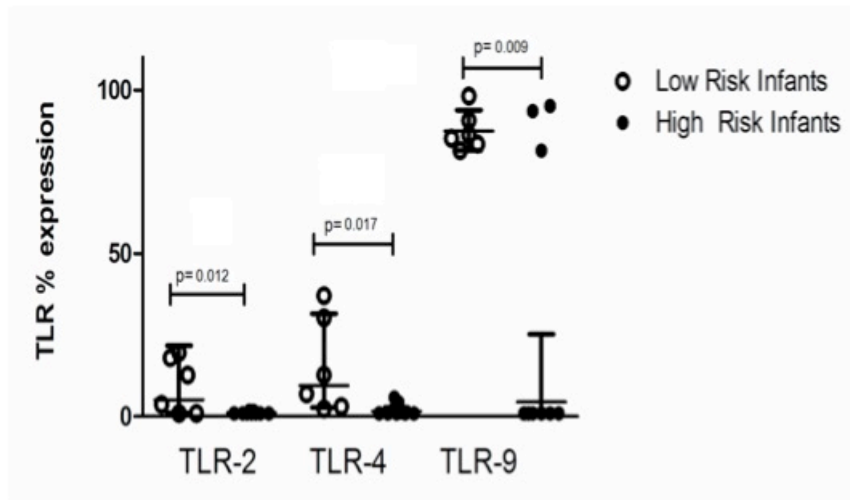


Figure 2

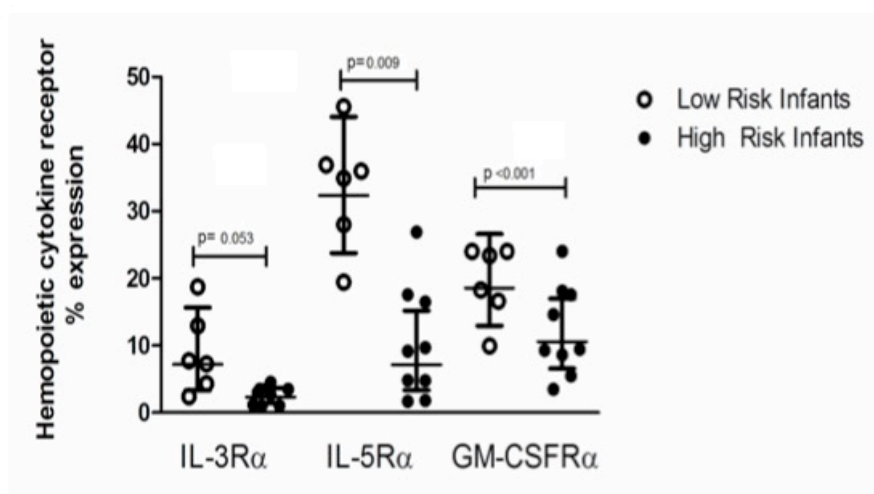


Figure 3

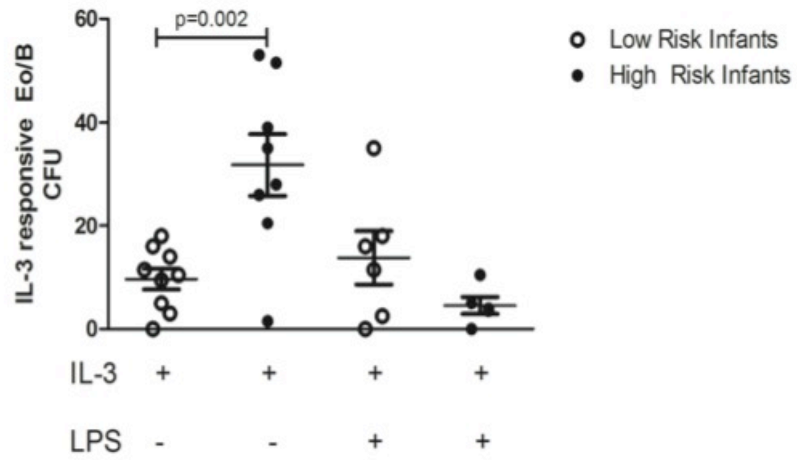


Figure 4

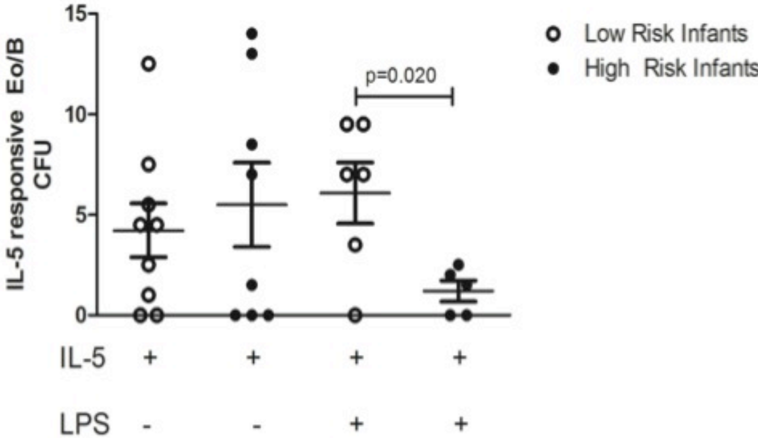
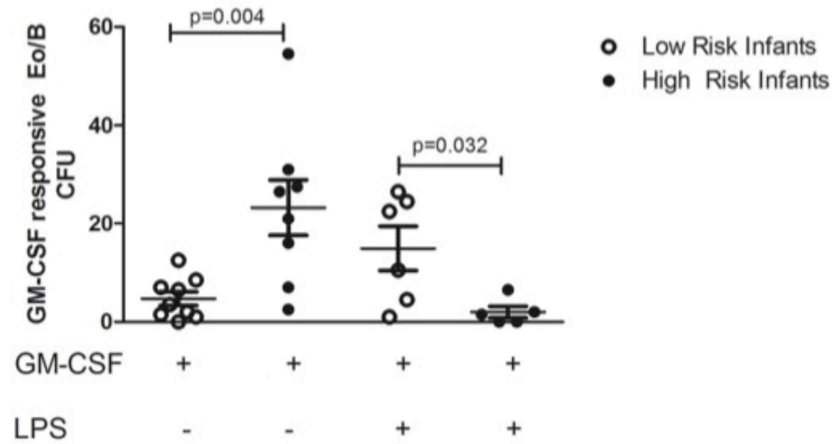


Figure 5



CHAPTER 4

IL-4 and IL-13 Differentially Regulate Toll-Like Receptor Induced Eosinophil-Basophil Differentiation of Cord Blood $CD34^+$ Progenitor Cells

Submitted

This paper has been submitted. In the previous chapter we reported that at-risk infants have reduced LPS-induced Eo/B CFU formation compared to low-risk infants. These findings may imply that infant allergy (as defined by maternal SPT) is associated with dysregulated LPS signalling [154,155]; however, the factor(s) which influence neonatal LPS signalling are unknown. As allergy is associated with a T_H2 driven pathology, the studies in this chapter investigated whether T_H2 cytokines, which are present in placental tissue [164,165] and shown to influence innate immunity [166], can modulate neonatal $CD34^+$ cell LPS-induced Eo/B CFU formation. We show that CB progenitors isolated from healthy pregnant women have reduced LPS-induced Eo/B CFU in response to IL-4:IL-4Rα signalling via inhibition of ERK 1/2 signalling. These findings not only expand on those of chapter 2, but may also provide a mechanistic basis for the findings in chapter 3.

Drs. Judah Denburg and Roma Sehmi, and I were responsible for the experimental design and interpretation of the results obtained. I was responsible for generating the data and for writing the article. Both Drs. Denburg and Sehmi were involved in the editing process.

