

DENDRITIC CELL PROFILES
IN ATOPIC AT RISK INFANTS AT BIRTH

CORD BLOOD DENDRITIC CELL POPULATIONS
IN ATOPIC-AT-RISK AND NOT-AT-RISK INFANTS

By

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Lay Abstract

Allergic disease development typically begins in infancy, progressing classically in a series of stages from early life through adulthood. Currently, there is a lack of reliable predictive tests for the development of atopic sensitization and disease. This has slowed efforts to intercept and prevent allergy development at its earliest stages. Dendritic cells (DCs) link innate and adaptive immunity and are thought to be key players in the development of allergic disease. However, the low numbers of DCs in blood make them challenging to study. Methods such as inducing the differentiation of DCs from progenitors are often utilized to obtain a sufficient number of cells. This project investigates whether receptor expression of cord blood-derived DCs grown *ex vivo* are comparable to the profiles of *in vivo* DCs at birth. Furthermore, the expression of key receptors on DCs grown *in vivo/ex vivo* are compared in atopic at-risk, not-at-risk infants.

Abstract

Allergic disease encompasses multiple complex syndromes including hayfever, food allergies, eczema and asthma. Atopy is the genetic predisposition towards an IgE-driven immune response in reaction to environmental stimuli, and often serves as a predictor for the development of allergies in the future. While disease etiology is not yet fully understood, many factors including genetics and the environment play a role in the development of allergic disease. Reliable methods for predicting atopic disease development are crucial in emerging therapeutic approaches, which aim to decrease allergic disease severity and clinical progression through early detection and preventative measures. While DCs are emerging as key players in the development of allergic disease, they are challenging to study *in vivo* due to their low numbers, and *ex vivo* methods remain relatively unstudied.

In this project, receptor expression profiles of atopic-at-risk infants compared to not-at-risk infants were examined in DCs found in cord-blood at birth and CD34⁺-derived DCs cultured *ex vivo*. Atopic-at-risks exhibited a higher percentage of *ex vivo* pDCs expressing TSLPR when compared to not-at-risks. Additionally, an increase of FcεRI expression in atopic-at-risks was found approaching significance in *in vivo* mDCs.

Furthermore, DC differentiation in culture from hematopoietic progenitors and the differences between *in vivo* and *ex vivo* DCs were studied. Results indicated a consistent 10-fold increase in the DC population after a 12-day culture compared to cord blood DC numbers. Additionally, a distinct DC population emerged as early as Day 3 with a substantial increase in the percentage of mDCs relative to pDCs. A trend of increased TSLP, CD80, CD86 receptor expression and decreased TLR-5, ST2, FcεRI receptor expression after culture in both mDCs and pDCs was also noted.

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List of Abbreviations

IgE- immunoglobulin E

FcεRI- the high-affinity IgE receptor

TH- T helper cell

IL- interleukin

TNF- tumour-necrosis factor

HPC- CD34+ hematopoietic progenitor cell

DC- dendritic cell

MHC- major histocompatibility complex

TLR- toll-like receptor

RAS–MAPK- rapidly accelerated fibrosarcoma-mitogen activated protein kinase

GM-CSF- granulocyte macrophage colony stimulating factor IFN- interferon

pDC- plasmacytoid DC

mDC- myeloid DC

cDC- conventional DC

HLA-DR- human leukocyte antigen-D related

ST2- suppression of tumorigenicity 2

MyD88- myeloid differentiation primary response 88

TSLP- thymic stromal lymphopoietin

TSLPR- thymic stromal lymphopoietin receptor

Lin- human lineage cocktail including CD3, CD14, CD16, CD19, CD20, CD56

SCF- stem cell factor

LPS- lipopolysaccharide

Flt3L- fms-related tyrosine kinase 3 ligand

FT- flow through

IMDM- Iscove's Modified Dulbecco Medium

FCS- fetal calf serum

BSA- bovine serum albumin

PBS- phosphate-buffered saline

MNC- mononuclear cell

SEM- standard error of the mean

BDCA- blood dendritic cell antigen

CLEC9A- C-type lectin domain containing 9A

1. INTRODUCTION

1.1 Allergic Disease

The term “allergy” was developed in the early 1900s to describe the tendency of some people to exhibit immune hypersensitivity when exposed to certain substances. In scientific terms, an allergy is an abnormal adaptive immune response to an otherwise harmless environmental stimulus, and includes asthma, allergic rhinoconjunctivitis (known as hayfever), atopic dermatitis (known as eczema), and food allergies (Fernandes et al., 2008; Galli et al., 2008). The production of immunoglobulin E (IgE) and the expansion of allergy-specific T-cell populations is a characteristic of many allergic responses, but not all. The stimulus, called an allergen, is an environmental protein that doesn't elicit an allergic response in a healthy individual. When first introduced, an allergen acts by inducing IgE production and “sensitizing” the individual to the stimulus (Chad, 2001). Re-exposure to the same allergen induces an allergic reaction. Common allergens include latex, grass, pollen, animal proteins, house dust mite particles, and foods (e.g. peanuts, shellfish, milk, eggs) (Jaén et al., 2002). Allergen involves a T_H2 -type immune response, where immunoglobulin class-switch recombination in B cells drives IgE secretion (Galli et al., 2008; Gauchat et al., 1990). An environmental particle may also induce a hypersensitive immune response on contact, for example contact dermatitis after exposure to nickel, however this kind of reaction is not thought to be IgE-mediated (Antico and Soana, 2015; Kaplan et al., 2012; Novak et al., 2008).

Allergic disease remains the most common immunological disease and is rapidly growing in the adult population, especially in industrialized countries (De Martinis et al., 2017). It is also the most widespread chronic health condition, with a global reach (De Martinis et al., 2017). The costs associated with allergies are significant: child food allergies alone in the United States were estimated to cost \$24.8 billion annually, or \$4184 per year per child (Gupta et al., 2013).

1.2 Atopy

The term atopy was coined by Coca and Cooke in 1923, before the advent of IgE-associated classification of allergic inflammation (Bellanti and Setticone, 2017). Atopy refers to the genetic predisposition to produce IgE antibodies in response to an allergen, and is associated with an increased risk of developing allergic reactions and related disorders (Thomsen, 2015). Atopic manifestations are usually characterized by an overabundance of IgE antibodies in the serum, thought to be the result of increased B-cell stimulation from elevated T-cell cytokine secretion. (Janeway et al., 2001). Notably, B-cell stimulation with IL-4 is crucial in the regulation of immunoglobulin class switching to IgE, contributing to the development of allergic disease (Nies et al., 2002). It is important to note that not all instances of atopy result in allergic disease and not all allergic-type disease stems from atopic sensitization, but the presence of atopic sensitization correlates to the increased risk of developing an allergic disease in the future (Thomsen, 2015).

1.3 The Atopic March

Atopic sensitization occurs most frequently during childhood and the subsequent development of one or more atopic syndromes usually follows a well-known sequence of events, called the atopic march. The first step in the atopic march is the development of atopic dermatitis; occurring in roughly 20% of all children (Berger, 2000; Thomsen, 2015). Bouts of atopic dermatitis are characterized by the inflammation of the skin in response to allergens causing dry, scaly patches, but is usually outgrown by adolescence (Berger, 2000). About 30% of children with atopic dermatitis also experience allergies to food (Thomsen, 2015). Food allergies develop according to the exposure to the allergen; allergies to milk and eggs usually develop in early childhood while allergies to wheat develop later. Globally, confirmed food allergies with an IgE-mediated response occur in 3-5% of the population, with anaphylaxis occurring in less than 0.1% of the population (Thomsen, 2015). In Canada food allergy is even more prevalent, with an estimated 6.9% of children under 18 years and 7.7% of adult Canadians reactive to some food allergen (Soller et al., 2015). Many children allergic to milk, eggs, and wheat will eventually tolerate those foods, fewer will outgrow nut allergies, and food allergies developed in adulthood have the worst prognosis (Thomsen, 2015). 40% of children with food allergies also have IgE-mediated inflammation of the lung bronchi; asthma (Thomsen, 2015). While asthma itself is not prevalent in infants, the development of asthma is commonly preceded by recurrent instances of respiratory infections and wheeze in infancy, as well as other atopic disease like eczema. Allergic rhinoconjunctivitis develops last, in late childhood, and is most frequent in patients with ages ranging from 20-40 years old

(Strachan, 1989). Today, it is prevalent in roughly 1/5 of the population in the West (Strachan, 1989; Thomsen, 2015).

1.4 The T_H1/T_H2 Paradigm in Allergy

Allergic disease has been historically considered to be mediated by a dominant T helper cell (T_H) type 2 immune response where CD4⁺ helper T cells secrete interleukin-4 (IL-4), IL-5, IL-6, IL-9, IL-10 and IL-13 cytokines, stimulating the IgE secretion and eosinophil infiltration that are hallmarks of allergic inflammation (Berger, 2000; Janeway et al., 2001). Thymic stromal lymphopoietin (TSLP) has been heavily associated with dendritic cell-mediated T_H2 responses; it has even been suggested that TSLP acts as a master switch for allergic inflammation at the epithelium-dendritic level (He and Geha, 2010; Ito et al., 2005; Liu, 2006; Watanabe et al., 2004, 2005). In contrast, a T_H1 response is characterized by production of T_H1 cytokines, such as interferon-gamma (IFN γ), IL-2 & tumour necrosis factor (TNF), that correspond to a variety of pro-inflammatory responses against viral and bacterial pathogens (Berger, 2000). A strong T_H1-type response is not commonly associated with allergy cases. There is usually a balance between T_H1 and T_H2 responses because the activation of one pathway tends to inhibit the other. A third type of immunological response, T_H17, is characterized by secretion of IL-17 and IL-22 cytokines (Waite and Skokos, 2012). This response is beneficial during bacterial and fungal infections but may become detrimental during viral and parasitic infections (Waite and Skokos, 2012). It is now known that not all instances of atopic disease are characterized by a T_H2-dominant response. Some studies have shown the

involvement of T_H17 pathways in allergic disease, as individuals with severe asthma have been observed with neutrophilia and increased IL-17 production, characteristic of a T_H17 -type response (Al-Ramli et al., 2009). This has shed new light on the immunopathogenesis of atopic disease and revised the idea of the allergic T_H1/T_H2 paradigm.

1.5 The “Hygiene Hypothesis”

Instances of atopic disease have drastically increased worldwide in predominantly Western countries from the 1960s-onwards (Thomsen, 2015). The “hygiene hypothesis” attempted to address this trend by suggesting that the emphasis on cleanliness in Western countries resulted in the decrease of infant exposure to pathogens, hindering T_H1 -immune development and leading to increased susceptibility of allergic disease (Strachan, 1989; Thomsen, 2015). It was thought that better hygiene practices and antibiotic use decreased the frequency of T_H1 -stimulating infections such as typhus & tuberculosis (Thomsen, 2015). In turn, weakened T_H1 -type responses disrupted the balance of T_H1/T_H2 immunity, contributing to a heightened T_H2 response and the development (or increased severity) of allergic disease. Supporting the “hygiene hypothesis”, children growing up in a rural farm environment where they are exposed to a variety of animal proteins and microbiota develop allergic diseases at dramatically lower levels than children in suburban environments (von Mutius, 2012). Our current understanding of allergy development suggests that microbial gut or respiratory colonization rather than microbial infection during early infancy is crucial in the development of persistent

allergic disease. A multitude of birth-cohort studies provide evidence that a deficiency in microbial colonization by some commensal microbes during early infancy results in the development of altered immune function, heightening allergic disease susceptibility (Björkstén et al., 1999, 2001; Dominguez-Bello et al., 2010; Kalliomäki et al., 2001).

1.6 Cord-Blood Hematopoietic Progenitor Cells

Hematopoietic progenitor cells (HPCs) are CD34⁺ multipotent cells that give rise to all blood cells, including T-cells, blood granulocytes, and DCs, as well as are capable of self-renewal. Found in the bone marrow, peripheral and cord blood of humans, they account for 0.05-0.2% of peripheral blood and 0.1-0.5% of cord blood (Denburg et al., 1996). Hematopoiesis is the development of blood cells, occurring during embryonic development and during adulthood (Jagannathan-Bogdan and Zon, 2013). In gestation, hematopoiesis occurs in the yolk sac. It temporarily occurs in the liver before definitive hematopoiesis is established in the bone marrow and thymus (Jagannathan-Bogdan and Zon, 2013).

Previous studies have shown that there are increased numbers and altered types of HPCs in the blood and bone marrow of adults with atopic diseases (Denburg et al., 1985, 1989, 1996; Gibson et al., 1991; Otsuka et al., 1986). Subsequently, the “bone marrow hypothesis” of allergy suggests the increased numbers of inflammatory cells observed in people with atopy arise, at least in part, from increased differentiation of inflammatory cell progenitors. Studies using cord-blood samples from the Canadian Healthy Infant Longitudinal Development (CHILD) and Family Atherosclerosis Monitoring In Early

Life (FAMILY) birth-cohort studies have further demonstrated that CD34⁺ progenitors in cord blood at birth have altered numbers and profiles in infants at high risk for allergic disease, which may be useful in predicting the development of allergy (Fernandes et al., 2008; Morrison et al., 2009; Subbarao et al., 2015).

1.7 Hematopoiesis and In-Utero “Priming”

There is an accumulation of evidence that the development of allergic sensitization is initiated prior to birth. For one thing, maternal atopic status remains one of the best predictors of the child’s atopic status later in life (Abbott et al., 2012). The differences found in HPCs at birth in infants who develop allergies later in life suggests that infants who will become allergic are already primed at birth to generate cells involved in the inflammatory response. The same phenomenon can be seen further down the differentiation pathway; there is evidence that allergen-specific helper T cells can be primed *in utero* (Devereux et al., 2001). Whether this priming is the result of the environment of the uterus, the genetics of the fetus, or a mix of both, remains unclear.

The consequences of maternal T_H2-type responses during pregnancy on the fetus immune development remain somewhat mysterious. Some studies have successfully proven that a mother's immune system during pregnancy has an important role in fetal development. Maternal exposure to the common allergen ovalbumin during pregnancy in mice has been shown to cause altered developmental trajectories in the offspring (Schwartz et al., 2015). In human longitudinal studies there is evidence for systemic maternal immune changes during a healthy pregnancy, with an increasingly profound

anti-inflammatory phenotype developing from the second trimester onwards (Graham et al., 2017). Furthermore, a newborn does not possess a completely undeveloped immune system. Immune development during gestation is proliferative and well underway by the time of birth. B-cells develop in the fetus by 9 week's gestation time, entering circulation by 12 weeks (Hayward, 1983). T-cells begin exiting the thymus around 14 weeks gestation, after which helper T cells can be observed in the spleen (Hayward, 1983). While there is a clear lack of secondary lymphoid tissues in the fetus, this is thought to reflect an absence of antigen stimulation instead of immune underdevelopment (Hayward, 1983).

1.8 The “Early Phase” Response

An individual sensitized to an allergen experiences allergic inflammation following exposure to the stimulus. An early phase response is the immediate immune reaction occurring after a single case of exposure, which may be followed by a late-phase response in many individuals. (Galli et al., 2008). Activation and degranulation of mast cells is central to the pathogenesis of allergic disease. Mast cells are tissue-based inflammatory cells that arise from pluripotent cells in the bone marrow before being released into the bloodstream. Mast cells mature in the tissue and are primarily tissue-residents, but the mechanisms of their tissue infiltration remain unclear (Stone et al., 2010). A growing body of evidence including models and animal studies have found a role of mast cells in maintaining body homeostasis, wound healing, as well as in innate and adaptive immunity (Artuc et al., 2007; Lewis and Austen, 1981; Wedemeyer et al.,

2000). The allergic immune response also involves the recruitment of other effectors to the site of inflammation, notably eosinophils and basophils (Janeway et al., 2001). The early-phase response is initiated by activation and subsequent degranulation of mast cells that occurs when adjacent IgE molecules cross-link with allergens on the FcεRI receptor (Janeway et al., 2001). The accumulation of crosslinked IgE molecules activates the protein tyrosine kinases LYN and FYN, which triggers an intracellular signalling cascade causing RAS–MAPK (mitogen-activated protein kinase), phospholipase C-γ and PI(3)K pathway activation (Cargnello and Roux, 2011; Gilfillan and Rivera, 2009; Gilfillan and Tkaczyk, 2006; Marshall, 2004; Rivera and Gilfillan, 2006). These signalling cascades result in the release of a variety of cytokines, prostaglandins, leukotrienes, biogenic amines (e.g. histamine), proteoglycans (e.g. heparin, chondroitin sulphate), proteases (e.g. tryptases, chymases, carboxypeptidases) and other compounds that cause the symptoms of an allergic reaction (Boyce, 2007; Bradding and Holgate, 1996; Caughey, 2007; Finkelman, 2007; Pejler et al., 2007). Symptoms include vasodilation (causing reddening of the skin or conjunctiva), increased vascular permeability (leading to swelling in the tissue, watery eyes), contraction of bronchial smooth muscle (causing wheezing by obstructing the airway), and increased secretion of mucus (causing a runny nose), while stimulation of sensory nerves in the airway, skin, and nose cause coughing, itchiness, and sneezing (Galli et al., 2008).

