CHANGES IN CORD BLOOD DENDRITIC CELLS AS BIOMARKERS OF FETAL EXPOSURE TO STRESSOR STIMULI

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Submitted in accordance with the requirements of Imperial College London for the degree of MD(Res)

The candidate confirms that the work submitted in this thesis is the result of her own investigation except where reference has been made to published literature and that appropriate credit has been given where reference has been made to the work of others

2014

ABSTRACT

DCs are central to fetal defences and it was postulated that phenotypic changes on CBDCs in response to infectious/stressor stimuli could identify compromised fetuses.

Investigations were performed on whole blood using monoclonal antibody labelling and flow cytometry. Functional studies included endocytosis of Dextran particles and MLR.

Both plasmacytoid (HLA-DR^+CD11c^-) and myeloid (HLA-DR^+CD11c^+) DCs were identified in CB. Additionally CB contained a DC subset with a HLA-$DR^+CD11c^-CD45^{intermediate(inm)}$ phenotype. This population expressed lower levels of CD45 and HLA-DR and did not express plasmacytoid (CD123, BDCA2, and CD45RA) or myeloid (CD33 and CD13) markers. All subsets exhibited endocytosis and unlabelled CBDCs exhibited lymphocytic stimulatory capacity.

Both myeloid and plasmacytoid CBDC subsets showed no change with gestation. The $CD11c^-CD45^{inm}$ subset decreased with increasing gestation representing 31.33% of total DCs in preterm, 21.26% in term CB and 1.54% in adult PB. $CD11c^-CD45^{inm}$ DC numbers expressing CD40, CD86 and production of IL-12 increased significantly with stressors. The myeloid and the plasmacytoid subsets showed no upregulation of CD40 and CD86 with stressors. The myeloid subset decreased while the plasmacytoid subset increased IL-12 production with stressors.

Neutrophilic activation markers of CD11b and CD16 showed significant correlation with the stressed CB samples which exhibited proinflammatory DC responses, thus validating the clinical classification.

These data indicate that CB contains plasmacytoid and myeloid DC populations as seen in adult PB. Additionally this study has identified a hitherto unreported CBDC subset with an immature phenotype, exhibiting endocytosis and phenotypically distinct from plasmacytoid DCs. Of the

three subsets, only the $CD11c^{-}CD45^{inm}$ subset showed a costimulatory response to stressors suggesting this subset to be the most kinetic; changing with advancing gestation as well as exposure to stressors. Thus investigating phenotypic changes on CBDC subsets, especially on the $CD11c^{-}CD45^{inm}$ subset, could serve to identify fetuses exposed to stressor stimuli and at risk of adverse sequelae.

ACKNOWLEDGEMENTS

It is with immense gratitude that I acknowledge the support and help of my supervisors Professors Stella Knight and Ronald Lamont in the long and arduous conception and birth of this thesis. Their clarity of vision and perseverance in the face of adversities has been a constant source of inspiration and encouragement to me.

I am indebted to all my colleagues who supported me in the A.P.R.G, Dr Andy Stagg for his excellent tutorial and guidance and Alison Scoggins who was always there with a strong shoulder to lean on.

I am mindful that this work would have remained a dream had it not been for my many patients who found the courage in the midst of their grief and stress to consider the greater good and agreed to donate the samples. I remain always grateful to them and their babies, some of whom are not with us anymore.

Finally, I owe my deepest gratitude towards my parents without whose help none of this would have been possible and to my son Nathaniel who makes all my endeavours worthwhile.

'Nisi Dominus aedificaverit domum in vanum laboraverunt qui aedificant eam nisi'

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ABBREVIATIONS

ANOVA	Analysis of Variance
APC	Allophycocyanin
APC	Antigen presenting cell
BSA	Bovine Serum Albumin
^0C	Degree Celsius
CB	Cord blood
CD	Cluster of differentiation
cDC	Conventional/myeloid DC
CDP	Common DC progenitor
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CO_2	Carbon di-oxide
COX	Cyclo-oxygenase
CP	Cerebral Palsy
cpm	Counts per minute
CpG	Cytosine-poly-Guanine
CRP	C-reactive protein
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DDC IDC	Dermal DC-interstitial DC
DNA	Deoxyribonucleic acid

EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence Activated Cell Sorting
FCS	Fetal Calf Serum
FIRS	Fetal inflammatory response syndrome
FITC	Fluorescein isothiocyanate
FITC-dextran	Fluoresceinisothiocyanato-dextran
Flt3L (FL)	Fms-like tyrosine kinase 3 ligand
FSC	Forward scatter channel
GM-CSF	Granulocyte macrophage colony-stimulating factor
gm	Gram
HLA	Human Leukocyte antigen
HSC	Haemopoietic stem cells
ICAM	Inter cellular adhesion molecule
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
KL	c-kit ligand
LC	Langerhans cell
LDC	Low density cell
LIN-CK	Lineage Cocktail
LPS	Lipopolysaccharide

MDP	Macrophage/DC progenitor
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
µl	Microlitre
ml	Millilitre
MLR	Mixed Leukocyte Reaction
µm	Micrometre
MMP	Matrix metalloproteinases
moDC	Monocyte-derived DC
ng	Nanogram
NK	Natural killer
No:of	Number of
PAMP	Pathogen associated molecular patterns
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PC5	Phycoerythrin-cyanine5
PCI	Positive control intensity
pDC	Plasmacytoid/lymphoid DC
PE	Phycoerythrin
PE-Cy5	PE-indotricarbocyanine
PGE2	Prostaglandin E2

PROM	Premature rupture of membranes
PVL	Periventricular leukomalacia
PRR	Pattern recognition receptors
RANKL	Receptor activator of nuclear factor-κB ligand
RBC	Red blood cell
RNA	Ribonucleic acid
SED	Super-enhanced Dmax normalized subtraction
SCF	Stem cell factor
SSC	Side scatter channel
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Treg	Regulatory T cell

CHAPTER 1

INTRODUCTION

INTRODUCTION

Evolution over the millennia has equipped the human body with a multifaceted defence system effective against assault by external pathogens. The immune system protects the host from pathogens as well as distinguishes self antigens from foreign antigens thus forestalling self destruction. These multiple almost contrary functions of the immune system have interesting consequences for the development of the fetus whose tissues are essentially a conglomeration of maternal antigens recognised as self antigens and paternal antigens normally recognised as foreign. The biology of the pregnancy is unique as it supports the co-existence of two immunologically diverse hosts. The uterus which harbours the fetus and provides nutrients is also the site of a unique immunological phenomenon whereby maternal tolerance to fetal antigens is developed, while allowing immunogenic activation to prevent infection. The antigen presenting dendritic cell (DC) is the initiator and modulator of the immune response and plays a central role in orchestrating both immunogenic and tolerogenic effects. The DC's unique plasticity of function enables the modulation of the fine balance between fetal immune competence and immunotolerance, which is determined by the maturation status of the DC. Thus the *raison d'être* for the contrary requirements of the immune system is acceptance of the fetal allograft by the maternal immune system while providing protection from a hostile pathogen filled micro environment.

The fetal immune system has developed the ability to mount a response to immunogenic stimuli from a very early period in the gestation, even though evidence so far points to a reduced ability to mount an effective response. Evidence of this reduced response is best available from the phenotypic and functional changes on the fetal DCs. Thus it follows that evidence of an

immunogenic response mounted by the fetal DC reflects exposure of that fetal immune system to antigenic/pathogenic stimuli. Although occurring in response to infectious threats, release of the fetal immune system from the tolerogenic placental control and mounting of an immunogenic response has been proven to have wide reaching adverse effects on the pregnancy such as preterm labour and delivery and long term fetal neurological and respiratory morbidity.

Understanding this immunological paradox is essential in identifying the aetiology and pathogenesis of such adverse outcomes. The role of this cardinal cell of the immune system, the DC, in modulating and mounting an immunogenic fetal response and thereby enabling identification of the immunocompromised and at risk fetus is the main focus of this work.