IL-4 and IL-13 Differentially Regulate Toll-Like Receptor-Induced Eosinophil-Basophil Differentiation of Cord Blood CD34^{+} Progenitor Cells

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Short title: IL-4 inhibits LPS-induced Eo/B differentiation

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Summary

Intrauterine environmental exposures have been shown to influence neonatal immunity and subsequent allergic development. Since we have previously shown that high-atopic risk infants have decreased eosinophil-basophil (Eo/B) differentiation after stimulation with lipopolysaccharide (LPS), we investigated whether a T_H2 milieu (IL-4 or IL-13), found in placental and cord blood (CB), could influence LPS-induced Eo/B colony formation (CFU) and the mechanism(s) through which this might occur. CB $CD34^+$ cells from healthy donors were stimulated with IL-4 or IL-13 (in combination with LPS) and assessed for Eo/B differentiation using methylcellulose cultures or Eo/B differentiation-related intracellular signalling pathways, using flow cytometry. Additionally, pharmacological inhibitors were added to the methylcellulose cultures to determine the effect of blocking intracellular signalling in $CD34^+$ cells in relation to Eo/B CFU formation. We found that stimulation of $CD34^+$ cells with IL-4, but not IL-13, reduced Eo/B CFU formation in the presence of LPS; this was found to be dependent on IL-4Rα and not IL-13Rα1. Additionally, IL-4 reduced the expression of ERK 1/2 after LPS stimulation, which was recovered by inhibition of IL-4Rα. Addition of IL-13 did not have an inhibitory effect on ERK 1/2 expression. Functionally, *ex vivo* methylcellulose cultures demonstrated that inhibition of ERK 1/2 significantly reduced Eo/B CFU formation. We thus show for the first time that the responsiveness of CB $CD34^+$ progenitor cells to LPS is differentially regulated by the T_H2 cytokines IL-4 and IL-13. This may have implications for *in utero* interactions between placental-derived pro-allergic cytokines

and neonatal progenitor cells, which by regulating Eo/B differentiation, could influence the development of allergic inflammation in early life.

Abbreviations

TLR	toll-like receptor
LPS	lipopolysacchride
BM	bone marrow
CB	cord blood
Eo/B	eosinophil-basophil
sMFI	specific median fluorescence intensity
CFU	colony forming units
GM-CSF	granulocyte-macrophage colony-stimulating factor
PB	peripheral blood
JAK	janus kinase
STAT	signal transducer and activator of transcription
SOCS	suppressor of cytokine signalling
MAPK	mitogen activated protein kinase
ERK	extracellular regulate kinase
CFU	colony forming unit

Introduction

The dramatic and recent rise in allergies, along with their early onset suggests that *in utero* events are critical to the development of allergies [1]. Environments rich in microbes, such as farming environments, appear to protect against the development of allergies in children, especially when the exposure is pre-natal [2]. These protective effects are associated with alterations in both the neonatal innate [3,4] and adaptive [5] immune systems. These studies suggest that the microenvironment of the uterus plays a key role in shaping the infant's response to environmental stimuli, which subsequently influences the development of allergy [1]. Although it is unknown how the maternal environment may exert such effects, it is tempting to speculate that the fetal immune system interacts with the cytokine milieu prevailing in the mother through the fetal-placental interface [6].

Our group has extensively investigated the role of hematopoietic progenitor cells in infant CB in relation to allergic risk [7-10]. We have recently shown that the presence of maternal atopy alters CB progenitor toll-like receptor (TLR) phenotype and function; at-risk infant $CD34^+$ cells express reduced TLRs with muted LPS-induced Eo/B CFU [10], compared to low-risk infants. Investigation into the protective effect afforded by farming environments [2] has shown that the outcome of gene-environment interactions may be related to epigenetic mechanisms; both placental CD14 expression [11] and CB T regulatory FOXP3 expression [5] are under epigenetic control. Therefore our previous findings suggest that a genetic predisposition to allergy (as assessed by maternal allergic sensitization), and/ or epigenetic effects related to the maternal environment, [5,11] are

associated with altered LPS signalling in the neonate. Given the importance of maternal factors, including cytokines [6], in altering neonatal immune responses, we investigated the effect of a prototypical atopic 'T_H2 milieu' on hematopoietic progenitor cell responses to LPS.

The T_H2 cytokines IL-4 and IL-13 are secreted by variety of leukocytes and play an important role in the development of allergic responses. These cytokines are involved in IgE production [12] and eosinophil recruitment to the airways [13]. Not only is the expression of these cytokines found to be increased in the airways of allergic subjects [14] but high serum levels of IL-4 have been detected in CB of at-risk infants who subsequently develop atopic disease [15,16]. Although these cytokines have been recently also shown to influence human CB $CD34^+$ cell chemotaxis [17] and murine bone marrow (BM) Eo/B CFU formation *ex vivo* [18], it is unclear what effects IL-4 or IL-13 might have on LPS-induced Eo/B CFU formation from human CB $CD34^+$ cells.

In this study, we investigated whether the T_H2 cytokines IL-4 and IL-13 influence LPS-induced Eo/B CFU of CB progenitor cells. We *hypothesized* that TLR-induced signalling may be altered by T_H2 cytokines, representative of an "atopic milieu", resulting in reduced Eo/B CFU [10]. We show that IL-4:IL-4Rα inhibits LPS-induced Eo/B CFU by blocking ERK 1/2 signalling in CB $CD34^+$ cells. Since Eo/B differentiation is altered in children at risk for allergy [7-10], improved understanding of Eo/B differentiation processes may permit novel approaches targeting the regulation of these cells and the modulation of Eo/B-mediated allergic inflammation in early life.

Materials and Methods

Cord blood collection

Pregnant mothers admitted to the Labour and Delivery ward at McMaster University Medical Centre, Hamilton, ON, Canada provided informed consent for CB donation prior to delivery. The CB samples were collected from otherwise healthy pregnant women. Upon delivery, each CB sample was collected in a 60mL syringe containing 2mL of heparin (1000 units/mL; Sigma, Mississauga, ON), stored at 4°C until processing. This study was approved by the Hamilton Health Sciences/ McMaster Faculty of Health Sciences Research Ethics Board.

Cord blood processing and CD34$^+$ cell enrichment

CB samples were depleted of erythrocytes using gravity sedimentation as previously described [10]. To enrich the sample for CD34$^+$ cells, the pellet was resuspended at a concentration of 5 x 10^7 cells/mL in RoboSep Buffer (PBS containing 2% FBS and 1mM EDTA; Stem Cell Technologies, Vancouver, BC). The cells were transferred to a 5mL falcon polystyrene round-bottom tube (Becton Dickenson 2058, Franklin Lakes, NJ) and EasySep Negative Selection Human Progenitor Cell Enrichment Cocktail with CD41 depletion (Stem Cell Technologies) at a concentration of 50µL/mL cells was added. The solution was mixed and incubated for 15 min at room temperature. The magnetic nanoparticles (Stem Cell Technologies) were added at a concentration of 50µL/mL cells and incubated for 15 min at room temperature. The cell suspension was then brought to a total volume of 2.5 mL by adding RoboSep Buffer and the tube was placed inside the

RoboSep Magnet (Stem Cell Technologies) for 10 min at room temperature. This sample was further enriched by placing the liquid portion in a new 5mL tube and re-incubating the sample in the magnet for 10 min. The purity of $CD34^+$ cells was between 85-90%.

Methylcellulose cultures

Methylcellulose colony assays were completed as previously described [10] using enriched CB $CD34^+$ at a plating concentration of 2×10^4 cells/ 35mm x 10 mm culture dish (Falcon Plastics, CA) in duplicate. In addition, cultures were supplemented with IL-4 or IL-13 (50ng/mL) to assess their role in Eo/B CFU formation. For receptor blocking/neutralization experiments, anti-IL-4Rα or -IL-13Rα1 (5 and 50μg/mL; R&D Systems) were added to the methylcellulose cultures supplemented with IL-4, LPS and hematopoietic cytokines.

The role of LPS-induced signalling on Eo/B CFU formation was investigated by adding the specific inhibitors to the methylcellulose culture assay: 5 or 50 μM STAT5 inhibitor [19] or PD98059 (ERK 1/2 inhibitor) [20], or 2 or 20μM SB203580 (p38 MAPK inhibitor) [21] (Calbiochem, Cambridge, MA) or dimethylsulfoxide (DMSO) vehicle control. These concentrations were found to be non-toxic to cells.