1.9 The “Late Phase” and “Chronic” Responses

A late phase reaction involves recruitment of eosinophils, basophils, T_H2 cells, and other leukocytes to the site of the allergic response (mediated by IgE-activated residents such as mast cells) as well as airway hyperresponsiveness to non-specific bronchial stimuli (e.g. histamine, methacholine) that is a defining characteristic of allergic asthma (Charlesworth et al., 1989; Cockcroft et al., 1977a, 1977b; Galli et al., 2005). This phase occurs 2h-6h after a clinically evident early-phase response, peaking 6h–9 h after allergen exposure and fully resolving in a few days (Galli et al., 2008). Persistent re-exposure to the allergen can cause the development of chronic allergic inflammation and associated tissue changes (Leung et al., 2004; Pawankar et al., 2004). Instances of chronic allergic inflammation are characterized by innate and adaptive immune cell, eosinophil & basophil infiltration into the tissue from the bloodstream, structural changes, and may result in tissue or organ damage (Doherty and Broide, 2007; Galli et al., 2008; Mauad et al., 2007).

1.10 Eosinophils/Basophils in Allergy

Eosinophils are granulocytes that arise from hematopoietic progenitors and are a staple feature of the allergic response. They express a variety of cell surface molecules including receptors for IgG, IgA; cytokine receptors for IL-3, IL-5, granulocyte macrophage colony stimulating factor (GM-CSF), IL-1 α , IL-2, IL-4, interferon (IFN)- α , and TNF- α ; chemokines; adhesion molecules; leukotriene receptors; platelet activating factor receptor; and toll-like receptors including TLR7, TLR8 (Stone et al., 2010). Fc ϵ RI

is generally not recognized to activate eosinophils nor be expressed, although this is controversial. The development of eosinophils from hematopoietic progenitor cells (HPCs) is promoted through GM-CSF, IL-3, and notably IL-5 secretion, which are often co-expressed in T_H2-type cells (Stone et al., 2010). Other mediators including TSLP, IL-25, IL-33 are known to contribute to eosinophil-mediated airway inflammation. IL-33 has been shown to directly stimulate eosinophil differentiation from progenitors, and similar to IL-33, TSLP is crucial for the development of T_H2-type airway eosinophilia (Morita et al., 2015; Stolarski et al., 2010).

Eosinophils develop in the bone marrow before entering the bloodstream but are primarily tissue-resident cells. Eosinophil main function is the release of pro-inflammatory mediators; the production of cytotoxic proteins is of distinguishable importance. (Stone et al., 2010). Cytotoxic proteins released by eosinophils can create pores in the membranes of target cells, suppress T-cell proliferation, suppress B-cell antibody production, and induce mast cell degranulation (Venge et al., 2001). Eosinophil counts of $500/\text{mm}^3$ in peripheral blood are considered normal; higher-than-average eosinophil count in blood (eosinophilia) is associated with a number of diseases, infections and autoimmune disorders, including allergy and asthma (Stone et al., 2010).

Basophils share many similarities with mast cells but arise from a distinct HPC-derived lineage and possess other unique functions (Stone et al., 2010). Similar to eosinophils, they express IL-3, some IL-5 & GM-CSF, receptors including CD11b, CD11c, CD35, CD88, immunoglobulin Fc receptors (FcεRI and FcγRIIb), and TLRs (Stone et al., 2010). However, basophils are mainly circulating cells, notable in their rapid

and abundant expression of IL-4 and IL-13. Studies modelling non-IgE mediated IL-4 production by basophils have shown that they contribute to the priming of T_H2-type cell differentiation (Stone et al., 2010; Sullivan and Locksley, 2009). Basophils also store histamine in granules, which is released following activation, similar to mast cells. While mast cells, eosinophils, and basophils express many of the same receptors and cytokines, they each have notable and unique roles in the context of allergic pathogenesis.

1.11 Treatments Options for Allergic Disease

Many advancements in therapies for allergic disease have been made in the past decade. Early-phase allergic reactions respond well to antihistamine treatment. Antihistamines are available over-the-counter and temporarily relieve the symptoms of mild allergic reactions due to their antagonistic ability on receptor H₁ (De Vos, 1992). Treatment for more severe, chronic allergic disease such as asthma are commonly steroid-based, targeting the late-phase response. According to the 2018 GINA guidelines, the goals of asthma treatment are symptom control and the reduction of exacerbation risk (Global Initiative for Asthma., 2018). Inhaled corticosteroids (alone or in conjunction with long-acting beta agonists) and leukotriene receptor antagonists are common medications prescribed long-term for patients with mild to moderate asthma; these treatments control asthma symptoms and prevent further attacks (Global Initiative for Asthma., 2018; McCracken et al., 2017). Intravenous administration of cortical steroids, as well as short-acting beta agonists, are administered as “rescue” medications for the rapid relief of sudden allergic exacerbations (Global Initiative for Asthma., 2018;

McCracken et al., 2017). Other therapies, including immunotherapy and Omalizumab, have been designed to reduce immune sensitivity to the allergen. Omalizumab, approved for the treatment of allergic asthma and rhinitis in patients not responding to standard therapy, decreases total IgE levels using anti-IgE monoclonal antibodies to prevent IgE from binding to the high-affinity Immunoglobulin E receptor (FcεRI) (McCracken et al., 2017; Stone et al., 2010). Oral immunotherapy for food allergies involves the controlled introduction of the allergen to the patient in increasing amounts, with the aim of reducing or eliminating the allergic reaction (Keet and Wood, 2014). Novel strategies such as modified protein immunotherapy and the use of adjuvants attempt to improve on these treatment options, using similar mechanisms of therapy (e.g. introducing modified proteins of the allergen to the patient) but with a safer, more tolerogenic approach (Keet and Wood, 2014). Furthermore, a subset of asthma patients with a specific asthmatic subtype (“eosinophilic asthma”) who exhibited resistance to traditional corticosteroid treatment responded to anti-IL-5 treatment, which decreased the eosinophil count in the blood and tissue and improved control of the asthma symptoms (Haldar et al., 2009; Nair et al., 2009). With the advent of personalized medicine, reliable methods for predicting allergic disease are central to emerging therapeutic approaches, which aim to decrease disease severity and clinical progression through early detection and preventative measures (Gordon, 2011).

1.12 Dendritic Cells and the Allergic Response

DCs are the most potent antigen-presenting cells, as well as the link between innate and adaptive immunity. The main function of mature DCs is to activate naive CD4⁺ T-cells through MHC class II antigen presentation (Janeway et al., 2001). As such, they are one of the first cells to come in contact with a potential allergen. Two commonly distinguished DC subtypes include plasmacytoid DCs (pDCs) and myeloid DCs (mDCs), also known as conventional DCs (cDCs), characterized by whether they arose through a lymphoid or myeloid lineage as well as by their functional differences (Wallet et al., 2005) (Figure 1).

Plasmacytoid DCs are present in 0.2-0.8% of peripheral blood and highly express the cell surface markers CD123, BDCA-2 and the MHC class II receptor HLA-DR (Schoeters et al., 2007). Mature pDCs are distinguishable by their high interferon-alpha and interferon-beta expression through the activation of TLR9 and TLR7 signaling pathways (McKenna et al., 2005; Sallusto and Lanzavecchia, 2002). In contrast, mature mDCs highly express the cell surface markers CD11c, CD1c, as well as human leukocyte antigen-D related (HLA-DR) (Qu et al., 2014; Schoeters et al., 2007). They notably secrete abundant IL-12 after pathogen recognition via TLR2 and TLR4 activation (Qu et al., 2014). mDCs are further divided into two subcategories based on further differences in function, with the markers BDCA-1, BDCA-3, and clec9A commonly used to distinguish between them (Qu et al., 2014).

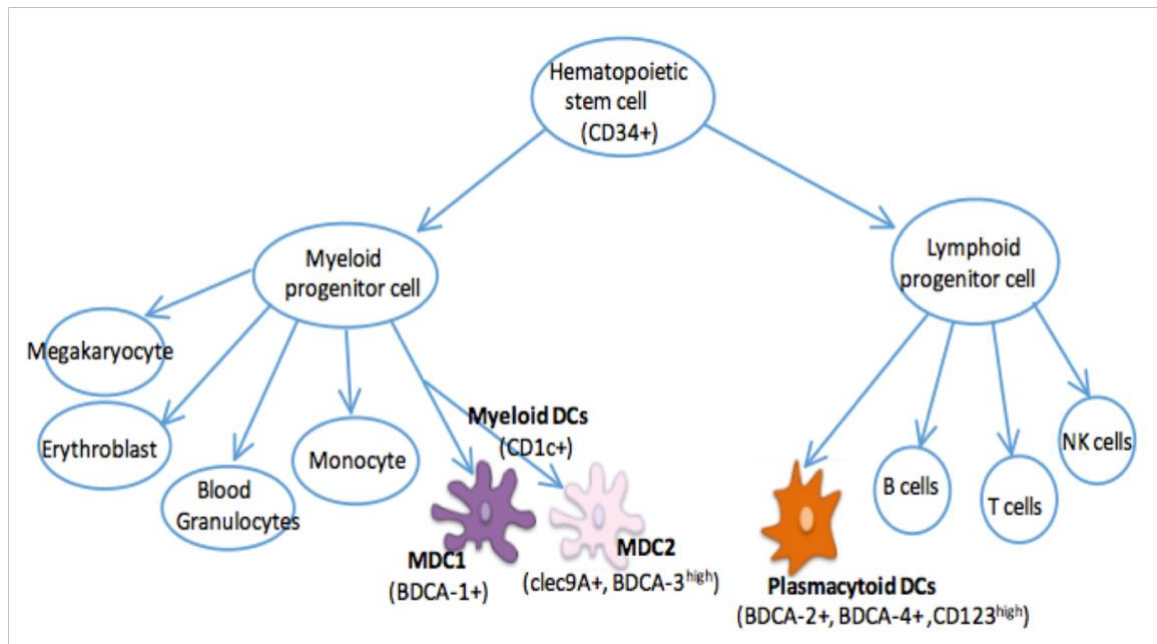


Figure 1: Hematopoietic stem cell cascade & dendritic cell markers. Two distinct populations of DCs (myeloid or plasmacytoid) differentiate from CD34⁺ hematopoietic stem cells through myeloid or lymphoid progenitors. There are also two subtypes of myeloid DCs (MDC1, MDC2), and various markers distinguishing each DC subtype. Adapted from Miltenyi Biotec Human Cell Detection poster.

Arising from CD34⁺ progenitors in the bone marrow, immature unstimulated DCs initially have low T-cell activation potential but high phagocytic capability, constantly sampling the environment around them (Wallet et al., 2005). Potential allergens come in contact with DCs in the airway, or through disrupted epithelium (Galli et al., 2008). Recognition of pathogens by the DCs is achieved through phagocytosis and pattern recognition receptors on the DC membrane, such as TLRs, which recognize a variety of chemical signatures and molecules found on microbes and other pathogens (Mahla et al., 2013; Wallet et al., 2005). TLR5 recognizes bacterial flagellin on the dendritic cell surface, and TLR9 is activated by unmethylated CpG sequences on DNA molecules (Christensen et al., 2006; Miao et al., 2007). Loss of TLR9 has also been shown to increase the activation of DCs and exacerbate the progression of lupus (Christensen et al., 2006). Additionally, the “suppression of tumorigenicity 2” receptor (ST2) is a member of the Toll-like/IL-1-receptor superfamily, specifically an interleukin-1 receptor that binds with the IL-33 ligand (Kakkar and Lee, 2008; Piehler et al., 2016). Activated TLR5, TLR9, and ST2 receptors all interact with the adaptor protein “myeloid differentiation primary response 88” (MyD88), resulting in downstream secretion of pro-inflammatory cytokines and other mediators (Christensen et al., 2006; Kakkar and Lee, 2008; Miao et al., 2007).

Immature DCs circulate in non-lymphatic peripheral tissues, and experience increased expression of MHC-associated molecules once they come in contact with a pathogen (Tan and O’Neill, 2005; Wallet et al., 2005). This activates the DCs, priming them for antigen presentation and T-cell activation (Tan and O’Neill, 2005).

Activated DCs migrate to the lymph nodes or local mucosa through the bloodstream and present the antigen to naive T-cell through MHC class II antigen presentation, subsequently maturing the DC and initiating T-cell responses. (Galli et al., 2008; Sallusto and Lanzavecchia, 1994). In a newborn, some DCs are already found in the spleen, but do not activate T-cells to the extent which adult DCs do (Sun et al., 2003).

T-cell stimulation with IL-4 and IL-13 causes effector B-cells to secrete IgE (Douglass and O'Hehir, 2006; Janeway et al., 2001) (Figure 2). IgE levels in blood are low at birth in healthy individuals and gradually increase during childhood, with IgE serum levels peaking during adolescence and decreasing through adulthood (Stone et al., 2010). While blood IgE levels are the lowest compared to other immunoglobulin subtypes, the total concentration of IgE in serum is influenced by the atopic status of the individual, as well as environmental and genetic factors (Adkinson et al., 2008; Gould and Sutton, 2008).

IgE crosslinks the allergen to the FcεRI receptor on mast cells, causing sensitization to the allergen (Figure 2). Many factors can determine the extent and likelihood of allergic sensitization, including genetics, the environment, allergen load, among others. The epithelium is the primary physical barrier against allergen exposure. The epithelium is selectively permeable towards potential allergens through the presence of receptors, transporter proteins and junctions (van Ree et al., 2014; Steed et al., 2010). Thus, many allergens first come in contact with receptors on epithelial cells, stimulating them to secrete T_H2-associated cytokines and instigate the related immune responses (Swindle et al., 2009). The presence of pathogen recognition receptors in the airway epithelium, for

example, leads to the recruitment of immune cells such as DCs, and the initiation of an inflammatory response following pathogen exposure (van Ree et al., 2014; Willart et al., 2012). DCs themselves are one of the first cells to come in contact with an allergen, since they are present just underneath the epithelial barrier and are known for their phagocytic abilities, as well as the presence of pathogen recognition receptors including TLRs (Maverakis et al., 2015). The allergic response is illustrated in Figure 2.

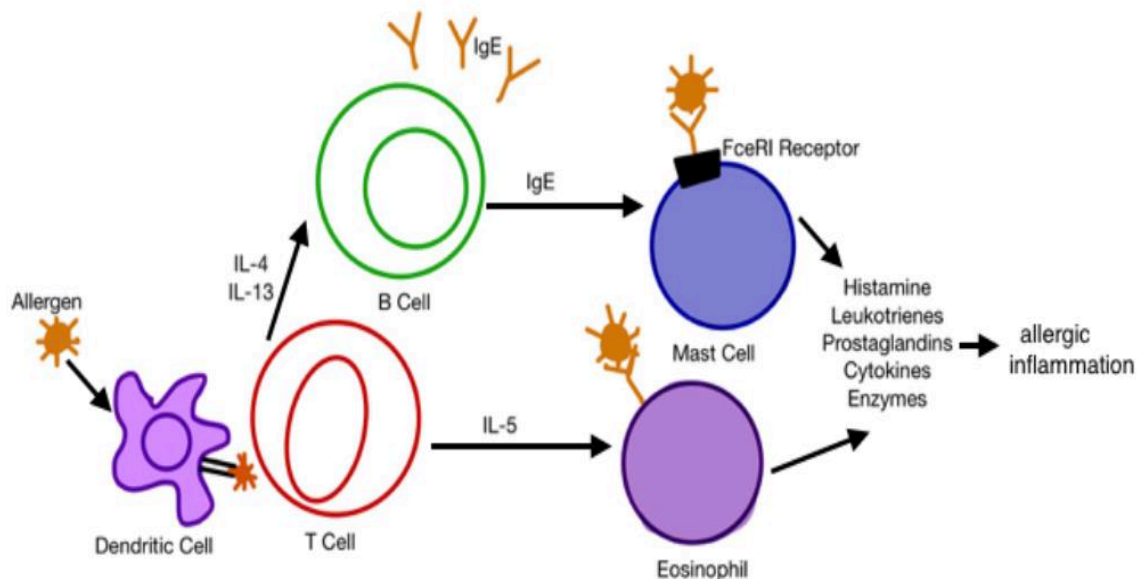


Figure 2: Schematic of allergic response pathway, and the complex interplay between T-cells, antigen presenting cells, B cells, and mast cells. DCs capture and present allergens to naive T-cells, which in turn activate B-cell secretion of IgE. In combination with co-stimulatory factors, IgE stimulates mast cells and other effector cells, such as eosinophils, to secrete histamine and proteins responsible for allergic symptoms. Adapted from Biback, Conte, James & Ng, October 27, 2015 "The Biochemistry of Asthma" (presentation)

TSLP is a hematopoietic cytokine with a role in activating transcription factors STAT1, STAT3, STAT5, JAK1 and JAK2 to promote the development of T_H2 cells. (Borowski et al., 2013; Kitajima et al., 2011; Wang et al., 2006). It is expressed in the thymus, skin, airway epithelium and gut epithelium, and forms a heterodimer with its receptor, TSLPR, and the IL-7 α receptor, found on a multitude of adaptive and innate immune cells (He and Geha, 2010). TSLP is known to activate DCs when bound, with notable roles in enhancing mDC maturation and leading to the production of T_H2-type pro-inflammatory cytokines in both DC subsets that induces naïve T-cell differentiation into T_H2 cells (Reche et al., 2001; Soumelis et al., 2002). It has been suggested that TSLP-activated DCs have a role beyond T_H2-type response priming, affecting the maintenance of T_H2 memory cell polarization during allergic disease as well (Wang et al., 2006).