1.1 DENDRITIC CELLS

1.1.1 Introduction

DCs are bone marrow derived antigen presenting cells (APC), which are found both in lymphoid tissues where they act as initiators of immune responses and in non-lymphoid peripheral tissues where they fulfil a sentinel like function both creating and curtailing the immune response (Steinman, 1991; Banchereau and Steinman, 1998; Banchereau, *et al.,* 2000). DCs are the gatekeepers of the immune system and are widely distributed throughout the body especially at portals of pathogen entry. In the immature state DCs actively uptake and process antigen in their environment and when stimulated by appropriate cytokine and chemokine signals, mature into potent APCs orchestrating an immune response (Hart, 1997). The DC liberates cytokines (Reid, *et al.,* 1990; Wan and Bramson, 2001) and chemokines (Cyster, 1999) to summon and co-ordinate different effector cells of the immune system, including T-cells (Knight, *et al.,* 1982), natural killer (NK) cells (Fernandez, *et al.,* 1999), macrophages and monocytes (Steinman, 1991) and other DCs (Knight, *et al.,* 1998). DCs play a pivotal role at the interface between innate and adaptive immunity as innate pattern recognition pathways trigger DC activation which determines the adaptive immune responses (Medzhitov and Janeway, 1999). They are central in the ability to discriminate between self and non self antigens and maintain the dual role of immune tolerance to self antigens as well as mounting an immune response against foreign antigens (Hawiger, *et al*., 2001; Bonifaz, *et al*., 2002; Knight, *et al*., 2002).

When first observed in the late nineteenth century, DCs were mistaken to be of neural origin due to the dendritic like projections displayed by their cytoplasm and described as Langerhan cells by

Paul Langerhans. (Langerhans, 1868). The renewal of interest in DC research was initiated with the discovery of Birbeck granules in DC cytoplasm with the use of electron microscopy, leading to the identification of Langerhans cells *in situ* (Birbeck, *et al*., 1961; Zelcikson, 1966; Hoshino, *et al*., 1970). Almost 100 years passed before the term 'dendritic cells' was described by Ralph M Steinman and Zanvil A Cohn (Steinman, 1973). The immunological significance of DCs emerged only after the discovery of MHC class II antigens on the cell surface (Nagao, *et al*., 1976; Rowden, *et al*., 1977; Stingl, *et al*., 1978; Tamaki, *et al*., 1979). More extensive knowledge about them was curtailed in the 1970's due to the low numbers *in vivo* and ineffective generation techniques, they are less than 0.5% of all nucleated cells in adult peripheral blood (PB) and cord blood (CB) (Naderi, *et al*., 2009). The development of methods to isolate and generate DC's from blood and bone marrow precursor cells led to the explosion in the current knowledge. Inaba *et al* showed that rapid production of reasonable numbers of DCs was possible in a liquid culture medium with the addition of Granulocyte macrophage colony-stimulating factor (GM-CSF) (Inaba, *et al*., 1992). The use of this method provided DCs with the phenotypic and physical characteristics of DCs including mixed leukocyte reaction (MLR) and T cell area homing. But GM-CSF stimulates all the systems of myeloid cells including granulocytes and macrophages, and also causes DC maturation. Hence methods of freshly isolating DCs from non manipulated cultures have been developed (Naderi, *et al*., 2009).

1.1.2 DC phenotypical characteristics

Human DC is identified phenotypically based on their surface expression of major histocompatibility complex (MHC) class II protein Human Leukocyte antigen (HLA-DR) and lack of expression of lineage associated markers (CD3, CD14, CD16, CD19, CD34 and CD56) (Knight, 1984; Reid, 1997; Banchereau, *et al*., 2000). The term 'lineage' includes all cells that express markers for lymphocytes, granulocytes, monocytes, NK cells and stem cells.