Phospho-flow to detect intracellular activation of signalling pathway molecules

LPS from Escherichia coli 0111:B4 was purchased from Sigma and used at the optimal concentration of 10μg/ml as previously reported [10]. For stimulation studies, enriched $CD34^+$ cells were stimulated with LPS and IL-4 or IL-13 (50ng/mL) for 5, 15 or 30min at

37°C 5% CO_2 in tissue culture plates Multiwell (Falcon) with RPMI complete (RPMI plain, HEPES, Pen/ Strep and FBS). For receptor neutralization studies, cells were incubated with anti-IL-4Rα or -IL-13Rα1 (R&D Systems) for 15min before the addition of IL-4 or IL-13, respectively and LPS. Following the incubation, cells were fixed using PhosFlow CytoFix Buffer (BD Biosciences, Ontario, Canada), and then centrifuged for 10 min at 1500 RPM. After washing, cells were permeabilized (PhosFlow Perm Buffer III; BD Biosciences) for 30 min on ice, washed with FACS Buffer (PBS, 0.1% sodium azide) then stained with a PE-conjugated phospho-specific mAb against p38 MAPK (pT180/pY182), STAT5 (pY694) or ERK 1/2 (pT202/pY204), FITC conjugated CD45 and PerCP conjugated CD34 or isotype control, all purchased from BD Biosciences.

Acquisition and analysis

Acquisition was performed using a LSR II flow cytometer (BD Bioscience); 5×10^3 events were collected for analysis. To enumerate $CD34^+$ cells, we used an established multiparameter gating strategy as previously described [10]. The amount of phosphorylated p38, STAT5 or ERK 1/2 was calculated using an expression index of the specific median fluorescence intensity (sMFI): $sMFI_{stimulated\ cells}/sMFI_{unstimulated\ cells}$ [22].

Statistical analysis

Data were analyzed using IBM SPSS Statistics version 20.0 (Chicago, IL) and represented in figures as mean ± SEM. Data that were not normally distributed were log transformed and subsequently analyzed with the t- test to compare differences between two groups, or the ANOVA with a Dunnett *post hoc* analysis for many groups. We

further applied the Mann-Whitney U test to assess the sensitivity or robustness of the results, and the results were consistent. We set the criterion for statistical significance a priori at $\alpha = 0.05$. All p values were reported to two decimal places.

Results

IL-4 and IL-13 differentially influence GM-CSF- and IL-3-responsive Eo/B CFU formation after LPS stimulation

We were first interested in whether the T_H2 cytokines, IL-4 and IL-13, influence LPS-induced Eo/B CFU formation. To test this, IL-4 and IL-13 were added separately to methylcellulose cultures containing hematopoietic cytokine and LPS stimulated CB $CD34^+$ cells. As shown in Figure 1A-B, incubation with IL-4 or IL-13 alone was not sufficient to induce Eo/B CFU production. However, the addition of IL-4 to GM-CSF and LPS ($p = 0.028$) or IL-3 and LPS ($p=0.007$) stimulated cultures resulted in a significant decrease in Eo/B CFU, compared to these same cultures without IL-4 stimulation (Fig 1A). Conversely, the addition of IL-13 to any of the culture conditions did not reduce the number of Eo/B CFU compared to the LPS stimulated conditions (Fig 1B).

IL-4Rα is necessary for IL-4-induced reduction in GM-CSF- and IL-3-responsive Eo/B CFU

To determine whether the inhibitory effect of IL-4 on LPS-induced Eo/B CFU formation (Fig 1A) was dependent on IL-4Rα, CB enriched $CD34^+$ cells were incubated with anti-IL-4Rα or -IL-13Rα1 in the methylcellulose cultures for 14 days. As shown in Figure 2A, the addition of anti-IL-4Rα to the cultures resulted in an increase in the number of GM-CSF- ($p=0.015$) and IL-3-responsive ($p=0.033$) Eo/B CFU formed in the presence of LPS. Furthermore, the addition of anti-IL-13Rα1 did not restore the number of GM-CSF- or IL-3-responsive Eo/B CFU formed in the presence of LPS (Fig 2B); colony counts remained significantly reduced ($p=0.020$ and $p=0.040$ respectively) as compared to LPS

stimulated cultures. This supports a specific role for IL-4:IL-4Rα signalling, independent of IL-13Rα1, in the inhibition of LPS-induced Eo/B CFU.

IL-4 and IL-13 differentially influence LPS-induced phosphorylation of ERK 1/2

In order to investigate the potential mechanism of reduced LPS-induced GM-CSF- and IL-3-responsive Eo/B CFU formation in the presence of IL-4, we next determined if IL-4 had any effect on LPS-induced intracellular signalling [25] in CB $CD34^+$ cells. To do this, $CD34^+$ cells were incubated with LPS with our without IL-4 or IL-13 and analyzed using flow cytometry. As show in Figure 3A, the addition of IL-4 to LPS-stimulated $CD34^+$ cells resulted in a significant reduction in the expression of ERK 1/2 ($p= 0.015$), whereas the addition of IL-13 did not. Additionally, pre-incubation with anti-IL-4Rα to block IL-4 signalling recovered the expression of ERK 1/2 in the presence of LPS (Fig 3A). We were unable to detect any differences in p38 MAPK or STAT5 expression with the addition of IL-4 to LPS stimulated $CD34^+$ cells (Fig 4A and 5A respectively).

Inhibition of ERK 1/2 reduces GM-CSF-and IL-3-responsive Eo/B CFU formation after LPS stimulation

Eosinophilopoiesis is a highly regulated process that has been demonstrated to be dependent on ERK signalling of murine BM $CD34^+$ cells [20]. Since we found that IL-4 down-regulated LPS-induction of ERK 1/2 (Fig 3A), we next assessed the effects of blocking ERK 1/2 in CB $CD34^+$ cell Eo/B CFU formation in the presence of LPS. As shown in Figure 3B, the addition of PD98059, a specific pharmacological inhibitor of ERK 1/2 signalling [20], resulted in reduced GM-CSF- ($p= 0.021$) and IL-3-responsive

(p=0.008) Eo/B CFU formation in presence of LPS. No differences were observed with IL-5-responsive Eo/B CFU.

Although we did not observe significant differences in p38 MAPK (Fig 4A) or STAT5 (Fig 5A) after IL-4 stimulation, we did evaluate the effect of inhibiting these proteins since they are involved in CB [21,23] and PB [24] progenitor cell derived eosinophilopoiesis. As shown in Figure 4B, blocking p38 MAPK in the presence of LPS reduced GM-CSF-(p= 0.031), IL-3-(p=0.023) and IL-5- (p=0.006) responsive Eo/B CFU. As shown in Figure 5B, blocking STAT5 reduced GM-CSF- (p = 0.031), IL-3- (p=0.011) and IL-5-responsive (p=0.034) Eo/B CFU in the presence of LPS.

Discussion

Prenatal exposure to microbes has a protective effect on the development of allergy in children [2]. We have previously shown that a genetic predisposition to allergy reduces the Eo/B differentiative response of CB progenitor cells to LPS, whereas low-atopic risk infants produce more Eo/B colonies [10]. These findings suggest that maternal atopy is associated with deficient LPS signalling in infant CB [25-28]; however, the factor(s) that influence this relative deficiency are unclear. In the present report, we investigated how a T_H2 microenvironment could influence CB progenitor cell Eo/B differentiation responses to LPS . We show for the first time that IL-4, but not IL-13, reduces CB progenitor Eo/B differentiation responses to LPS *in vitro*.

The cytokines IL-4 and IL-13 have been shown to influence hematopoietic progenitor cell chemotaxis [17] and Eo/B CFU [18] in human and murine systems, respectively. In these models, IL-13 was found to have an enhancing effect on both migrational responses to stromal-derived factor 1α, and on Eo/B differentiation. Our observation that IL-13 did not have an inhibitory effect on Eo/B CFU (Fig 1B) is in line with those of others [18] demonstrating promotion of eosinophilopoiesis. Conversely, there are reports in which IL-4 has an inhibitory role in both eosinophilopoiesis of CB-derived progenitor cells [29,30], and peripheral blood eosinophil survival [31]. Interestingly, work by the latter group found that patients with atopic dermatitis, a T_H2-driven disease, had significantly greater eosinophil apoptosis in response to IL-4 [31]. Our findings therefore agree and extend the former studies [29,30] by demonstrating an inhibitory effect of IL-4 on LPS-induced Eo/B CFU (Fig 1A), which is dependent on IL-

4Rα (Fig 2A) and not IL-13Rα1 (Fig 2B). Our results demonstrate that IL-4 and IL-13, which generally have redundant signalling responses due to shared receptor subunits, can stimulate divergent responses when they ligate their cognate receptors [32]: inhibition of Eo/B CFU in the presence of LPS is dependent on Type I (IL-4Rα:γc), as opposed to Type II (IL-4Rα:IL-13Rα1) signalling, accounting for non-redundant effects [32] on Eo/B CFU formation.