IL-4 is a cytokine that stimulates naïve helper T-cells to differentiate into pro-inflammatory T_H2 cells, which further secrete IL-4 in a positive feedback system. It can be derived from an array of cells including mast cells, eosinophils, basophils, and T-cells (Galli et al., 2008). Overabundance of IL-4 is associated with allergic disease (Hershey et al., 1997).

Activated DCs are frequently recognized by expression of ligands associated with T-cell stimulation, which include CD80 & CD86. CD80 and CD86 stimulate IgE release from B-cells in the presence of co-stimulatory factors including IL-4 & IL-13 secreted by T_H2 cells (Galli et al., 2008). This is achieved through immunoglobulin class-switch recombination, where gene segments encoding the immunoglobulin heavy chain are

rearranged so that the antibody of the IgE class is produced (Galli et al., 2008). IgE disperses on site, entering the lymphatic system before entering the blood, where it is distributed system-wide and binds to FcεRI on mast cells.

FcεRI is a high-affinity receptor for the Fc region of immunoglobulin E, well known for its role in the inflammation response signaling cascade (Shin and Greer, 2015). FcεRI is expressed by both pDCs and mDCs, with a role in promoting inflammation by up-regulating DC production of pro-inflammatory cytokines (Novak et al., 2004; Shin and Greer, 2015). It has also been suggested that FcεRI signaling can induce an anti-inflammatory response in some cases, as FcεRI signaling was shown to interfere with TLR signaling in pDCs and possibly mDCs (Platzer et al., 2015).

1.13 Differences in Dendritic Cell Phenotypes Linked to Allergic Manifestation

While mechanisms of DC involvement in allergies are well-researched, data available on the DC phenotype in the context of allergic/non-allergic subjects is lagging. A recent study showed differences in MHC-II expression and an exaggerated T_H2 response of DCs present in the airway of mice naturally susceptible to allergic sensitization, compared to naturally resistant mice (Leffler et al., 2018). These findings provide evidence of DC ability to induce allergic sensitization or resistance, and indicate a genetically-based susceptibility for allergic sensitization in the airway (Leffler et al., 2018). TSLP has also been shown to push DC polarization towards the initiation of T_H2-type responses (Ito et al., 2005; Wang et al., 2006, 2007; Watanabe et al., 2004,

2005). Human studies on DC phenotypes in severe steroid-resistant asthmatics have shown differences in mDC frequency and therapy response compared to steroid sensitive asthmatics (Chambers et al., 2018). Other studies have shown no significant differences in receptor expression between asthmatics and non-asthmatics, but finding differences in the functional ability of FcεRI in blood DCs instead (Holloway et al., 2001).

Maternal atopic status has been linked to differences in DC phenotype in the offspring at birth, implicating a non-genetic, non-environmental factor involving DCs in the development of allergy. In a murine model, neonates of asthmatic mothers were more susceptible to future allergic sensitization compared to mice with non-atopic mothers (Hamada et al., 2003). Furthermore, adoptive transfer of DCs from allergen-naive murine neonates with asthmatic mothers to control neonates conferred increased airway responsiveness and allergic inflammation, further suggesting that maternal atopic status is linked to the offspring's atopic status, and is independent of allergen exposure (Fedulov and Kobzik, 2011).

1.14 Identification of Dendritic Cell Populations by Flow Cytometry

Flow cytometry is a popular immunophenotyping method, particularly useful for the study of cells without the need to isolate a pure cell population. Cell size and granularity are one of the first parameters examined when locating a certain cell type during flow cytometry data analysis because they provide the means to detect singlets and exclude debris from the gating strategy. There are also negative controls in place, to exclude certain cell types that co-express important markers with your target cell

population. DC surface markers, phenotypes in relation to disease, and cell expression have widely been observed using flow cytometry methods, with the markers utilized for distinguishing different DC subtypes varying between groups. Across the board, DC populations are recognized by their high MHC II molecule-associated expression (HLA-DR⁺) and lack of T-cell, natural killer cell, B-cell, granulocyte, and erythrocyte lineage markers (Lin⁻) (MacDonald et al., 2002; Merad et al., 2013). As a cell type derived from bone-marrow progenitors, DCs can also be distinguished by CD45, a leukocyte marker found on such cells with a hematopoietic origin (Altin and Sloan, 1997). These parameters are much too broad to distinguish DC subtypes, thus more refined and precise markers are utilized, each with unique advantages and limitations. CD11c is an integrin protein historically used as a marker for “conventional” mDCs, however it is also expressed on some lung and intestinal macrophage populations as well as on pDCs in low levels, so should be used in combination with other markers (Merad et al., 2013). Non-overlapping markers are used to distinguish between the mDC subtypes; CD141⁺ (BDCA3⁺) mDCs account for 10% of mDCs found in the blood while CD1c⁺ (BDCA1⁺) mDCs account for the majority (Collin et al., 2013). Plasmacytoid DCs are distinguishable from mDCs by low expression of CD11c, combined with positive expression of CD123, or positive expression of CD303 (BDCA-2) and CD304 (BDCA-4) (Autissier et al., 2010; Collin et al., 2013). The appropriate use of BDCAs versus CD11c/CD123 in a panel to differentiate between mDCs and pDCs is subjective, as each method is proper for different aims. BDCAs are thought to be more specific in identifying DC subtypes, often utilized when the purity of rare DC subpopulations is particularly

important. However, no significant differences were found when comparing the BDCA method and the CD11c/CD123 method for the purpose of distinguishing between mDCs and pDCs (Rovati et al., 2008).

1.15 Dendritic Cells Generated *Ex Vivo*

Due to the limited numbers of DCs found in the blood compared to other cell types, alternative methods are often utilized to obtain sufficient numbers of DCs for study. The *ex vivo* differentiation of DCs from monocytes or bone-marrow progenitors are widely referenced culturing techniques used in murine and human studies. Both methods involve the culture of DC progenitors in an appropriate rich medium, with the supplementation of select cytokines to encourage differentiation (O'Neill and Wilson, 2004). While the principle and methodology of monocyte or HPC-derived DCs are similar amongst the available protocols, it is acknowledged that each method possesses its unique usefulness in different contexts of study. DCs derived *ex vivo* from monocytes have been found to resemble an independent category of DCs that differentiate during an inflammatory immune response (Nair et al., 2009). Alternately, bone-marrow-derived DC culture systems attempt to mimic the natural generation of DCs that occurs in a non-inflammatory state (Inaba et al., 1992). Different kinds of rich medium have been successfully used for the generation of DCs; the medium is often supplemented with fetal calf serum and antibiotics (e.g. penicillin, streptomycin) to serve as a bystander antigen with growth-promoting properties and reduce culture contamination, respectively (Lardon et al., 1997; Lutz and Rössner, 2007). Furthermore,

supplementation with cytokines is essential to the growth of DC populations in culture, discussed as follows.

The culture of progenitor cells with GM-CSF, TNF- α , stem cell factor (SCF), and IL-4 supplementation, as demonstrated with HPCs by Lardon et. al, are among the common techniques used to generate DCs *ex vivo*, (Lardon et al., 1997). GM-CSF is an essential growth factor for the generation of all cells in the myeloid lineage. While it is shown to be highly effective in the generation of antigen-presenting myeloid-type DCs in culture, it also generates significant sub-populations of granulocytes and adherent macrophages (Inaba et al., 1992). The combination of TNF- α with GM-CSF was shown to yield higher numbers of DCs than supplementation of GM-CSF alone, with TNF- α inhibiting the formation of granulocytes in the colony as well as activating the DCs, in a gentler fashion than seen with a general stimulant such as lipopolysaccharide (LPS). (Santiago-Schwarz et al., 1993). SCF has been shown to supplement cell proliferation and increase yield of DCs generated from HPCs in culture, without modifying the commitment to DC differentiation established by GM-CSF+TNF- α (Santiago-Schwarz et al., 1995). Supplementation with IL-4 was also added to various protocols since it had been associated with a reduction in the amount of CD14⁺ monocytes present following culture, and was shown to further supplement the growth of HLA-DR^{high} cells with DC morphology and antigen-presenting capabilities (Lardon et al., 1997; Rosenzweig et al., 1996). Fms-related tyrosine kinase 3 ligand (Flt3L) supplementation can also dramatically increase the numbers of both myeloid-type and lymphoid-type DCs in culture, but harbours the disadvantage of not acting solely on DC generation

(Maraskovsky et al., 1996). *In vivo* injection of Flt3L in mice expanded the populations of an assortment of hematopoietic cells (Brasel et al., 1996; Shaw et al., 1998). While there is limited available information on the differentiation progress in respect to cytokine-associated changes in DC immunophenotype during culture, there are multiple successful studies on the identification of DC subtypes and precursors generated from these *ex vivo* methods (Breton et al., 2015; Wang et al., 2016).

2. HYPOTHESIS

This project investigates the profiles of cord blood-derived dendritic cell populations isolated *in vivo* at birth or generated *ex vivo* from CD34⁺ hematopoietic progenitor cells (HPCs). The immunophenotype of DC subsets and expression of key receptors of interest will be compared in atopic at-risk and not-at-risk groups. Furthermore, the expression profiles of DCs generated *ex vivo* will be compared to DCs *in vivo*, with the purpose of gaining insight into DC differentiation *ex vivo*.

Specific Hypotheses:

- 2.1 Cultured cord-blood CD34⁺ progenitor-derived DCs are comparable to DCs present in cord blood at birth.
- 2.2 Cultured cord-blood CD34⁺-derived DCs from atopic at-risk infants have a different immunophenotype than DCs from not-at-risk infants.
- 2.3 DCs found in cord blood at birth from atopic at-risk infants have a different immunophenotype than DCs from not-at-risk infants.

3. AIMS

Aim #1. Optimization of the DC culturing procedure, development of antibody panel and optimization of DC gating strategy for flow cytometry

Aim #2. Flow-cytometry data acquisition and analysis of receptor expression of *in vivo* DCs present in cord blood at birth

Aim #3. Flow-cytometry data acquisition and analysis of receptor expression of DCs generated *ex vivo*, and subsequent comparison to the *in vivo* DCs obtained from the same sample.

Aim #4. Comparison of the immunophenotype of *in vivo* and *ex vivo* DC's in atopic-at-risk and not-at-risk newborns.

4. MATERIALS AND METHODOLOGY

4.1 Obtaining Samples. Cord blood samples were obtained from newborns at McMaster Children's Hospital, following parental consent. Atopic risk status was determined based on a questionnaire completed by the mother. Atopic-at-risk (n=16) and not-at-risk (n=7) infants were determined based on the mother's self-reported atopic status.

4.2 Processing Cord Blood. Cord blood was transferred into 50 mL tube and mixed at a 1:5 ratio of 6% dextran (Sigma) to cord blood. The sample was mixed thoroughly before incubating in a water bath at 37°C water bath for 30 minutes. The clear top layer was transferred to fresh 50 mL tube, where it was diluted 1:1 with McCoys 5A media (Gibco). 12 mL of Lymphoprep (Stemcell Technologies) was previously aliquoted into a 50 mL tube, and 20mL of the diluted blood was carefully layered on top of the density gradient medium. The sample was then centrifuged at 2200rpm, R/T for 20 min (Allegra X15R centrifuge, SX4750A rotor, Beckman). Next, the mononuclear cell layer was transfer to fresh 50 mL tube and top up to the 50 mL mark with McCoys 5A media. After thorough mixing, a 100uL sample was taken for a cell viability count. The cell suspension was centrifuged at 1500rpm for 10 min, and the supernatant aspirated completely before proceeding to magnetic labeling and cell separation.

4.3 Magnetic Labelling. The cell pellet was resuspended in 300uL of autoMACS rinsing solution (Miltenyi Biotec) for up to 10^8 cells. If more than 10^8 cells, were acquired, two tubes with 300uL were utilized. Next, 100uL of FcR Blocking reagent and 100uL of CD34 microbeads from the MACS CD34⁺ Microbead Kit (Miltenyi Biotec) were added to the cell suspension. The sample was pipetted to ensure thorough mixing, and incubated at 4°C for 30 min. Next, the cells were washed by adding 5-10 mL of rinsing solution, followed by centrifuging at 1500 rpm for 10 min at 4°C. The supernatant was aspirated, and the cells resuspended in 500uL of rinsing solution.

4.4 Isolating CD34⁺ Cells. Either an MS column (max 2×10^8 cells) or LS column (max 2×10^9 cells) was placed into MACs magnetic cell separator (Miltenyi Biotec) with a filter on top, and a tube to catch the “flow through” (FT). The column was prepared by running with the appropriate amount of rinsing solution (MS: 500uL, LS: 3mL). Next, the cell suspension was added through the column, followed by 3 washes with appropriate amount of rinsing solution (MS: 3 x 500uL, LS: 3 x 3mL). Rinsing solution (MS: 1mL, LS: 5 mL) was then pipetted directly into the column and immediately flushed out into a fresh 15mL tube and topped up to 10mL with desired media, collecting the magnetically labelled CD34⁺ cells. The “flow through” from the cell separation was also collected in a fresh 50mL tube and topped up with PBS. A sample of FT and CD34⁺ cells were taken for a cell count, while the remainder were each centrifuged at 1500 rpm for 10 min, R/T. The CD34⁺ cells proceeded to cell culturing while the FT cells were stained for flow

cytometry, as illustrated in Figure 3. With this strategy, isolated mononuclear cells were optimized to maximize yield of CD34⁺ cells to be isolated for DC culturing while ensuring sufficient presence of DCs in the FT for *in vivo* DC data collection.

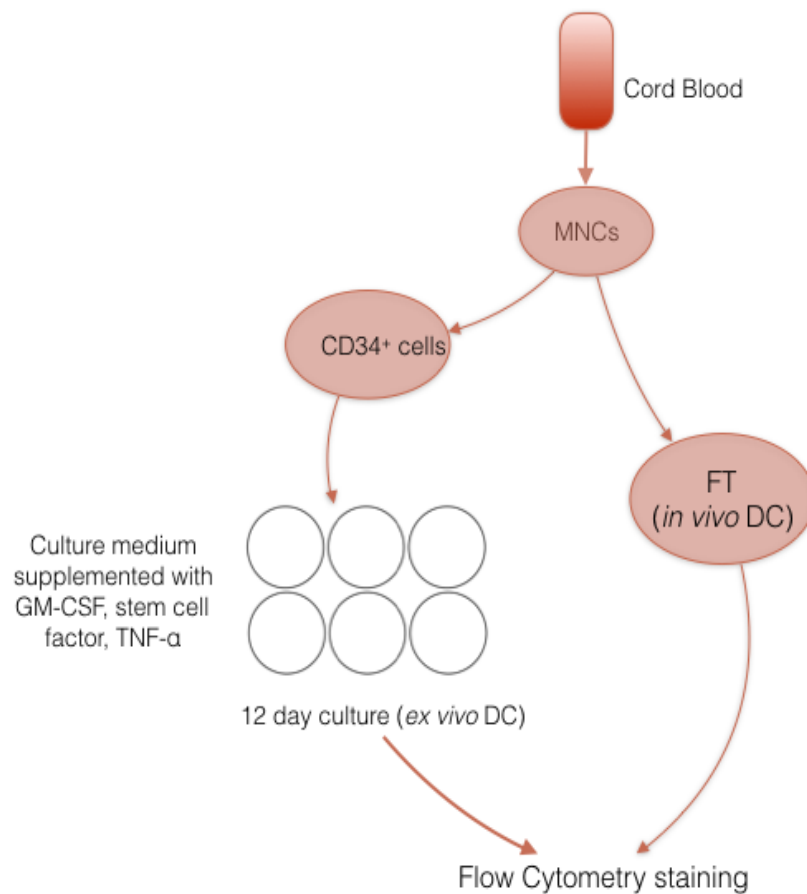


Figure 3: Schematic of the distribution of cell isolation from cord blood, illustrating how a single sample will be optimized for maximum yield.