It has recently been shown that maternal allergic history is associated with impaired LPS-induced phosphorylation of p38 MAPK and ERK 1/2 in PB monocytes [25,27]. This can be attributed to the activation of JAK-3 [33] and SOCS-1 [34], both of which are activated by IL-4 signalling and involved in suppression of LPS signalling. The fact that decreased LPS signalling was reproduced by pre-incubation of control (non-atopic) cells with IL-4 [25] is similar to our use of IL-4 to recapitulate the maternal allergic microenvironment *in utero,* and also in line with our data demonstrating the downregulatory role of IL-4 (pseudo maternal allergy) on $CD34^+$ cell LPS signalling. Therefore, our present findings suggest that in at-risk infants, with increased CB levels of IL-4 [15,16] have an inhibitory effect on Eo/B CFU [29,30] through altered CB $CD34^+$ cell responsiveness to LPS [10].

Our findings of suppressive effects of IL-4 on LPS signalling are in line with previous reports in the literature. For example, Hunt et al., [35] showed that exposure of IL-4 to murine macrophages suppressed LPS upregulation of MAPK expression; findings which were repeated in neutrophils [36] and microgial cells [37]. We show here that IL-4 also downregulates LPS-induced MAPK signalling in $CD34^+$ cells which not only has

implications for Eo/B CFU in *vitro* (Fig 3A) but is opposite to the effect of IL-13 (Fig 1B). Furthermore, studies showing that IL-13 activates MAPKs [38] agree with our observations that IL-13 does not inhibit LPS signalling.

The use of pharmacological inhibitors in the methylcellulose assay demonstrated the biological implications of IL-4 inhibition of ERK 1/2 signalling in CB $CD34^+$ cells. Our colony data (Fig 3B) not only reinforce the importance of MAPK signalling [20] in eosinophil differentiation, but also corroborate our phospho-flow analysis illustrating reduced ERK 1/2 expression after IL-4 stimulation (Fig 3A). The addition of IL-4 to the cultures not only reduced both GM-CSF- and IL-3-responsive Eo/B CFU in the presence of LPS (Fig 1A), but reduced LPS-induced ERK 1/2 signalling (Fig 3A), a finding supported by others [25]. Furthermore, *in vitro* inhibition of ERK 1/2 significantly reduced both GM-CSF- and IL-3-responsive Eo/B CFU (as opposed to IL-5 responsive colonies), which not only reinforces the specificity for ERK signalling in IL-4 inhibitory responses, but also provides a mechanistic basis for this inhibitory effect.

Both the *in utero* environment in which the fetus develops [11] and the neonatal immune system [39] are under epigenetic control. Studies involving pregnant mothers living in rural (farming) areas, where the incidence of allergies are low [2], compared to those living in urban areas, have demonstrated that both placental tissue CD14 expression [11] and CB T regulatory cell FOXP3 expression [5] are influenced epigenetically. Additionally, *in utero* smoke exposure has been associated with altered DNA methylation in CB cells [40]. In relation to allergy, murine models have demonstrated that dendritic cells from neonates born to asthmatic mothers have broadly increased DNA methylation

from birth, which allows these cells to mediate asthma susceptibility through increased *in vitro* allergen presentation [39]. In human studies, it has been shown that PB cells from children with atopic disease have altered methylation [41] and acetylation [42] patterns. Despite maternal allergy conferring an altered epigenetic profile in neonatal cells [39], and the importance of maternal cytokines in regulating neonatal immune responses [6], a specific role for epigenetic regulation by the intrauterine cytokine environment has not been demonstrated [43]. Emerging data supporting the role of epigenetics in both hematopoiesis [44] and allergic-risk [39], suggest that identification of epigenetic effects induced by intrauterine cytokines could be a target of fruitful investigation.

Differential responses of CB $CD34^+$ cells between low- and high-atopic risk neonates may depend on cytokine influences *in utero*, suggesting that an *in utero* atopic (T_H2) cytokine milieu can influence the innate immune responses of neonatal $CD34^+$ cells [10] and thus the development of allergic inflammatory responses in early life.

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Figure Legends

Figure 1A -B. IL-4, but not IL-13, inhibits LPS-induced Eo/B CFU. CB CD34+ cells were cultured for 14 days (5% CO_2, 37°C) with hematopoietic cytokines IL-5 (1 ng/mL), IL-3 (1 ng/mL) or GM-CSF (10ng/mL) with or without LPS, IL-4 (A), or IL-13 (B). Data represented as mean ± SEM and significant findings presented by an asterisk (*), $p \leq 0.05$.

Figure 2A-B. Inhibition of IL-4Rα, but not IL-13Rα1, recovers LPS-induced Eo/B CFU. CB CB CD34+ cells were cultured for 14 days (5% CO_2, 37°C) with hematopoietic cytokines IL-5 (1 ng/mL), IL-3 (1 ng/mL) or GM-CSF (10ng/mL) with or without LPS, IL-4 and anti-IL-4Rα (A), or anti-IL-13Rα1 (B). Data represented as mean ± SEM and significant findings presented by an asterisk (*), $p \leq 0.05$.

Figure 3A-B. IL-4, but not IL-13, inhibits LPS-induced ERK 1/2. A) CB CD34+ cells were pre-incubated with anti-IL-4Rα or anti-IL-13Rα1 Abs and stimulated with LPS and IL-4 or IL-13 respectively. Cells were stained with antibodies to intracellular ERK 1/2 and analyzed using flow cytometry. B) CD34+ cells were incubated with a pharmacological inhibitor ERK1/2 (PD98059) or DMSO vehicle control in methylcellulose cultures stimulated hematopoietic cytokines IL-5 (1 ng/mL), IL-3 (1 ng/mL) or GM-CSF (10ng/mL) with or without LPS for 14 days (37°C and 5% CO_2). Eo/B cultures are tight, granular clusters of 40 cells or more. Data presented as mean ± SEM and significant findings presented by an asterisk (*), $p \leq 0.05$.

Figure 4A-B. Effect of IL-4 or IL-13 on LPS-induced p38 MAPK expression. A) CB CD34+ cells were pre-incubated with anti-IL-4Rα or anti-IL-13Rα1 Abs and stimulated

with LPS and IL-4 or IL-13 respectively. Cells were stained with antibodies to intracellular p38 MAPK and analyzed using flow cytometry. B) $CD34^+$ cells were incubated with a pharmacological inhibitor p38 MAPK (SB203580) or DMSO vehicle control in methylcellulose cultures stimulated hematopoietic cytokines IL-5 (1 ng/mL), IL-3 (1 ng/mL) or GM-CSF (10ng/mL) with or without LPS for 14 days (37°C and 5% CO_2). Eo/B cultures are tight, granular clusters of 40 cells or more. Data presented as mean ± SEM and significant findings presented by an asterisk (*), $p \leq 0.05$.

Figure 5A-B. Effect of IL-4 or IL-13 on STAT5 expression after LPS stimulation. A) CB $CD34^+$ cells were pre-incubated with anti-IL-4Rα or anti-IL-13Rα1 Abs and stimulated with LPS and IL-4 or IL-13 respectively. Cells were stained with antibodies to intracellular STAT5 and analyzed using flow cytometry. B) $CD34^+$ cells were incubated with a pharmacological inhibitor to STAT5 or DMSO vehicle control in methylcellulose cultures stimulated hematopoietic cytokines IL-5 (1 ng/mL), IL-3 (1 ng/mL) or GM-CSF (10ng/mL) with or without LPS for 14 days (37°C and 5% CO_2). Eo/B cultures are tight, granular clusters of 40 cells or more. Data presented as mean ± SEM and significant findings presented by an asterisk (*), $p \leq 0.05$.