4.5 Inducing HPC differentiation into DCs in culture. DC culture methodology is summarized in Figure 4 and was based on the original method described in Lardon et. al. (1997). Prior to commencement of cell culture, culture medium containing Iscove's Modified Dulbecco Medium (IMDM) (Stemcell Technologies) with 10% fetal calf serum (FCS) (Gibco), 2% penicillin-streptomycin (Gibco- 10,000 units/mL penicillin, 10,000 µg/mL streptomycin), 1% bovine serum albumin (BSA) (MACs stock BSA, Miltenyi Biotec) was prepared. The CD34⁺ cell pellet was resuspended at 50 000 cells/mL in culturing medium and plated on a 6-well plate, with 2 mL per well (10⁵ CD34⁺ cells per well). One well on each plate contained 2 mL of distilled water for humidity. Next, each well was supplemented with the appropriate amount of cytokines; 50 ng/mL GM-CSF (Peprotech) 50 ng/mL SCF (Cedarlane), 2.5 ng/mL TNF- α (Peprotech), and incubated at 37°C, 5% CO₂. At day 5 and onwards, the culture was supplemented with 1000 U/mL IL-4 (Peprotech). Half-medium changes occurred every 3-4 days. The culture was scraped on day 12, with both the adherent cells and supernatant collected for immunophenotyping.

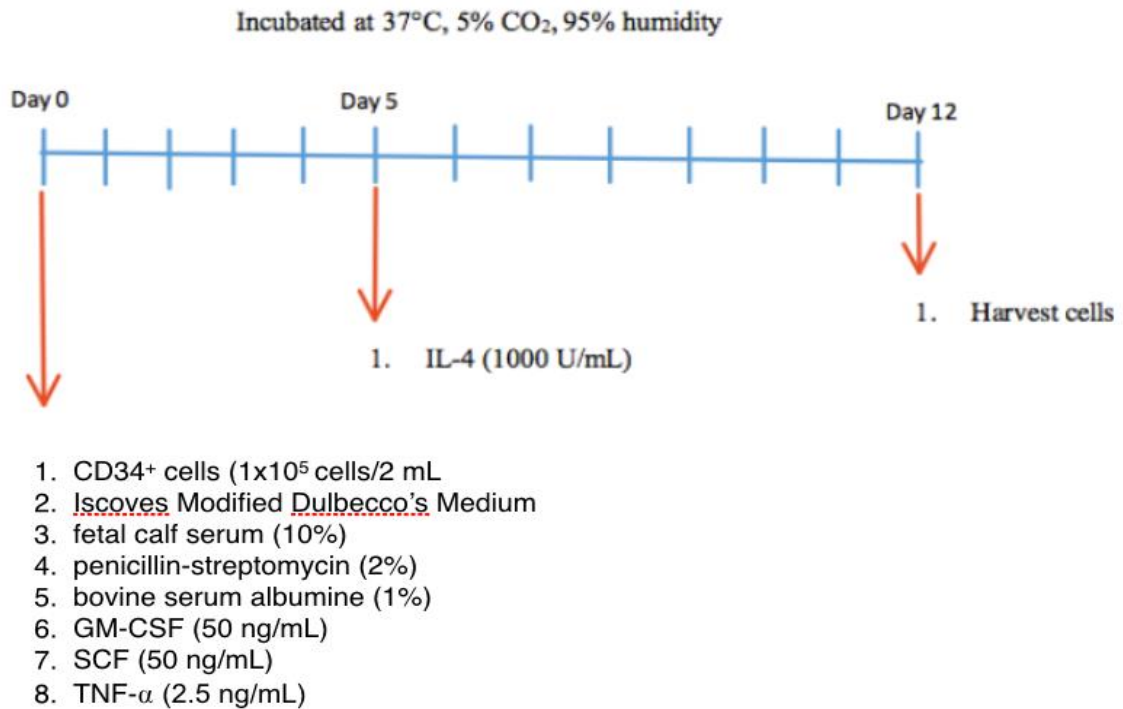
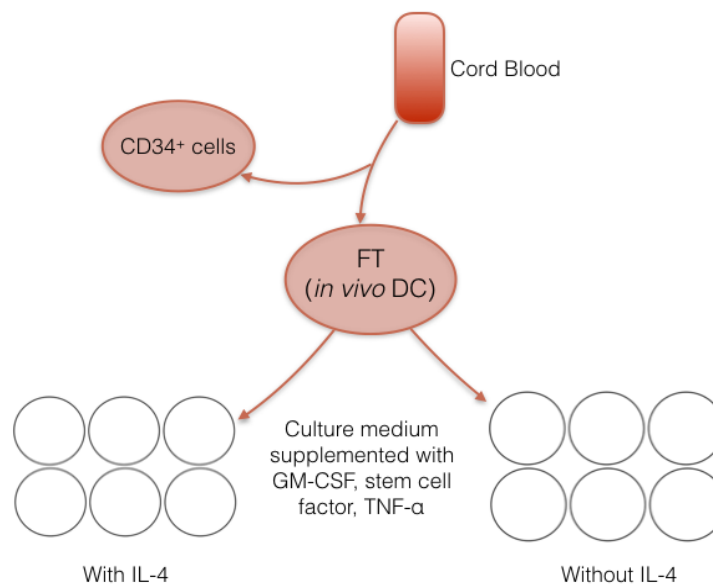


Figure 4: Timeline schematic of DC culturing procedure. Isolation of CD34⁺ cells from cord blood began on Day 0, prior to culturing procedure. Flow cytometry staining was conducted on day 12.

4.6 Exposing “in vivo” DCs to the culture environment. DCs present *in vivo* at birth were cultured for 3 days to gain better understanding of the differences between DCs in the FT and CD34⁺- derived DCs. FT cells (n=2) were washed with culture medium and plated at a concentration of 250 000 cells/mL in two 6-well plates with the appropriate cytokines in each well (50 ng/mL GM-CSF, 50 ng/mL stem cell factor, and 2.5 ng/mL TNF- α). One plate was supplemented with 1000 U/mL IL-4 at day 0 onwards, while the other plate contained no IL-4. The culture was incubated at 37°C, 5% CO₂ for 3 days. The cells were collected on Day 3, preceding flow cytometry analysis. Culture protocol for “in vivo” DCs is summarized in Figure 5.



Flow Cytometry staining after 3 days in culture

Figure 5: Schematic of *in vivo* DC culturing procedure. Isolation of FT cells from cord blood is conducted as previously described. Flow cytometry staining occurs on Day 3 of culture using the typical antibody panel.

4.7 Flow Cytometry. *In vivo* and *ex vivo* DCs were analyzed with flow cytometry to determine the expression of key receptors. 5-10 uL of fluorescently-labelled mouse anti-human monoclonal antibodies were added to previously prepared aliquots of FT or cultured DCs. The monoclonal antibodies used for staining include anti-TLR9 (eBioscience), anti-TLR5 (RD systems), anti-ST2 (MBL), anti-TSLP (eBioscience), anti-FcεRI (eBioscience) and their corresponding IgG isotype controls, as well as anti-CD80 (BD Pharmingen), anti-CD86 (BD Pharmingen), anti-HLA-DR (BD Pharmingen), anti-CD11c (Biolegend), anti-CD123 (Biolegend), anti-CD45 (eBioscience), & Lin (BioRad). Methodology and antibody panel used were the same for flow cytometry on the FT cells and the CD34⁺-derived cells cultured *ex vivo*.

Flow buffer (phosphate-buffered saline (PBS)+ 5% FBS) was prepared prior to the commencement of antibody staining protocol. Cells were distributed into labelled polystyrene round-bottom tubes, including 3 “experimental” tubes, 3 “isotype control” tubes, and 1 “unstained cells” tube. FT cells were distributed at 2x10⁶ cells/tube. Next, 1-2 mL of flow buffer was added to each tube, then the samples were centrifuged at 1500 rpm for 10 min at 4°C. Supernatant was discarded, tubes were blot-dried and resuspended. The pellets were resuspended in 20-50uL fc block and left in the fridge for 30 min. After the samples were removed from incubation, 2 mL flow buffer was added to each tube, and left in the fridge overnight. The next day, 1 mL flow buffer was added to each sample and they were centrifuged at 1500 rpm for 10 min at 4°C. Supernatant was discarded, tubes were blot-dried and resuspended.

4.7.1 Cell Surface Staining. Surface antibodies were added to each cell as described in Table 1. 30uL of flow buffer was added to “Unstained Cells” tube instead of antibodies. Compensation tubes were prepared using OneComp eBeads (Invitrogen) prior to data acquisition. These samples were stored in the fridge, covered with aluminium foil, prior to data acquisition. Tubes 1 and Iso 1 proceeded to intracellular staining.

Table 1: DC antibody panel and the corresponding fluorochrome. Tubes 1-3 are experimental, while tubes Iso 1-3 serve as controls.

	<i>Tube 1</i>	<i>Iso 1</i>	<i>Tube 2</i>	<i>Iso 2</i>	<i>Tube 3</i>	<i>Iso 3</i>
<i>EF450</i>	CD45 0.5uL	CD45 0.5uL	CD45 0.5uL	CD45 0.5uL	CD45 0.5uL	CD45 0.5uL
<i>AF700</i>	Lin 5uL	Lin 5uL	Lin 5uL	Lin 5uL	Lin 5uL	Lin 5uL
<i>APC h7</i>	HLA-DR 2.5uL	HLA-DR 2.5uL	HLA-DR 2.5uL	HLA-DR 2.5uL	HLA-DR 2.5uL	HLA-DR 2.5uL
<i>PerCP 5.5</i>	CD11c 5uL	CD11c 5uL	CD11c 5uL	CD11c 5uL	CD11c 5uL	CD11c 5uL
<i>PeCy7</i>	CD123 5uL	CD123 5uL	CD123 5uL	CD123 5uL	CD123 5uL	CD123 5uL
<i>FITC</i>	TLR5 5uL	Mouse IgG1 5uL	ST2 5uL	Mouse IgG1 5uL	FceRI 5uL	Mouse IgG2b 5uL
<i>PE</i>	<i>TLR9</i> 5uL	<i>Rat IgG2a</i> 5uL	TSLP 5uL	Mouse IgG2a 5uL	CD80 5uL	Mouse IgG1 5uL
<i>APC</i>	—	—	—	—	CD86 10uL	Mouse IgG1 10uL

After the addition of antibodies, the samples were gently vortexed, covered with aluminum foil and incubated in the fridge for 30 min. Next, the samples were washed with 1-2 mL flow buffer and centrifuged at 1500 rpm for 10 min at 4°C. Supernatant was discarded, tubes were blot-dried and resuspended. The cell pellets in tubes 2, iso 2, 3, and iso 3 were resuspended in 300 uL of flow buffer while the pellet in “unstained” tube was resuspended in 500uL buffer.

4.7.2 Intracellular Staining. 300 uL IC fixation buffer (eBioscience) was added to Tubes 1+ Iso 1, which were then incubated in the fridge for 20 min. Samples were washed with flow buffer, centrifuged at 1500 rpm for 10 min at 4°C, supernatant was discarded, tubes were blot-dried and resuspended. Next, 2 mL of 1x permeabilization buffer (eBioscience) was added to tubes 1+ Iso 1. Following incubation in the fridge for 20 min, the tubes were centrifuged at 1500 rpm for 10 min at 4°C, supernatant was discarded, tubes were blot-dried and resuspended. Intracellular antibodies (TLR9, Rat IgG2a) were now added, followed by 20 min incubation in the fridge. Lastly, 2mL of flow buffer was added to tubes 1+ Iso 1, which were then centrifuged at 1500 rpm for 10 min at 4°C. The supernatant was discarded, tubes were blot-dried and resuspended, and resuspend in 300uL of flow buffer prior to data acquisition.

4.8 Flow Cytometry Data Acquisition. Immunophenotypic data was acquired on a BD LSR II flow cytometer (BD Biosciences) in the McMaster Immunology Research Centre, while data analysis was conducted using Flow Jo software (Tree Star). The DC gating strategy was developed based on a DC gating method previously established by the Denburg lab (Figure 6).

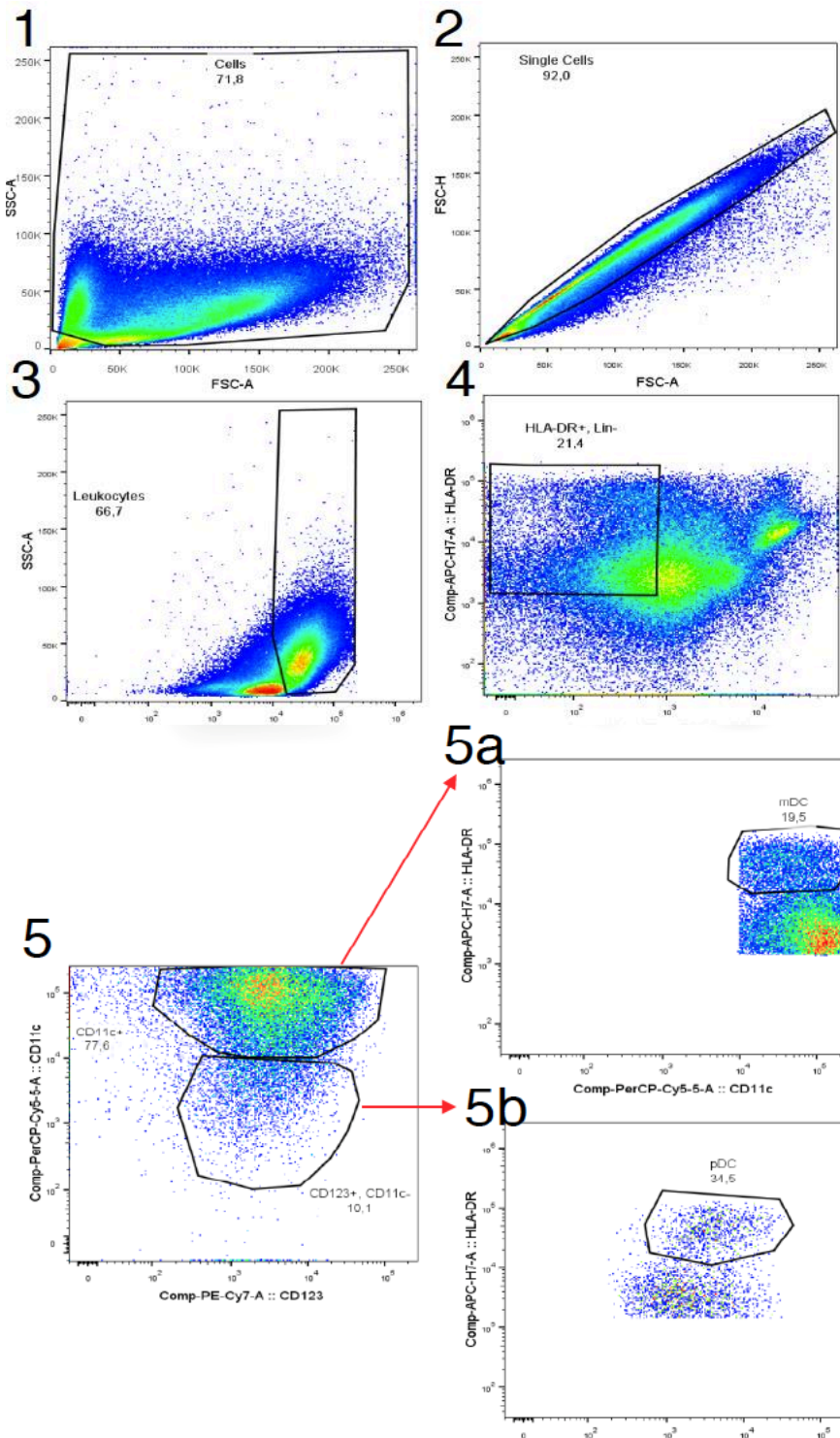


Figure 6: DC gating strategy. 1) cell gate (FSC-a x SSC-a) with debris gated out, 2) single cell gate (FSC-h x FSC-a), 3) leukocyte gate (SSC-a x CD45⁺), 4) DC-like gate (HLA-DR⁺ x Lin⁻), 5) mDC-like gate (CD11c⁺) and pDC-like gate (CD123⁺, CD11c⁻), 5a) mDC gate (CD11c⁺, HLA-DR^{high}), 5b) pDC gate (CD123⁺, CD11c⁻, HLA-DR^{high})

5. RESULTS

5.1 DC differentiation in culture from hematopoietic progenitors.

The starting concentration of CD34⁺ progenitors, culture plate size, culturing duration, medium change frequency and cytokine concentrations were all optimized and the methodology was modified accordingly. Culturing the progenitors consistently yielded a 10-fold increase in cell number and an increased proportion of DCs relative to other cells in the sample, corroborating the results in Larden et. al (1997) and Schoeters et. al (2007).