Figure 6. Schematic diagram of IL-4 inhibition of LPS-induced Eo/B CFU. This study demonstrates that LPS-induced GM-CSF- and IL-3-responsive Eo/B CFU is dependent on ERK 1/2 signalling. The addition of IL-4 to LPS stimulated $CD34^+$ cells inhibits LPS-induced ERK 1/2 signalling thereby reducing Eo/B CFU formation.

Figure 1

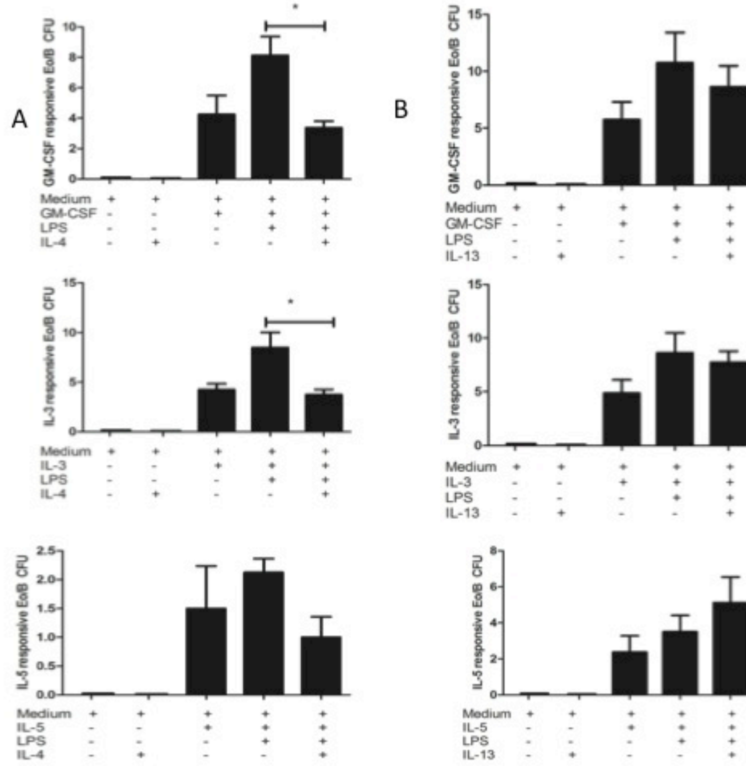


Figure 2

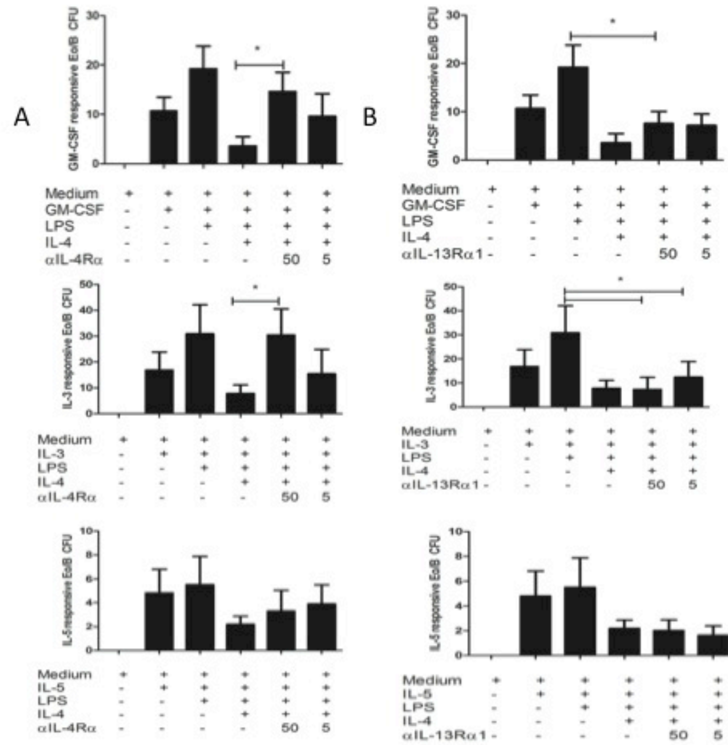


Figure 3

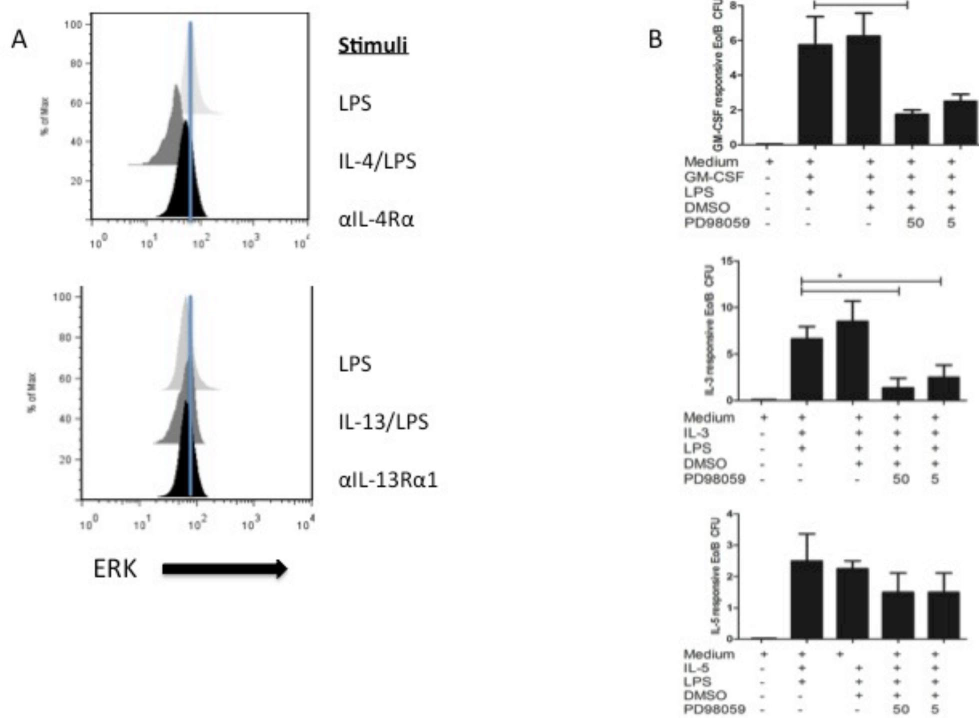
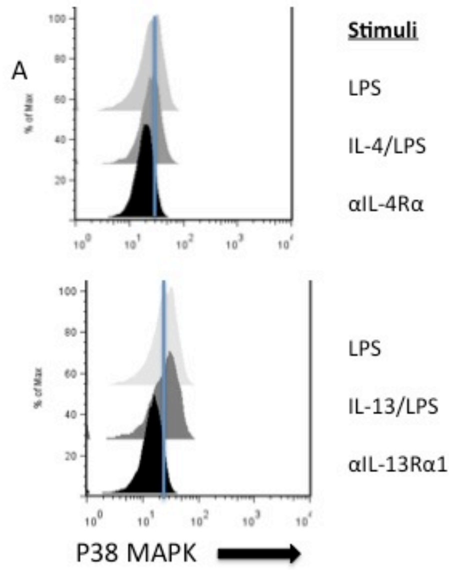