Morphologically, the cells appear larger in size and with distinguishable dendrites by day 12 (Figure 7.3). Some clustering is observed to begin as early as Day 3, prior to IL-4 supplementation (Figure 7.1). After IL-4 supplementation, cell proliferation and aggregation into large clusters increased dramatically (Figure 7.2).

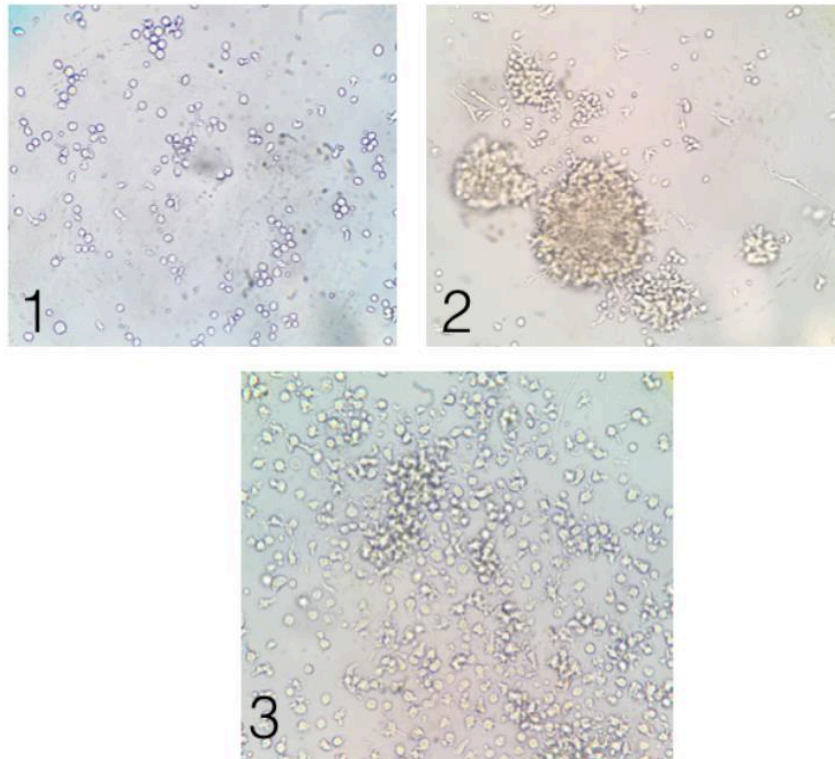


Figure 7: DC culture images at various timepoints, as viewed on an Olympus IMT microscope at 400x magnification. **1)** Day 3, prior to IL-4 supplementation. **2)** Day 8, post-IL-4 supplementation. **3)** Day 12.

Additional flow cytometry analysis (n=1) was conducted on cord blood mononuclear cells (with and without LPS stimulation), CD34⁺ cells, “flow-through” (FT) runoff from CD34⁺ positive selection, and CD34⁺-derived DCs after a 12 day culture to compare the distribution of DCs at different stages of sample processing on a single subject (Table 2). The purpose was to verify if FT cells could be used to study DCs *in vivo* a birth instead of mononuclear cells (MNCs), which would correspond to a larger yield of CD34⁺ cells available to proceed to culture. LPS stimulation was included in the experiment as a positive control of *in vivo* DCs, since LPS acts as a potent DC stimulator. High DC numbers were observed in the FT while isolated CD34⁺ cells exhibited low DC

numbers, indicating that the FT runoff did indeed collect a majority of the DCs present in the mononuclear cell layer. LPS stimulation was observed to increase the amount of DCs among mononuclear cells, as expected, strengthening the conclusion that DC populations were being correctly gated during FlowJo analysis. Lastly, CD34⁺-derived DCs after 12-day culture exhibited a 9-fold increase in the proportion of DCs in the leukocyte population. While the proportion of pDCs increased 6X after culture compared to MNCs, a more dramatic increase is seen in the 16X increase of the cultured mDC population compared to the mDCs present in MNCs.

Table 2: Distribution of DCs as a percentage of “DC” gate and “leukocyte” gate at various stages of cord-blood processing and progenitor culturing procedure (n=1).

Sample Cells	%mDC in “DC” gate (cells/single cells/ Lin -/ CD45+ HLA-DR+)	%pDC in “DC” gate (cells/single cells/ Lin -/ CD45+ HLA-DR+)
MNC	3.08	3.46
MNC, LPS-stimulated	6.62	16.5
“Flow Through”	20.9	45.8
CD34 ⁺	0.38	0.70
CD34 ⁺ -derived DCs	60.3	19.8

Sample Cells	%DC in “leukocyte” gate (cells/single cells/CD45+)
“Flow Through”	1.92
CD34 ⁺ -derived DCs	17.3

To gain insight into the DC differentiation progression in culture, CD34⁺-derived cells proliferating in culture (n=5) were tracked through the 12-day period and analyzed for DC markers using flow cytometry at different time points. Samples were taken at Day 3, Day 8, and Day 12 of the culture period, and compared to *in vivo* DCs from the flow through (Day 0). The development of a distinct population within the leukocyte population exhibiting a DC immunophenotype (HLA-DR⁺, Lin⁻) is seen emerging by Day 3, a finding consistent across all samples (Figure 8). This population is seen increasing as culture progresses, with the emergence of a secondary HLA-DR^{high} population seen developing by Day 12 (Figure 8). In addition, the overall proportion of DCs in the leukocyte population is consistently higher after culturing when compared to *in vivo* DCs.

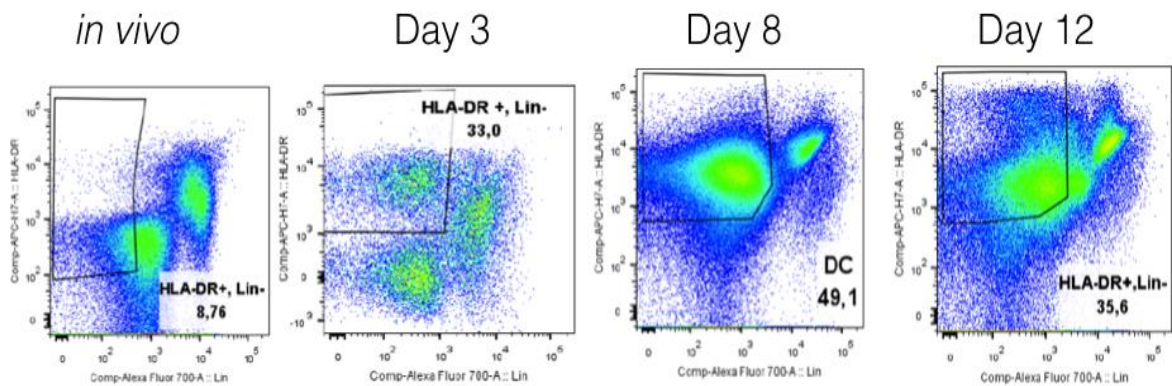


Figure 8: Leukocyte (CD45⁺) distribution during culture progression at Day 3, Day 8, Day 12, and *in vivo*. HLA-DR is on the y-axis, Lin is on the x-axis. DC population (HLA-DR⁺, Lin⁻) is shown in the gate. Further gating on DC population shown in Figure 9.

The ratio of mDCs to pDCs changes significantly during the culturing procedure. While the number of mDC-type (CD11c⁺) and pDC-type (CD11c⁻, CD123⁺) cells quickly and dramatically increased during culture, the emergence of distinct mDC (HLA-DR^{high}, CD11c⁺) and pDC (HLA-DR^{high}, CD11c⁻, CD123⁺) populations begins to be evident at Day 8 (Figure 9) (Figure 10). Notably, the mDC population experiences the largest growth at Day 12 when compared to the mDC population at Day 0 (Figure 10a).

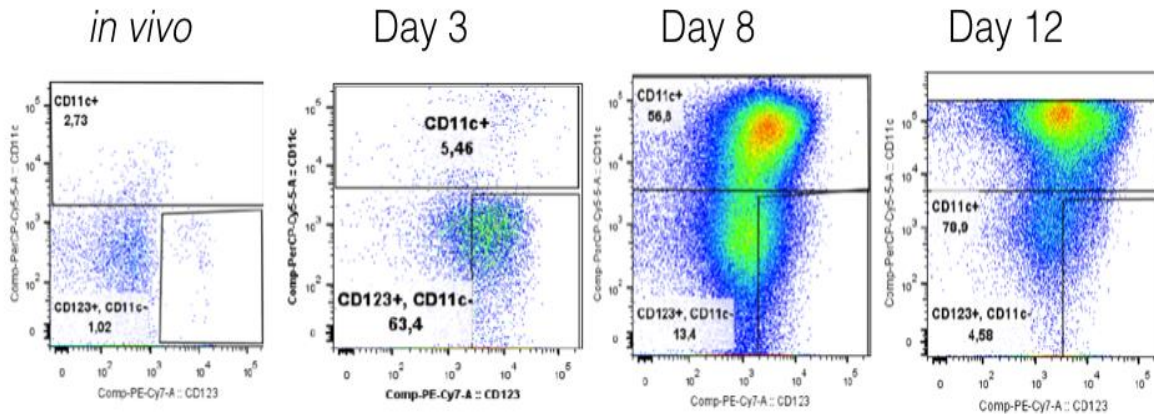


Figure 9: DC population (HLA-DR⁺, Lin⁻) distribution during culture progression at Day 3, Day 8, Day 12, and *in vivo*. CD11c is on the y-axis, CD123 is on the x-axis. mDC-like (HLA-DR⁺, Lin⁻, CD11c⁺) and pDC-like (HLA-DR⁺, Lin⁻, CD123⁺, CD11c⁻) gates are shown. Further DC subtype gating on these populations shown in Figure 10.

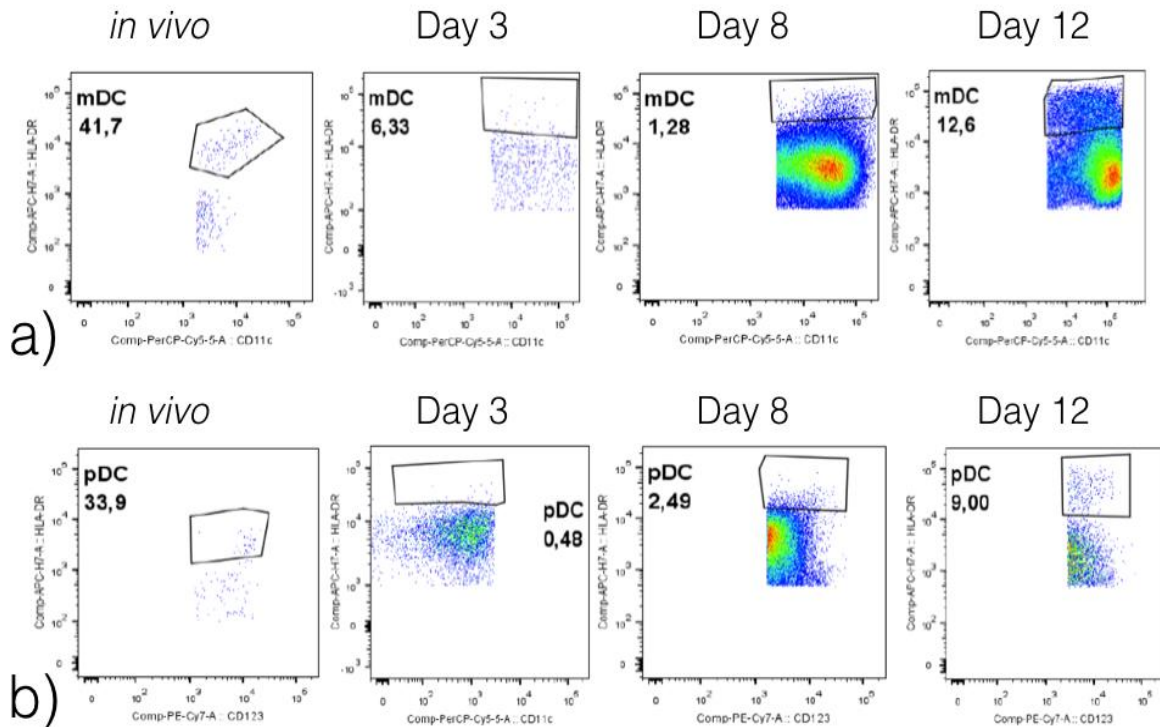


Figure 10: DC subtype distribution during culture progression at Day 3, Day 8, Day 12, and *in vivo*. **a)** mDC population (CD11c⁺, HLA-DR^{high}, Lin⁻) is gated. HLA-DR is on the y-axis, CD11c is on the x-axis. **b)** pDC population (CD123⁺, CD11c⁻, HLA-DR^{high}, Lin⁻) is gated. HLA-DR is on the y-axis, CD123 is on the x-axis.

5.2 Receptor expression profiles of DCs from atopic-at-risk infants compared to not-at-risk.

The percentage of cells expressing TLR9, TLR5, ST2, TSLP, FcεRI, CD80 & CD86 receptors were compared between atopic-at-risk (n=16) and not-at-risk (n=7) phenotypic groups in *in vivo* mDCs, *in vivo* pDCs, *ex vivo* mDCs & *ex vivo* pDCs. A normality test was performed to determine whether a T-test or Wilcoxon test would be most appropriate in the association analysis. When the data was found not distributed normally, the result from Wilcoxon test was considered instead of the T-test. Outliers were identified based on +/- 3 standard deviations and excluded from analysis.

A significant association was found in *ex vivo* pDC TSLPR expression, with atopic-at-risks expressing more TSLPR than not-at-risks (Figure 11). The outliers identified and removed during the association analysis are as follows: TLR5- 3 outliers; ST2-1 outlier; TSLPR-1 outlier; FcεRI-3 outliers. The data was not distributed normally, so a Wilcoxon test was used to obtain a p value of 0.04257 (Appendix 1). No statistically significant differences were found between the controls and cases in *ex vivo* mDCs, *in vivo* mDCs, and *in vivo* pDCs (Appendix 2). However, increased *in vivo* mDC FcεRI expression in atopic-at-risks compared to not-at-risks was found approaching significance, with a p value of 0.06697 (Figure 12).