Figure 4



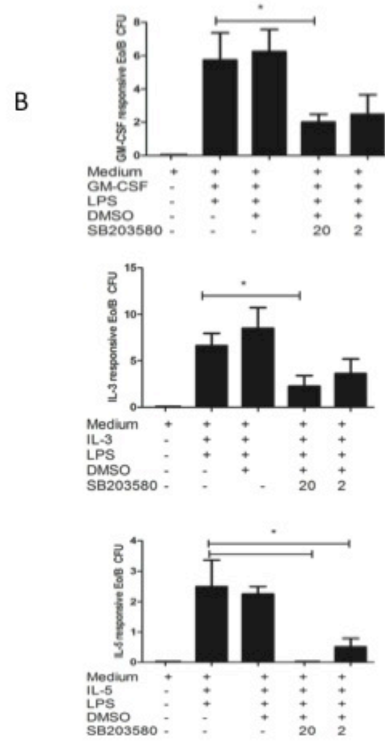
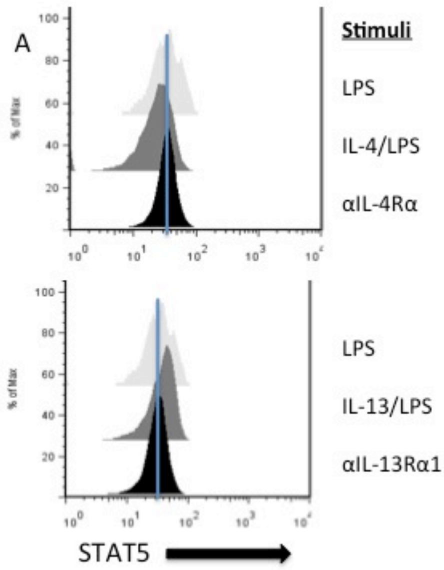


Figure 5



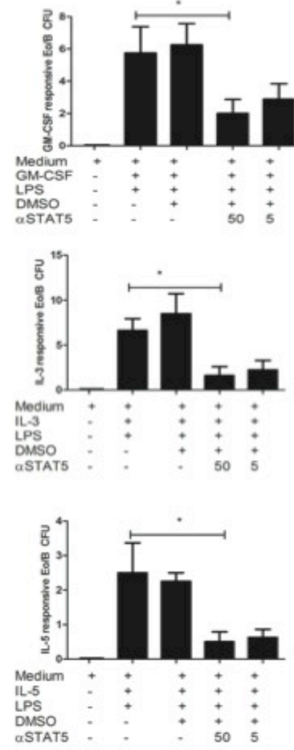
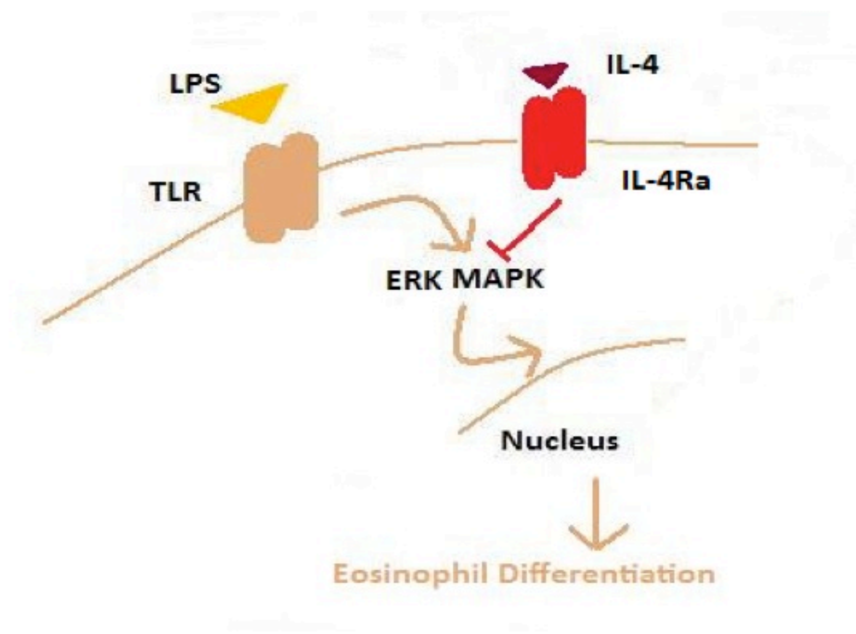


Figure 6



CHAPTER 5

Discussion

With the dramatic and recent rise in allergic diseases and asthma, and their manifestations in early life, *in utero* influence on neonatal immune development [4,167] and allergic propensity [132] represent prime areas for investigation. In our laboratory's work, CB progenitor cell phenotype and function have been shown to be altered with respect to maternal allergic predisposition [6] and diet [7], providing evidence of early life maternal (genetic and environmental) effects on neonatal immunity. With realisation of the increasing importance of innate immune stimulation in early life as a potential protecting factor against allergy [140], and evidence supporting a role for TLR-mediated differentiation of BM progenitor cells [10], the purpose of this thesis was therefore to examine the potential influence of maternal allergy, as well as innate (LPS and TLR-mediated) or adaptive (T_H2 cytokine-mediated) immune stimuli on CB progenitor cell Eo/B differentiation. The overarching hypothesis for the studies found herein is that *CB differentiation in response to LPS stimulation is altered by maternal atopy*, therefore influencing Eo/B development and thus, allergic inflammation, in early life.

The data presented in this thesis provide evidence for prenatal influences attributable to either genetic predisposition (e.g., maternal atopy and allergic sensitization), environment (as represented by surrogates, such as LPS- or T_H2 cytokine-stimulation), and/or gene-environment interactions (i.e., epigenetics) on the developmental decisions of CB progenitor cells. In this regard, the data described in chapter 2 demonstrate that CB progenitor cells are responsive to LPS stimulation through

increased GM-CSF-responsive Eo/B CFU. Chapter 3 explores the influence of maternal atopy on LPS-induced Eo/B CFU, finding that high-atopic risk infant CB progenitor cells produce less Eo/B CFU after stimulation with LPS, as compared to their low-atopic risk counterparts. Since these results suggest that maternal atopy influences neonatal immune responses *through* decreased LPS signalling [154,155], the data presented in chapter 4 show that T_H2 cytokines, which are increased in CB of at-risk infants [168-170] and probably secreted by the placenta [164,165] influence CB progenitor Eo/B differentiation. It was demonstrated that IL-4:IL-4Rα is involved in suppression of LPS signalling and Eo/B CFU formation, whereas IL-13 was not. These results not only provide a mechanistic basis for reduced Eo/B CFU in atopic-risk infants (chapter 3) but also show how cytokines found in the intrauterine environment [168-170] may modulate progenitor responses to LPS (chapter 2). Taken together, these findings support the concept that even before birth, maternal allergy ("genes") as well as *in utero* cytokines ("environment")—or interactions among these factors-- influence neonatal progenitor responses to immune stimuli. Such modulation of neonatal hematopoietic progenitor responses could then influence the development of allergic responses in early life.

5.1 Genetic Polymorphisms in TLRs and allergy

Allergic diseases are complex inflammatory disorders which are influenced by both genetic and environmental factors. Due to repeated correlation between higher TLR expression and lower incidence of allergy in farming environments, investigations into potential TLR polymorphisms in relation to allergy were first initiated [171]. With relevance to this thesis, specific polymorphisms in genes for many members of the TLR

family, including TLR-2, -4 and -9 [172], have been demonstrated to be associated with allergy and asthma. Additionally, beyond the actual TLR, polymorphisms in genes encoding downstream signalling molecules have also been linked to allergy [173] including IRAK-4 which is associated with serum IgE levels in asthmatics [174]. At least part of these gene-environment interactions may be mediated by epigenetic mechanisms (as further discussed below) [175]. For example, murine models demonstrate that IgE production correlates with hypomethylation of the IL-4 promoter [176].

Which precise TLR polymorphisms play functional roles in the hematopoietic progenitor responses observed in the high-atopic risk infants evaluated in our cohort are unknown, though potentially such polymorphisms could provide mechanistic explanations for our findings [177]. It is possible that the low- and high-risk infants assessed in one study in this thesis (Chapter 3) carry specific genotypes (or epigenetic changes in TLR genes, see below) which not only alter their allergic risk but also their responsiveness to LPS [177]. For example, a TLR-4 polymorphism is associated with asthma and reduced LPS-induced IL-12 responses *in vitro* [177]. Therefore, investigations into the potential functional consequences of TLR and/or downstream signalling molecule genetic polymorphisms or epigenetic alterations would be of interest to evaluate in subsequent cohort analyses, in order to provide more information regarding underlying mechanisms of reduced LPS-induced Eo/B differentiation observed in 'at-risk' infants.