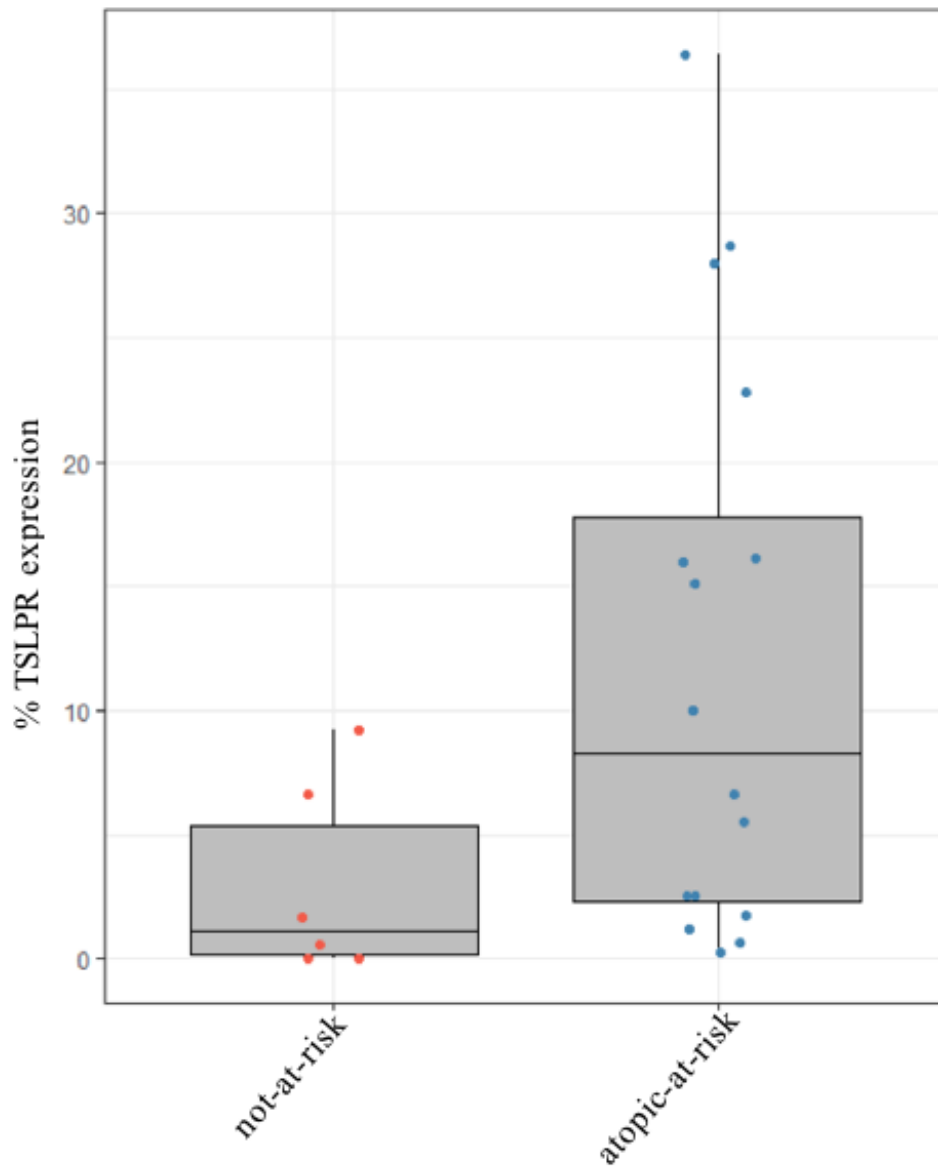


Figure 11: TSLPR expression of pDCs cultured *ex vivo* from cord-blood derived CD34⁺ cells. pDCs cultured *ex vivo* from cord-blood HPCs in atopic-at-risk infants have higher TSLPR expression than not-at-risk infants ($p < 0.05$). The experimental atopic-at-risk group ($n=16$) expresses TSLPR more than the not-at-risk ($n=7$) controls. Atopic risk was determined based on maternal self-reported atopic status. Mean \pm SEM is shown.

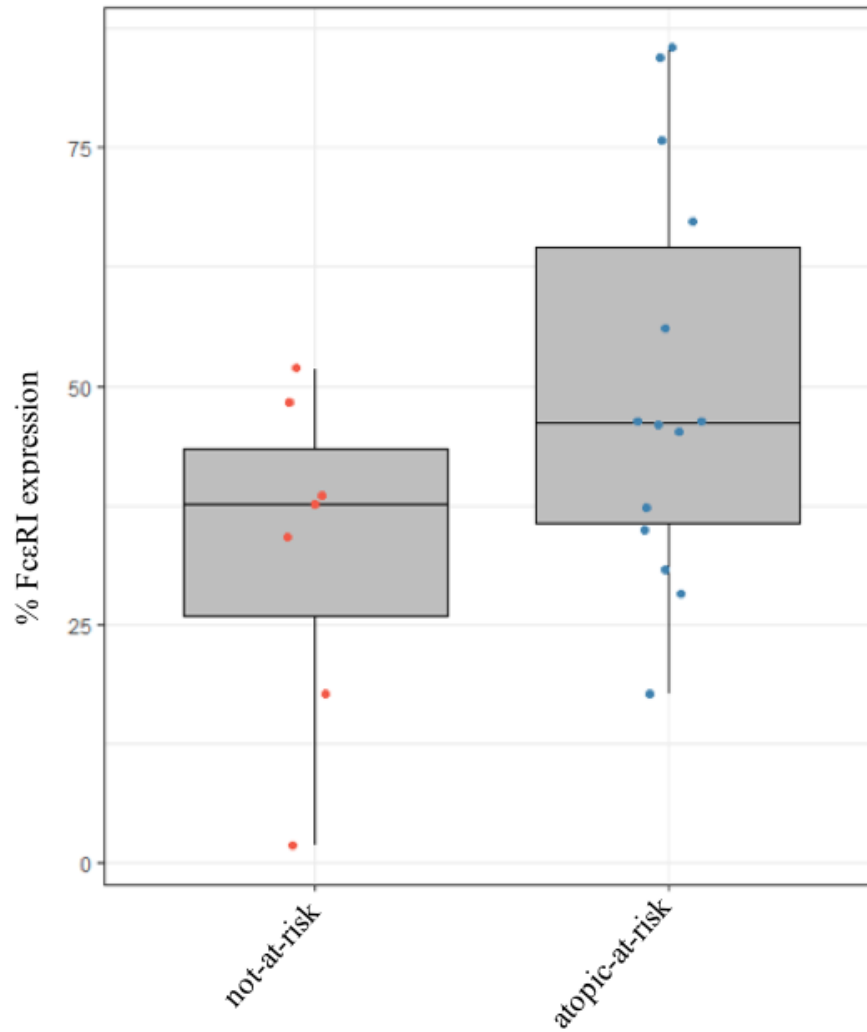


Figure 12: FcεRI expression of *in vivo* mDCs in atopic-at-risks compared to not-at-risks. FcεRI expression of mDCs present in the cord blood at birth is increased in atopic-at-risk infants ($p=0.06697$). *In vivo* mDCs obtained from the “flow through” of processed cord blood. Atopic-at-risk ($n=16$) is the experimental group, while not-at-risk ($n=7$) is the control group. Atopic risk was determined based on maternal atopic status. Mean \pm SEM is shown.

5.3 Receptor expression profiles of ex vivo DCs compared to in vivo DCs.

The percentage of cells expressing TLR9, TLR5, ST2, TSLP, FcεRI, CD80 & CD86 receptors were compared between DCs grown *ex vivo* (n=23) and DCs present *in vivo* (n=23) within the same subject. Both T tests and Wilcoxon tests were performed, and when the data was not distributed normally, the results from Wilcoxon test were considered instead of the T-test (Appendix 3). The data indicates that the proportion of mDCs expressing TLR5, TSLP, FcεRI and CD80 receptors are significantly different between DCs grown in culture and DCs present *in vivo* (Appendix 3). Proportion of cultured mDCs express more TSLP, CD80 receptors and less FcεRI and TLR5 than blood DCs (Figure 13) (Figure 14). Additionally, the proportion of pDCs expressing TLR5, ST2, TSLPR, CD80 and CD86 are significantly different between pDCs grown in culture compared to blood pDCs (Appendix 3). pDCs grown *ex vivo* in culture express less TLR5 & ST2, more TSLPR, CD80, CD86 than blood pDCs (Figure 13) (Figure 15). The general trend of increased TSLPR, CD80, CD86 and decreased ST2, FcεRI, TLR5 expression after culture is consistent in both mDCs and pDCs.

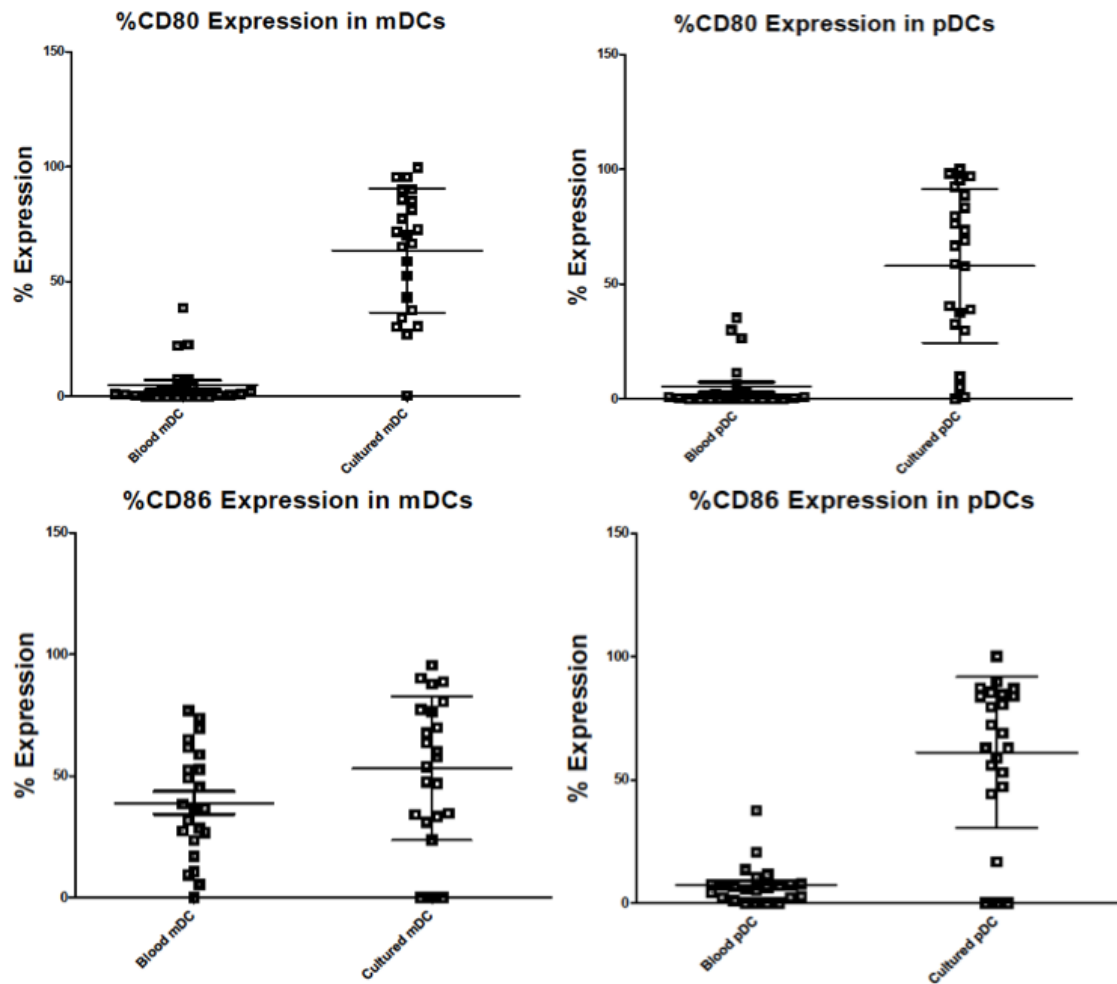


Figure 13: Comparison of % cells expressing receptors associated with DC activation between *in vivo* mDCs/pDCs (n=23) and *ex vivo* mDCs/pDCs (n=23). Differences in CD80 (mDCs and pDCs) and CD86 (pDCs) expression are statistically significant, with higher expression of both markers found in DCs grown in culture compared to blood DCs. Bars represent SEM. Further statistics found in the appendix.

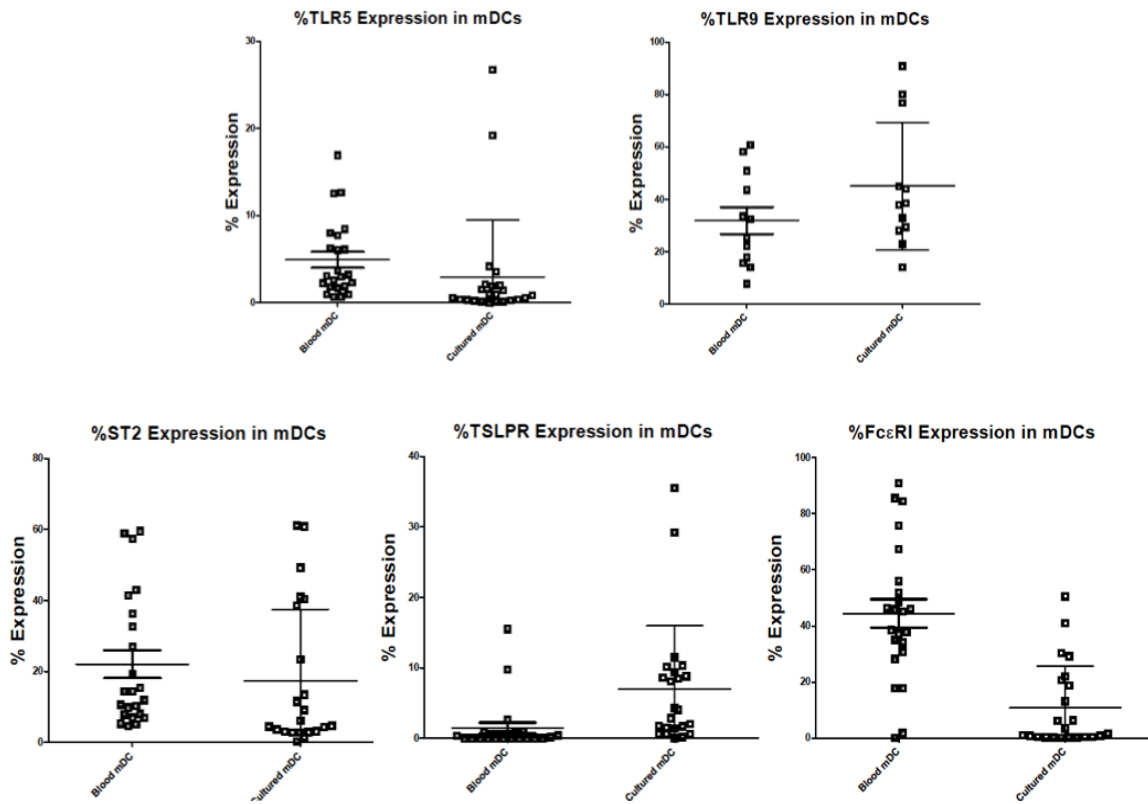


Figure 14: Comparison of % cells expressing immune receptors between *in vivo* cultured mDCs (n=23) and *ex vivo* blood mDCs (n=23). Differences in TLR5, TSLPR, FcεRI receptor expression are statistically significant, showing that mDCs grown in culture express TSLPR at higher levels, TLR5 & FcεRI at lower levels, than blood DCs. Bars represent SEM. Further statistics found in the appendix.

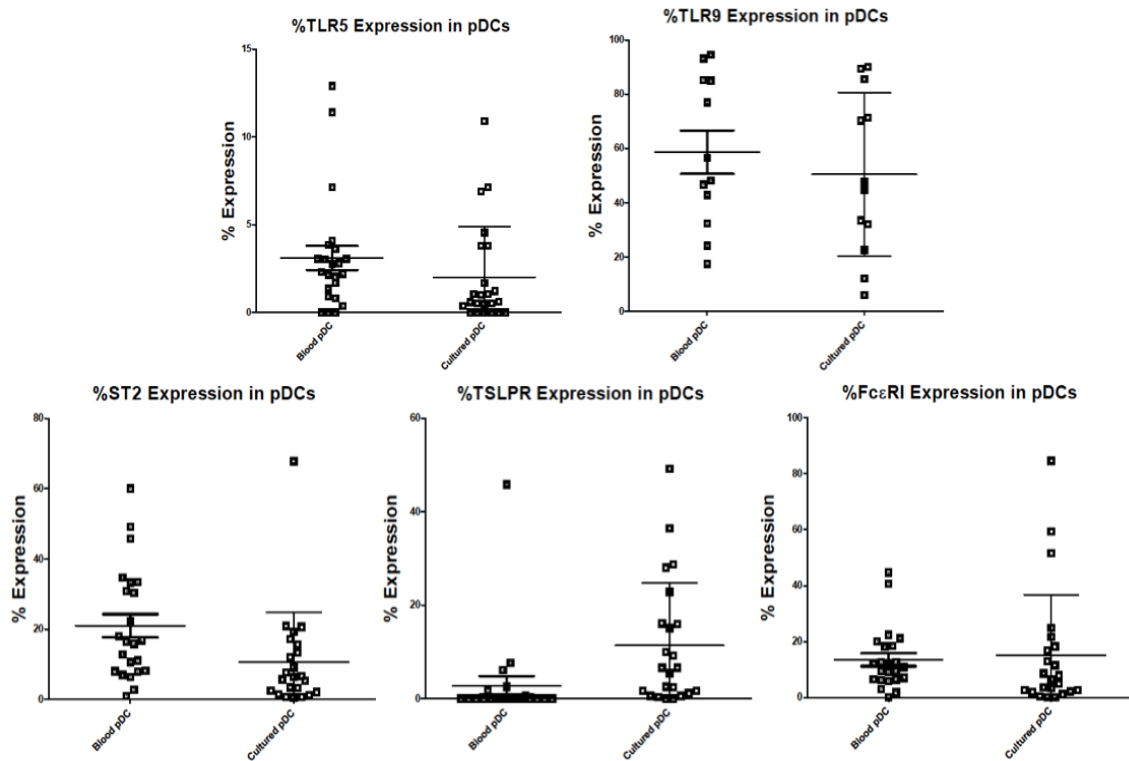


Figure 15: Comparison of % cells expressing immune receptors between *in vivo* pDCs (n=23) and *ex vivo* pDCs (n=23). *Ex vivo* pDCs exhibit a decrease in TLR5, ST2 expression & an increase in TSLPR expression compared to *in vivo* pDCs. Bars represent SEM. Further statistics found in the appendix.