Related to potential epigenetic effects, the genotype of the individual can also influence the epigenetic profile conferred by environmental exposures. It has been shown

that prenatal tobacco smoke exposure was associated with lower LINE-1 methylation in GSTM1-null, but higher methylation in GSTM1-present children, suggesting that exposure and genotype can influence susceptibility to DNA methylation [178]. Additionally, epigenetic regulation of TLR genes themselves is possible since it was recently shown that the methylation of the CD14 promoter was significantly lower in placental tissue from pregnant women living in rural (farming), compared to those in urban areas [175].

5.2 Going beyond TLRs on CB progenitor cells

Not only do the studies carried out in this thesis support investigations into other TLRs in relation to infant allergic risk, they also support investigating the role of other PRRs, including the NOD-like receptors (NLRs) and RIG-I like receptors (RLRs); each of these receptors has been implicated in allergic development. SNPs have been identified in NOD1 which are associated with increased risk of developing asthma and AD [179]. Interestingly, like the associations with TLR SNPs in farming environments, SNPs in NOD1 also contribute to the protective effect of farming environments [179].

Viral infections including rhinovirus and RSV [180], have been shown to be involved in asthma exacerbations [173]. In fact, it was recently demonstrated that early infection with RSV not only impairs T_{Reg} function, but also increases AHR and OVA-specific IgE upon OVA sensitization and challenge [181]. Since RLRs are involved in the detection of viruses, one could also presume that polymorphisms within these genes would be associated with allergy or asthma. However, no SNPs have been reported in

RLRs that are linked to allergic diseases. This could be simply related to the relatively new discovery of these receptors [173]. It nonetheless would be of interest to extend the results of this thesis, to not only other TLR ligands (e.g., viral detection by TLR-3), but also to other PRRs (e.g., NLRs). It has been recently shown that NLRs are involved in the differentiation of BM progenitors [182], which opens the door to exploring whether similar responses to those we have observed with TLR can be obtained with NLR in relation to CB progenitors eosinophil differentiation and ultimately, to infant allergic risk and disease development.

5.3 The influence of maternal allergy on CB progenitor responses

There is a burgeoning amount of data supporting the role of *in utero* exposures (including microbial exposures) in the development of allergy in early life [183]. We had previously demonstrated that CB progenitor cells are altered by both maternal atopy [6] and diet [7]; however, it remained unclear what effect early microbial stimulation might have on CB progenitor cells. Therefore, it was the goal of this thesis to evaluate CB progenitor cell responses to LPS, a prototypical bacterial ligand, in the context of a genetic predisposition to allergy or with *ex vivo* stimulation with T_H2 cytokines (representing an *in utero* atopic milieu).

The work in this thesis has contributed to our knowledge of prenatal mechanisms involving LPS stimulation of CB progenitor cells. Not only do we demonstrate that CB progenitor cells express TLRs, but depending on atopic-risk, infant progenitors have both altered expression of TLRs and differentially respond to LPS stimulation. Furthermore,

this work provides evidence of innate immune (i.e., LPS)-mediated mechanisms of Eo/B CFU formation, in conjunction with mechanistic insights into how an *in utero* atopic milieu (T_H2 cytokine) can modulate neonatal hematopoietic progenitor cell Eo/B differentiative responses. The conclusion derived from this body of work is that atopic-risk confers an altered capacity of fetal progenitor cells to respond to hematopoietic cytokines and LPS, possibly through prenatal exposure to "pro"-atopic cytokines (e.g., IL-4) or, as discussed below, due to gene-environment interactions and epigenetic modification of neonatal immune cells [184].

Concerning the Hygiene Hypothesis, our work points to the importance of early life microbial exposures in relation to maternal or fetal genetic predisposition to allergy. An immunological re-focus of the Hygiene Hypothesis may be appropriate, given our findings which highlight the emerging importance of the involvement of innate immune cells (e.g., progenitor cells), as opposed the Hypothesis' emphasis on classical adaptive immunity. The original Hygiene Hypothesis suggests that it is direct microbial/infectious stimulation of neonatal immunity (independent of the genetic factors or intrauterine environment) which is 'protective' against allergic development. Our studies add a new dimension to the Hygiene Hypothesis, suggesting that genetic background of the mother (atopy) and the intrauterine environment which the fetus develops (T_H2 cytokine milieu), or the interactions between genes and environmental exposures (through currently unexplored epigenetically regulated pathways), play critical roles in how microbial agents modulate neonatal progenitor responses.

5.4 Allergic development in early life

Our group has repeatedly shown that both the adult and neonatal hematopoietic progenitor system are involved in allergic inflammatory responses or are potentially related to infant allergic risk [95]. Increased hematopoietic cytokine-induced Eo/B CFU may have implications for the development of blood and tissue eosinophilia-associated with allergic disease in 'at-risk' infants, much like BM progenitor responses in adults with asthma [105]. The reversal of this 'hyperreactivity' by LPS exposure may suggest that, for those 'at-risk', prenatal exposure to bacterial products may be beneficial; in fact, these thesis findings suggest collectively that the combination of an atopic T_H2 milieu and microbial exposure *in utero* may work together to limit potentially harmful Eo/B inflammatory responses in infants most susceptible to them. Whether or not these responses persist throughout childhood remains to be investigated. Furthermore, since we only have information on neonates who are 'at risk' for the development of allergy, as opposed to clinically diagnosed allergic children, this constitutes a limitation of our work in relation to its potential therapeutic or diagnostic relevance. Clearly, these progenitor cell observations require validation in prospective cohorts, in which physician-diagnosed allergy and/or asthma is documented. Notwithstanding, differences in the responsiveness of progenitor cells exist prenatally, and these appear to be dependent on atopic-risk. This thesis work therefore highlights the possibility that infectious exposures in the neonatal period may decrease the likelihood of allergic inflammatory responses in 'at-risk' infants, through effects on innate immunity, including hematopoietic progenitor responses (e.g., Eo/B- mediated) shaped in early life.

5.5 In search for biomarkers for atopy in CB

Identification of high-risk infants is essential to studies involving atopy prevention [185]. Importantly, although family history of atopy is considered a very sensitive marker for the development of atopy in offspring [186], the fact that a number of children who develop atopy are from families without atopic background [187,188], makes it critical to determine whether immunological markers at the earliest time points (e.g., within CB, an early life biological window) could be utilised to predict atopic development in later life. By identifying this 'at-risk' population accurately, preventive measures could be formulated and applied with greater confidence [189]. The sensitivity and specificity of the methods used to identify these infants is therefore of utmost importance in determining the usefulness of a test to identify children at-risk for developing atopy [190]. Several *in vitro* tests have been evaluated for early diagnosis of atopic disease; most notable among these is the use of CB soluble mediators (IgE, cytokines), and cellular counts (eosinophils) [185].

Evaluation of the predictive capacity of CB IgE began over 20 years ago, the findings of which have been very controversial [190]. It is generally accepted that CB IgE alone is of less value than a positive family history in predicting allergy [191] and not a suitable screening test [186]. This mainly revolves around the issue that raised CB IgE levels are specific, but not sensitive, in the prediction of the development of allergic disorders [192]; i.e., low CB IgE does not necessarily rule out the development of allergy [192]. The earliest studies by Kjellman and Croner reported elevated CB IgE in 70% of infants at 18 months who developed atopic symptoms. The specificity of this test was

97%, but it was only 28% sensitive [193]. Additionally, a follow-up study by Magnusson *et al*., determined that CB IgE in 190 infants (at a cut-off value 1.2 kU/L) predicted atopy in 72% of infants at 18 months with 93% specificity and 68% sensitivity [194]. Another study by Edenharter *et al*., [195] found that elevated CB IgE (0.9kU/L) was associated with eczema and allergen sensitization at 1 year of age, but the sensitivity (10%) [195] and positive predictive value (PPV) (42%) [189] were poor. Subsequent studies have also failed to substantiate a significant predictive capacity of CB IgE [186,187,196]. Discrepancies among studies could be due to varying cut-off values, the short duration of follow-up and the definition and diagnosis of atopy/allergic disease [197]. With respect to longer follow-up studies, it was recently demonstrated that following infants from birth to 20 years did not greatly improve the sensitivity of this marker [198].