6. DISCUSSION

During the culture of cord blood-derived CD34⁺ progenitors, a consistent abundance of the mDC population compared to the pDC population by day 12 was observed. This is not unexpected, since the addition of GM-CSF to the medium was thought to push the progenitors towards the myeloid differentiation pathway. GM-CSF is a well-known growth factor which plays a role in the differentiation of cells with a myeloid lineage (Ushach and Zlotnik, 2016). As a general trend, the *ex vivo* DC subtypes express HLA-DR, CD11c, and CD123 higher than the *in vivo* DCs. One explanation for the difference is the culture environment itself, since the *ex vivo* cells are grown at dense conditions to maximize cell yield and cells in culture are more exposed to potential stimulants in the medium versus in the bloodstream (e.g. contact with airborne stimulants during medium changes). The cultured DC populations are then expected to be more activated than their *in vivo* counterparts due to their potential contact with stimulants, which expectantly translates to increased expression of antigen presentation and T-cell associated molecules such as HLA-DR, CD80, CD86. Furthermore, while the culture process succeeds at the proliferation of DC populations, it is known to yield a mixture of DCs and macrophages with a pro-inflammatory phenotype when supplemented with GM-CSF alongside TNF- α (Ushach and Zlotnik, 2016). TNF- α has also been shown to be necessary for the maturation of DCs grown *ex vivo* (Trevejo et al., 2001). Thus, *ex vivo* DCs supplemented with GM-CSF and TNF- α during culture are likely stimulated by the cytokines to activate antigen presentation mechanisms, as well as have contact with

pathogens, resulting in increased MHC-class II marker expression and a heightened state of maturity.

Culturing DCs present *in vivo* at birth was undertaken to gain better understanding of the differences observed in receptor expression between *in vivo* DCs and CD34⁺-derived DCs. In this supplementary experiment, cord blood was processed as usual and FT cells were suspended at a concentration of 250 000 cells/mL in regular culturing medium before being aliquoted in a 6-well plate. These cells were cultured for a total of 3 days, following the culturing conditions described previously, either in the presence or absence of IL-4 to account for the two phases of the culture procedure. The results (n=2) indicate that any trends observed occur cohesively in both mDCs and pDCs, with TSLPR, CD80, CD86 expression increasing and ST2, FcεRI expression decreasing after 3 days in culture (Figure 16). This is consistent with results from comparison of blood DC receptor expression to cultured DC expression, where cultured DCs had significantly increased TSLPR, CD80, CD86 expression, decreased FcεRI expression, and a trend towards decreased ST2 expression (Figure 13) (Figure 14) (Figure 15). These findings provide further evidence that the culture environment is likely the main contributing factor in the differences observed between *in vivo* and *ex vivo* DCs.

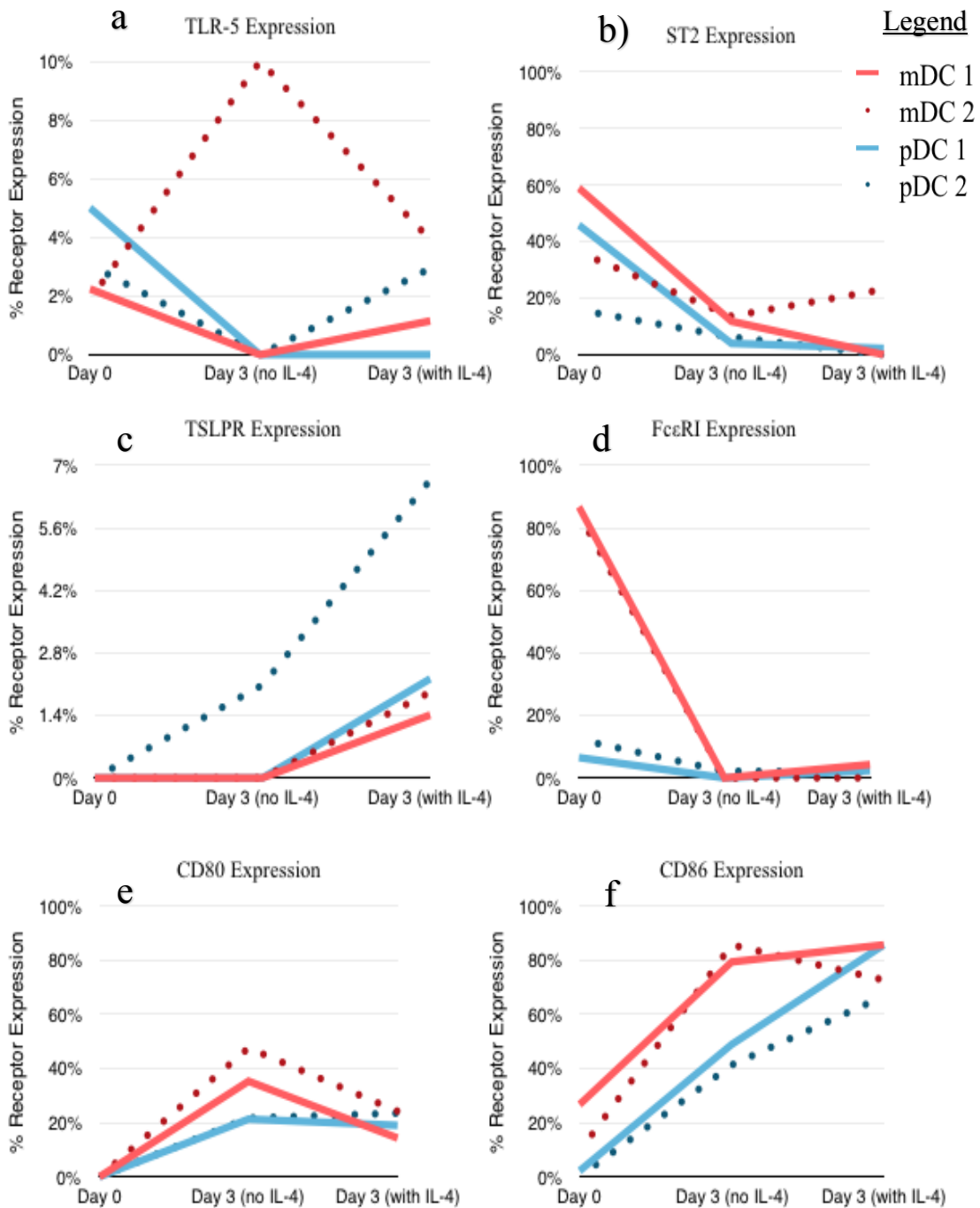


Figure 16: Graphs illustrating the change in receptor expression after *in vivo* DC exposure to culture environment (n=2). mDC 1 and pDC 1 refer to sample 1, while mDC 2 and pDC 2 refer to sample 2. DCs present in the blood at birth are cultured for 3 days according to CD34⁺ progenitor DC differentiation methodology, in the presence and absence of IL-4. In both mDCs and pDCs, TSLPR, CD80, CD86 expression increased and ST2, FcεRI expression decreased after 3 days in culture **a)** TLR5 expression, **b)** ST2 expression, **c)** TSLPR cell expression, **d)** FcεRI expression, **e)** CD80 expression, **f)** CD86 expression.

After supplementation with IL-4, the proportion of cells with a DC phenotype in culture was observed to increase (Figure 8). These results could be explained by the rapid differentiation of monocytes present into DCs after the addition of IL-4. IL-4 in the presence of GM-CSF has been shown to induce DC differentiation in monocytes by up-regulating TNF- α converting enzyme and activity in monocytes (Hiasa et al., 2009). Subsequently, the first phase of the culture procedure likely establishes a large number of myeloid-lineage cells (due to GM-CSF supplementation) including monocytes and DCs but limiting the generation of granulocytes (based on the effect of TNF- α supplementation). The second phase of culture, after IL-4 supplementation, probably increases the proportion of DCs by converting monocytes into DCs due to the potent effect of IL-4 on the polarity of monocyte differentiation.

DCs are frequently generated *ex vivo* from hematopoietic progenitors or monocytes using similar culture methodology, and the resulting *ex vivo* DCs are analyzed with the assumption that they originate and behave similarly to *in vivo* DCs. Overall, this project confirms that it is possible to generate high numbers of mDCs and pDCs from culture, but the subsequent DCs are at a different activation state than those present in the blood at birth, likely due to the culture environment. It is understood that any cell population generated *ex vivo* will not be an exact replica of their counterpart *in vivo*. An activated DC presenting pathogens certainly has initiated different cell pathways compared to an inactivated DC, however these differences in DC profile may be inconsequential depending on the research undertaken. In the context of this project, where the ultimate goal is to determine whether *ex vivo* DCs can be utilized to develop

predictive tests to determine the risk of allergic disease onset later in life, any differences in phenotype between *in vivo/ex vivo* DCs are insignificant in terms of project goals as long as *ex vivo* DCs retain their predictive power.

Unpublished observations from the Denburg lab demonstrate that the profiles of CHILD study derived cord-blood DCs are different between infants who develop atopic manifestations later in life compared to infants that do not. Specifically, the pDCs of atopic infants showed decreased TLR9 expression (Figure 17). Biases towards a Th2 response were also noted due to increased atopic DC expression of TLR5, FcεRI, and ST2, all relating to increased expression of Th2-type cytokines (Figure 18).

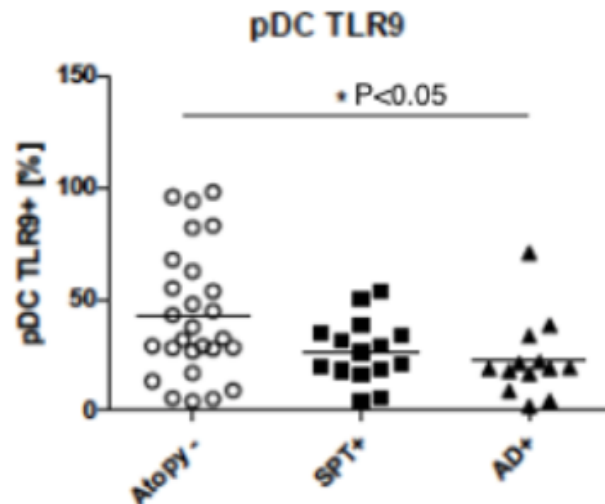


Figure 17: Unpublished observations illustrating pDC TLR9 receptor expression in cord blood of infants at 1 year of age. TLR9 expression is higher in infants without atopy (Atopy-) compared to those with positive skin-prick test (SPT+) or atopic dermatitis diagnosis (AD+). Tworek et. al. (2016, Sept). *Cord blood dendritic cell profiles and year one atopic manifestations in the Canadian Healthy Infant Longitudinal Development (CHILD) Study*. Canadian Society of Allergy and Clinical Immunology Annual Scientific Meeting, Montréal, Québec.

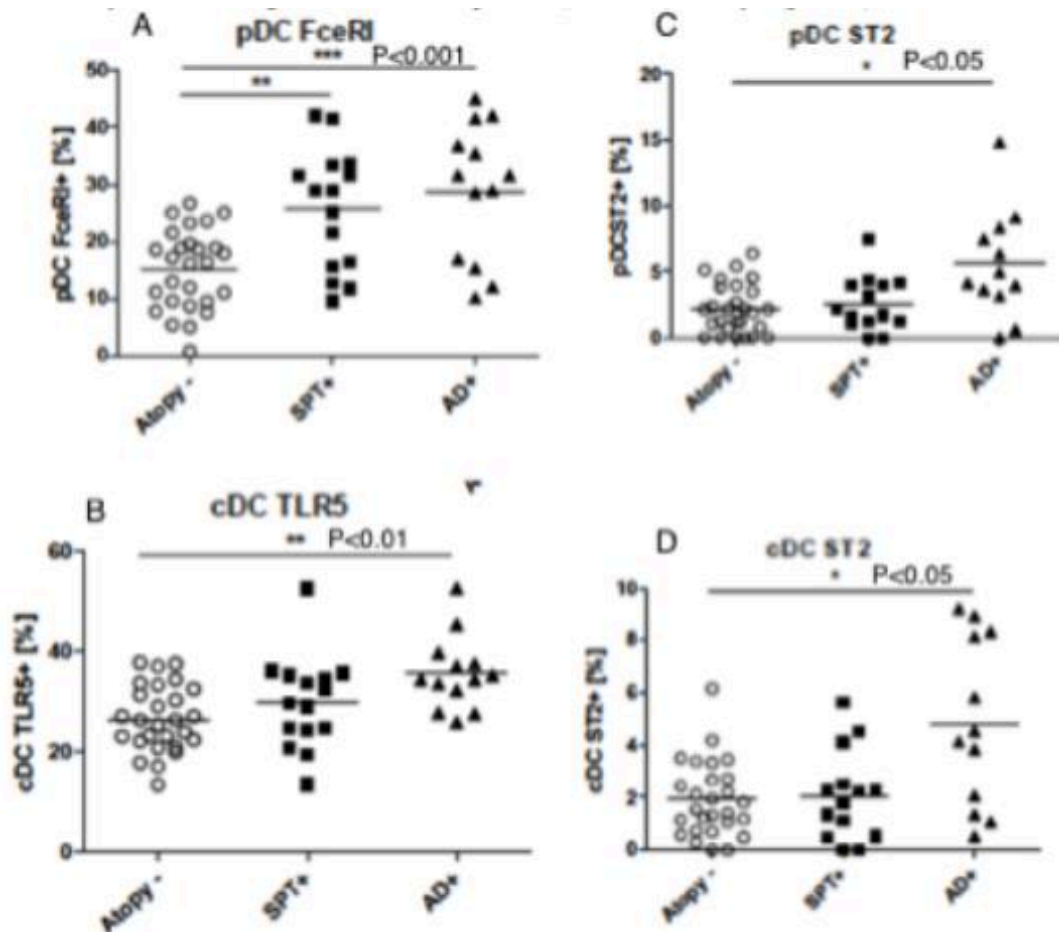


Figure 18: Unpublished observations illustrating FcεRI, TLR5, ST2 receptor expression in cord blood of infants at 1 year of age. Expression of **a)** pDC FcεRI **b)** mDC TLR5, **c)** pDC ST2 **d)** mDC ST2 is higher in infants without atopy (Atopy-) compared to those with positive skin-prick test (SPT+) or atopic dermatitis diagnosis (AD+). Tworek et. al. (2016, Sept). *Cord blood dendritic cell profiles and year one atopic manifestations in the Canadian Healthy Infant Longitudinal Development (CHILD) Study*. Canadian Society of Allergy and Clinical Immunology Annual Scientific Meeting, Montréal, Québec.

The receptors of interest in this project were selected based on these results, to examine whether these findings could be replicated in atopic-at-risk infants, as well as DCs cultured *ex vivo*. In both *in vivo* and *ex vivo* DCs, these results were not replicated, and in some cases the trend between atopic-at-risk/ not-at-risk was the opposite. For example, pDC TLR9 expression was higher in atopic-at-risks, while pDC TLR9 expression was significantly lower in confirmed atopics. TLR5 and ST2 expression in the mDCs of atopic-at-risks displayed similarly opposite trends. There was one similar trend found; pDCs in atopic-at-risks expressed more ST2 than in not-at-risks, comparable to the pDCs of confirmed atopics expressing significantly more ST2 than non-atopics. However, no comparable significant differences were found between atopic-at-risks/ not-at-risks in pDC TLR9, FcεRI, TLR5 and mDC TLR5, ST2.

The pDCs derived *ex vivo* in atopic-at-risks were found to have a statistically significant increase in TSLPR expression compared to not-at-risks (Figure 11). Another trend towards increased FcεRI expression in atopic-at-risks of *in vivo* mDCs compared to not-at-risks was also observed (Figure 12). The connection between TSLP, DCs and allergic disease is seen throughout literature. TSLP, in conjunction with the CD40 ligand, polarizes DCs to induce the differentiation of naïve CD4⁺ T cells as well as the production of Th2 cytokines (Watanabe et al., 2005). TSLP treatment of DCs *ex vivo* resulted in the upregulation of OX40 ligand (crucial for DCs treated with TSLP to polarize the differentiation of naïve T cells to Th2 cells) and blocked production of Th1-associated cytokine IL-12 (Ito et al., 2005). Additionally, the proliferation of naïve and

memory T-cells is thought to be mediated in part by the upregulation of MHC complexes and costimulatory markers in DCs activated by TSLP. (Watanabe et al., 2004). Further research established that DCs primed by TSLP are critical in maintaining homeostasis of T_H2 central memory T-cells, which could be further polarized to become T_H2 effector memory cells by TSLP (Wang et al., 2006). The association of allergic disease with increased FcεRI expression also concurs with current literature. FcεRI was shown to be upregulated in the airway tissues of adults with allergies as well as in skin-resident DCs in adults with atopic dermatitis (Bieber et al., 2000).