Although serum levels of T_H2 cytokines, such as IL-4, are elevated in CB of infants who develop AD [199-201], the most promising soluble mediator to date has been the T_H2-like chemokine, thymus and activation-regulated chemokine (TARC; CCL17). CB levels of CCL17 predicted development of allergic symptoms at 2 [169] and 6 [202] years of age, and were associated with infantile AD development in 62 neonates born to mothers without AD, and in 38 neonates born to mothers with no allergies [203]. The diagnostic potential of CCL17 for infantile AD was thus 85.7% sensitive, with a PPV of 68.6%. Based on these high values for sensitivity and predictive value, serum levels of CCL17 in CB may be a biomarker of AD in infancy.

Eosinophils (section 1.1.2) play a key role in allergic responses. As such, Calbi *et al*., reported that CB eosinophil count was significantly higher in newborns with an

atopic history [204]; however, when these infants were followed to 18 months, the PPV was only 41%. In this connection, Monti *et al*., found that atopic manifestations occurred in 62% of newborns with elevated CB ECP (> 18μU/L) compared to 27% with ECP below this value. Multi-variant analysis demonstrated that an infant's relative risk of developing atopy, when the infant has a family history and CB ECP > 18μU/L is 16.3, compared to 2.7 with family history alone [205]. Collectively, studies investigating predictors of atopy in CB suggest that it is unlikely that one mediator alone will accurately predict atopic development-- pointing to the necessity for combinational analyses and/or algorithms. These studies also open the door for exploration in other putative markers in CB that will identify infants at risk for atopy.

5.5.1 CB TLR expression: a potential biomarker of infant atopy?

The work of this thesis has demonstrated that infants stratified according to atopic risk--"high-risk" being defined as evidence for maternal allergic sensitization, *and* family history of allergic disease including asthma or rhinitis--display varying unique expression patterns of CB $CD34^+$ cell TLR-2, -4 and -9. A recent review by Chamlin *et al*., [190] revealed that over the last 20 years there have been 30 different definitions of atopic "high-risk" including, but not limited to: family history, CB IgE levels and/or sensitization to allergens in the mother or other first degree relatives [190]. These varied descriptions of atopic risk make the evaluation of potential biomarkers difficult [185,190]. Although we obtained information on family history of allergy, which is considered to be the most sensitive marker of infant risk [186], allergic disease in the family was not confirmed by a physician. Our utilisation of allergy SPT positivity of the

mother as the "gold standard" is only partly in line with previous definitions of "high-risk" [190].

Our finding that the expression of TLR-2, -4 and -9 is reduced on CB hematopoietic progenitor cells of high-risk infants suggests that these TLRs may represent early biomarkers of the development of allergic diseases. However, like the data regarding CB IgE, which in itself is not an adequate screening tool to predict the development of allergic disease [191], reduced TLR expression on CB progenitor cells might ultimately serve as an ancillary tool, in conjunction with markers of infant risk, including family history of atopy, in predicting the actual development of allergic disease. Utility of CB TLR phenotype for accurate prediction of allergic disease could be determined by clinical assessment children at 12 or 24 months, when the first manifestations of allergic disease begin [185].

Nonetheless, there is evidence in the literature that suggests TLRs may have a predictive capacity for AD in the first 2 years of life [9]. A recent study by Ruduit *et al*., [9] assessing 1063 children enrolled in the cohort, demonstrated that CB TLR-5 and TLR-9 expression was significantly reduced among children with AD compared with children without AD; similar trends were found for TLR-1-2, -4, -6, -7 and -8 [9]. Furthermore, survival analysis showed that children with a level of TLR-5 or TLR-9 expression in the upper tertile, as compared to the lower tertile, had the risk of developing AD reduced by 50% [9]. Although the authors did not conduct sensitivity analysis (i.e., assessing whether upper tertile expression of TLR-5 and -9 was found in the CB of all children who develop

AD), their findings do add some credence to the potential predictive capacity of TLR expression on CB cells.

Separately from TLR expression [9] but related to the prediction of AD using CB progenitor cells, our group has previously [7] and recently [206] shown that CB hematopoietic progenitor cell Eo/B CFU formation predicts the development of AD in the first year of life. The fact that the work done in this thesis was able to recapitulate previous findings relating to other phenotypic alterations (e.g., Eo/B CFU and hematopoietic cytokine receptor expression) in CB hematopoietic progenitor cells associated with maternal atopy [6,7] reinforces the potential predictive capacity of TLR expression on CB hematopoietic progenitor cells. Our results, are, however, based on small sample sizes, and thus require further validation in larger cohorts of mothers with well-defined allergic disease, accompanied by prospective analyses of infant development of allergic diseases and asthma.

5.6 Future directions: early life epigenetic regulation of allergy and hematopoiesis

Beyond specific alternations in the nucleotide sequence of specific genes implicated in allergy, the role of epigenetic regulation-- i.e., the silencing or activation of genes-- by environmental factors such as microbes has gained much interest of late [183]. Prenatal exposure to *A. lowffi*, a component of cowshed, is associated with reduced asthma development [140,207] in offspring through epigenetic mechanisms (histone acetylation) on the IFNγ and IL-4 promoters [141]; specifically, these promoters are acetylated (activated) or deacetylated (deactivated), respectively, in response to prenatal

A. lowffi exposure. Furthermore, in an experimental murine asthma model, OVA sensitization and challenge led to an increase in DNA methylation within the IFNγ promoter of $CD4^{+}$T cells, thereby silencing IFNγ expression; this finding supports the hypothesis that epigenetic modulation plays a role in asthma development [208].

Although the implications for human subjects are not clear, these findings provide a platform for investigating epigenetic pathways as a mechanism for gene-environment interactions in allergic disease. Interestingly, the repeated finding that prenatal LPS exposure is associated with prevention of allergic manifestations in offspring mice [138,207], may relate to the ability of endotoxin to bind histones, thereby disrupting chromatin remodelling and resulting in specific gene silencing [209]. In light of our findings demonstrating increased LPS-induced Eo/B CFU (chapter 2), which in turn is decreased in the presence of maternal atopy (chapter 3), it is tempting to speculate that early life exposure to LPS *epigenetically* regulates genes involved in Eo/B differentiation in high-atopic risk infants. This logic is in line with recent findings by Schaub et al., [137] who demonstrated that a natural mode of immunotherapy—i.e., farm exposures—correlated with epigenetic modifications in FOXP3 gene expression. Specifically, this group demonstrated that increased maternal farm exposure and consumption of unpasteurized milk was associated with increased T_{Reg} cells with demethylated (transcriptionally active) FOXP3 gene expression in neonatal CB [137].

Further studies have demonstrated that atopic status is associated with epigenetic signatures in immune cells, findings which correlate with disease severity. It was recently shown that atopic asthmatic children have decreased histone deacetylase

(HDAC) activity in their PBMCs [210], compared to nonasthmatic controls. This group of investigators also related these epigenetic findings to a clinical outcome, since reduced HDAC activity inversely correlated with BHR in these subjects [210]. Murine models have shown that neonates born to asthmatic mothers have broadly increased DNA methylation from birth in neonatal dendritic cells; indeed, these epigenetic changes are associated with a functional capacity to mediate asthma susceptibility, as evidenced by *in vitro* assays demonstrating increased allergen presentation by these cells [184]. According to these observations, maternal allergy results in an altered epigenetic profile in neonatal dendritic cells that is linked to pro-allergic function [184]. Such findings raise the possibility that high-risk infants may also have varied epigenetic profiles in their CB progenitor cells which differ from those of low-risk infants, and impact Eo/B differentiation. Since epigenetic modifications (including DNA methylation and histone modifications) are dynamic [176], and eosinophil-specific genes are under epigenetic control [211], early life exposure to bacterial species (e.g., LPS), could alter this epigenetic profile [212], with consequences for Eo/B CFU formation.

Future directions include an investigation into environmentally-induced epigenetic alterations in CB progenitor cell lineage-commitment. Given evidence supporting the transmission of maternal asthma to offspring in murine models, through epigenetic modifications of neonatal DCs [184], these may impact upon and modulate Eo/B differentiation [211] for example via modifications of the gene encoding IL-5Rα or in those connected to asthma by genome-wide association studies (e.g., those encoding IL1RL1 and IL-33) [213]. *Epigenetic modification of immune gene expression during*

critical periods of early development may provide new insights into the inception of allergy in early life [132,214].

CHAPTER 6

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