Overall, these findings are supported by the current literature and suggest that already at birth, there is a biological predisposition in some infants towards an allergic phenotype. The increase in DC TSLPR activity can be interpreted as an increased sensitivity to the effects of TSLP, resulting in more T-cell polarization towards a T_H2 phenotype. This may relate to an increased propensity for allergic immune responses and developing chronic allergic disease. There was also a discrepancy between *in vivo* and *ex vivo* TSLPR expression in atopic-at-risks/controls. Since the primary difference between the two sets of samples is the culture environment, perhaps the DCs in culture experience increased responses to potential allergens which amplifies some pathways and signals compared to DCs *in vivo*.

Sample size is the main limitation of this project. Sample acquisition relied on instances of labour and delivery, as well as the availability of medical professionals to properly prepare and preserve a cord-blood sample. Not enough samples were acquired to reach adequate statistical power, which has a substantial effect on how confidently the results can be interpreted. In this project, the effect size ranges from 1.25 to 2. This translates to a statistical power of 8% to detect small effect size differences to 33.4% power to detect large differences between receptor expression of atopic-at-risks and controls. Thus, this project is underpowered, and small differences between receptor expression of the experimental and control groups may have gone undetected. With this sample size, the p values of less than 0.05 (TSLPR expression) or approaching (FcεRI expression) could indicate highly significant findings but cannot be verified until reasonable power (80%) is achieved. While this data supported certain trends, more samples are needed to properly power this project and accurately detect the differences between atopic-at-risks/not-at-risks receptor expression in DCs.

Another source of discrepancy between the results of this project and the previous unpublished observations involves how each project determined atopic status. This project defined newborn atopic status as a risk based on maternal self-reported allergic status, while the previous study marked newborn atopic status as the confirmed positive skin-prick test result and physician diagnosed atopic dermatitis diagnosis later in life. Atopic risk and confirmed allergy are very different in terms of immunological response and predictive power of allergic disease. Atopic risk corresponds to an increased chance of developing allergies later in life, but does not indicate that the subject will positively

develop allergies. Similarly, an infant deemed not-at-risk may very well develop allergies later in life. As such, it is not unexpected that the trends found when examining the cord-blood of infants confirmed to develop allergies later in life are not present in atopic-risk infants. At this time, there is no way to determine which cases in the atopic-at-risk group will actually develop allergies, thus any trends observed would be expected to be weaker than in the pilot project due to “contamination” between the experimental and control groups.

Physician-diagnosed maternal allergic status was used for distinguishing atopic-at-risk and not-at-risks since this was shown to be a strong indicator of a child’s atopic status later in life (Fedulov and Kobzik, 2011; Hamada et al., 2003). However, many other factors contribute to atopic risk, including paternal atopic status, pets, mold exposure, among others. This project included some of these factors in the survey, including paternal atopic status, whether household has pets, and any recent dampness/mold problems. None were found to have any statistically significant effect on the differences between atopic-at-risks and not-at-risks.

Additionally, using a survey as a tool to gain information on a sample is imprecise, as the information provided cannot be confirmed and the survey may have some information left out. Human error during reporting can also contribute to error; for example, someone may incorrectly self-report that they have an allergy based on a non-allergic reaction to a metal. As such, the division of cord-blood samples into atopic-at-risk and non-atopic is indefinite, as some mothers who report themselves as atopic may not actually be so. In the future, implementing test of atopic status that can be controlled

by the experimenter are ideal, such as a skin prick test. However, such methods are more invasive than a survey and could result in a lower number of participants willing to take part in the study. In this project, the negatives associated with using a survey to determine mother's atopic status were carefully considered, but ultimately outweighed by the ability to acquire more samples.

The results of this Master's project illustrate that at birth there are already observable differences between infants who will develop allergic disease and infants who do not. The presence of these tendencies in atopic risk cases attests to the potential strength of the trends. Alternative methods to compare the expression profiles of TSLPR, FcεRI, and other receptors in atopics/non-atopics at birth are a natural next step to obtain more accuracy. Thus, this project provides the basis for follow-up studies on the gene expression of DCs in atopics/ non-atopics, where DCs can be accumulated in sufficient numbers through the differentiation of CD34⁺ cells in an *ex vivo* culture environment.

A major challenge in the study of DCs is their relatively low numbers, comprising roughly 1% of blood mononuclear cells (Hasskamp et al., 2005). While there are an increasing number of strategies and culturing methods of increasing DC numbers for research purposes, the profiles of these cultured DCs compared to their *in vivo* counterparts and the differentiation process from CD34⁺ progenitors to DCs have yet to be thoroughly studied. Furthermore, as DCs are key antigen-presenting cells within the immune system, they may be a crucial link between the environmental and genetic components that characterize allergic disease development. Available data on the DC phenotype with respect to allergic disease development remains scarce. It is imperative to

continue the study of atopic disease etiology since instances of allergic disease continue to increase worldwide. Not only will this research be useful in the development of reliable diagnostic tests for atopic disease, but it can also contribute to our understanding of DC differentiation as a whole. Better understanding of the complex interactions between immune development and the environment during the crucial stage of early infancy, and the subsequent effect on the development of allergic disease, is key to progressing towards new diagnostic biomarkers, novel preventive and therapeutic approaches.

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8. APPENDIX

Appendix 1: *Ex vivo* pDC association analysis between % cell expression and maternal atopy status. Outliers were determined and removed based on +/- 3 standard deviations. Significant values are shown in bold.

<i>Receptor</i>	<i>Normality test</i>	<i>T-test p value</i>	<i>Wilcoxon p value</i>
TLR5	0.000108	-	1
TLR9	0.3108	0.7569	-
ST2	0.009802	-	0.4475
TSLPR	0.001567	-	0.04257
FcεRI	0.008816	-	1
CD80	0.05125	0.3504	-
CD86	0.003608	-	0.7634

Appendix 2: Association analysis between % cell expression and maternal atopy status for **a) *in vivo*** mDCs; **b) *ex vivo*** mDCs; **c) *in vivo*** pDCs. Outliers were determined and removed based on +/- 3 standard deviations. No statistical significance was found. *denotes values approaching significance

a) <i>in vivo</i> mDC			
<i>Receptor</i>	<i>Normality test</i>	<i>T-test p</i>	<i>Wilcoxon p</i>
TLR5	0.004605	-	0.2938
TLR9	0.3935	0.3604	-
ST2	0.0007064	-	0.2042
TSLPR	0.0002867	-	0.1484
FcεRI	0.5026	*0.06697	-
CD80	0.0001384	-	1
CD86	0.6686	0.9341	-
b) <i>ex vivo</i> mDCs			
<i>Receptor</i>	<i>Normality test</i>	<i>T-test p</i>	<i>Wilcoxon p</i>
TLR5	0.001159	-	0.2588
TLR9	0.08814	0.7198	-
ST2	9.815 e-05	-	0.974
TSLPR	0.004667	-	0.3403
FcεRI	9.095 e-05	-	0.8019
CD80	0.1466	0.3624	-
CD86	0.1608	0.455	-
c) <i>in vivo</i> pDC			
<i>Receptor</i>	<i>Normality test</i>	<i>T-test p</i>	<i>Wilcoxon p</i>
TLR5	0.2395	0.2739	-
TLR9	0.2729	0.6825	-
ST2	0.03324	-	0.6714
TSLPR	1.214 e-07	-	0.7615
FcεRI	0.3656	0.7694	-
CD80	1.067 e-05	-	0.1765
CD86	0.1978	0.2428	-

Appendix 3: Association analysis between receptor expression of **a) *in vivo* mDCs** compared to *ex vivo* mDCs, **b) *in vivo* pDCs** compared to *ex vivo* pDCs. Statistically significant associations are bolded.

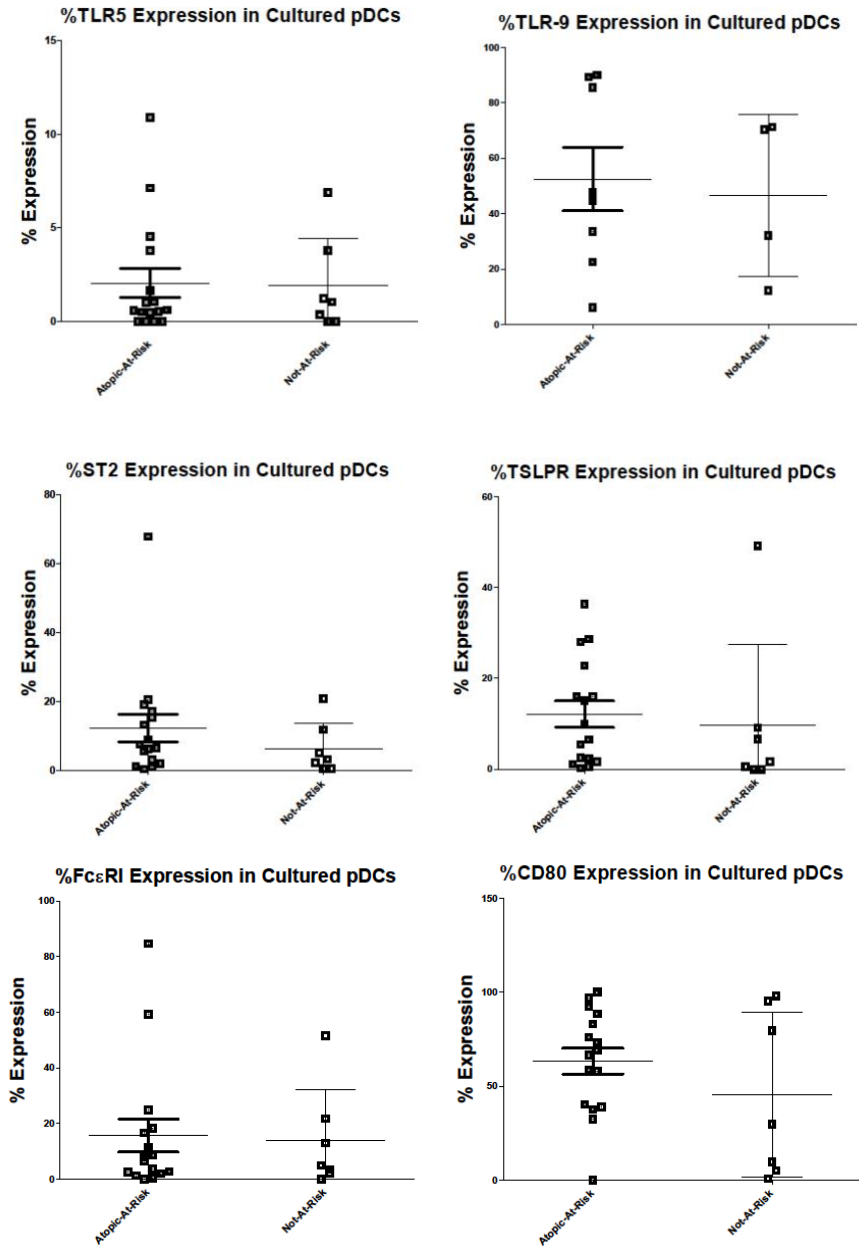
a)

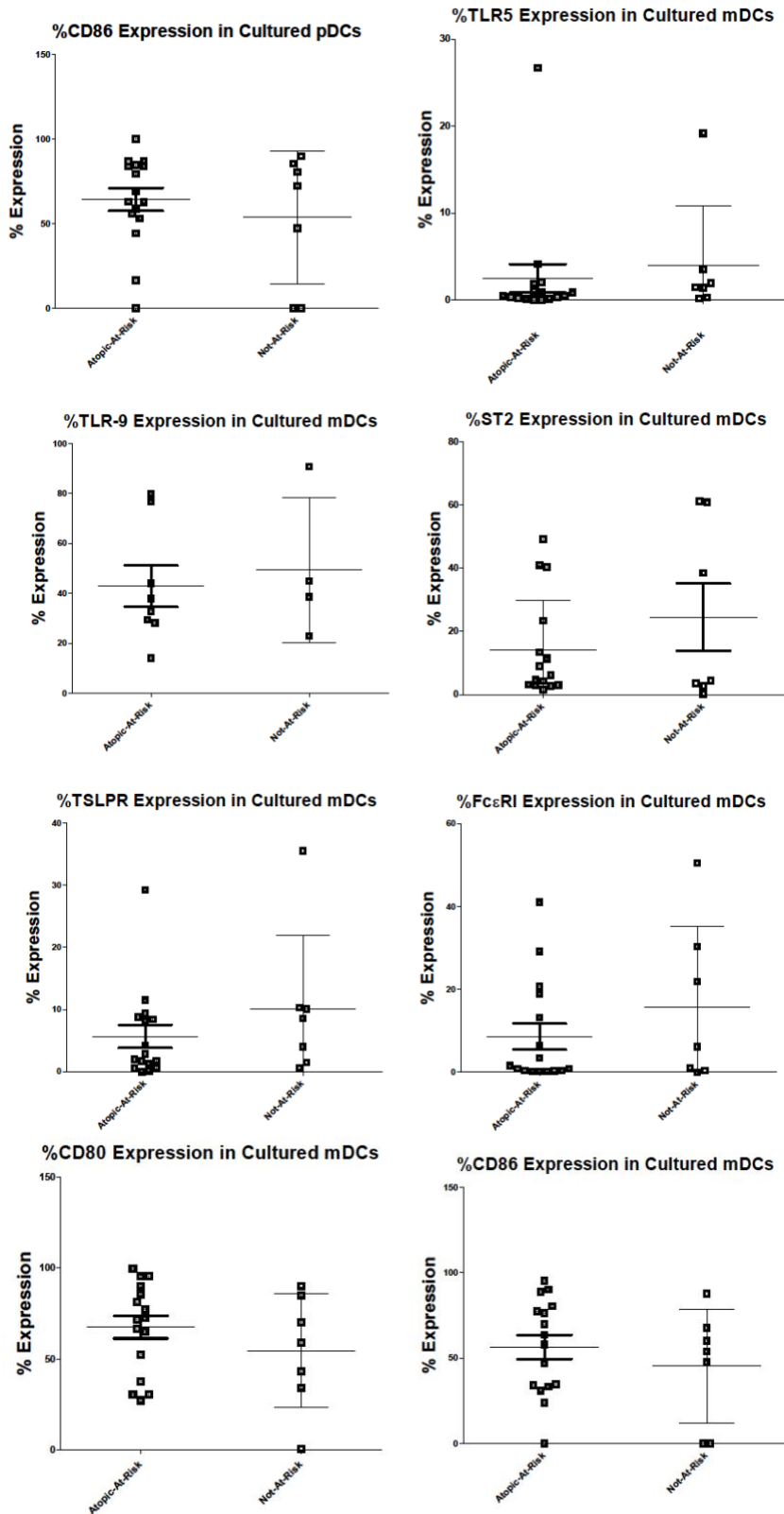
<i>Receptor</i>	<i>T-test p value</i>	<i>Wilcoxon p value</i>
pDC TLR5	0.03407	0.02951
pDC TLR9	0.4955	0.4776
pDC ST2	0.001229	0.001138
pDC TSLPR	0.0004579	6.869 e-07
pDC FcεRI	0.1633	0.08509
pDC CD80	4.066 e-08	5.799 e-07
pDC CD86	1.062 e-08	1.478 e-05

b)

<i>Receptor</i>	<i>T-test p value</i>	<i>Wilcoxon p value</i>
mDC TLR5	0.0003672	5.79 e-05
mDC TLR9	0.1468	0.1841
mDC ST2	0.4185	0.05596
mDC TSLPR	8.738 e-05	4.885 e-06
mDC FcεRI	1.777 e-07	1.251 e-06
mDC CD80	1.941 e-10	1.138 e-07
mDC CD86	0.076	0.07329

Appendix 4: Graphs illustrating *ex vivo* pDC, *ex vivo* mDC expression in atopic-at-risks and not-at-risks, including outliers. Mean \pm SEM is shown.





Appendix 5: Graphs illustrating *in vivo* pDC, *in vivo* mDC expression in atopic-at-risks and not-at-risks, including outliers. Mean \pm SEM is shown.