1.1.2.1 Morphology

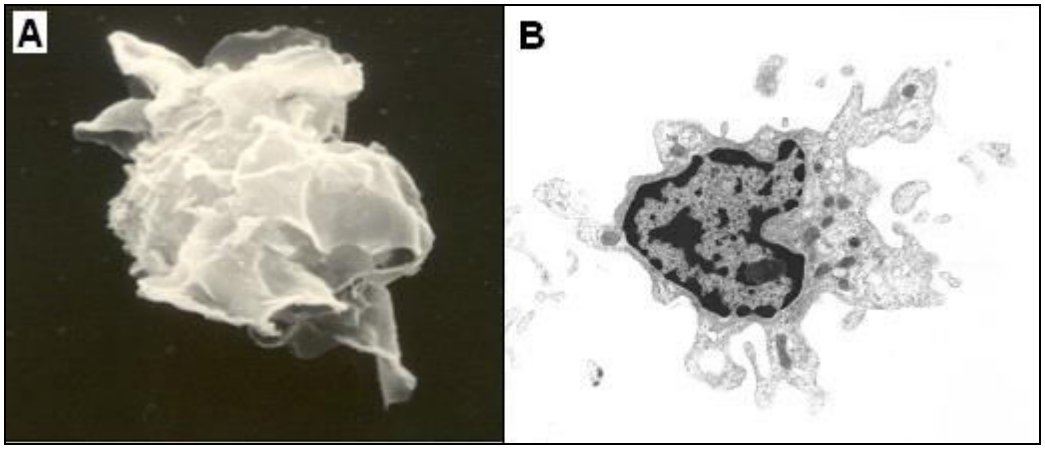


Figure 1.1 Electron micrograph of a human DC

Scanning (Figure 1.1A) and electron micrograph (Figure 1.1B) of a human DC (Type III) exhibiting long 'veil like' dendrites giving rise to the classical stellate appearance (from Prof S.C Knight and N English, A.P.R.G)

DCs were initially characterised and named on the identification of their multiple cytoplasmic processes which give rise to their classical stellate appearance. These cytoplasmic processes or

dendrites vary in size, length and number, and distinguish the DC from other mononuclear leukocytes (Steinman and Cohan, 1973). Electron microscopy delineates these processes as >10μm long and either 'veil like' or spiny. Initially DCs were classified as based on the cell surface membrane morphology of spiky projections, short bulbous extensions or thin cytoplasmic veils corresponding to Type I, II and III respectively (Knight and Stagg, 1993). Live DCs viewed by phase contrast microscopy exhibit extension and retraction of these 'veil like' projections continuously. Similar to mononuclear leukocytes, DCs have an irregular eccentric nucleus with heterochromatin arranged along the nuclear envelope. The cytoplasm has prominent mitochondria and an abundant number of endosomes (Kleijmeer, *et al*., 1995; Nijman, *et al*., 1995). Two physical properties of DCs were especially useful in carrying out early morphological studies. DCs adhere to glass and surface adherence of enriched populations yield cultures containing 50% DC preparations amenable to more detail analysis. DCs also have a buoyant density of less than 1.082 which is less than that exhibited by 90% of the other nucleated cells in spleen and lymph node and can be enriched 7-20 fold by centrifugation. These properties were used by Steinman and his colleagues to obtain relatively higher numbers of DCs which facilitated more detailed morphological and functional analyses (Steinman and Cohn, 1974).

1.1.2.2 DC classification

DCs are widely distributed in all human tissues and have multiple subsets exhibiting different characteristics and fulfilling varied functions. In adults, this classification is based on their sites of location as Tissue DCs in nonlymphoid and lymphoid tissues and PB DCs. Additionally DCs are classified based on ontogeny, as well as function.

Traditionally the following aspects have been used to identify DC lineages: morphology, phagocytic capacity, cell surface markers and antigen processing and presentation. Recent studies have established newer criteria that explain the development and functioning of DCs more succinctly. These include immune tolerance functions of DCs, identifying committed progenitors e.g. monocyte and DC progenitors, common DC progenitor (CDP) and preDCs (Onai, *et al*., 2007; Steinman and Idoyaga, 2010).

1.1.2.2.1 Classification based on phenotypic markers

Phenotypic markers on DCs vary between the steady state and the activated state attained after exposure to infectious/inflammatory stimuli. In adults in the non-inflammatory steady state, two major subsets of DC have been identified; the conventional/myeloid, usually called cDC or mDC and the plasmacytoid/lymphoid (pDC) (Shortman and Liu, 2002; Randolph, *et al*., 2005; Shortman and Naik, 2007; Wu and Liu, 2007; Hochrein and O'Keeffe, 2008). cDCs are lymphoid resident as well as non lymphoid resident and migratory. DCs that develop after an infection or inflammation include the monocyte derived DCs, the tumour necrosis factor producing DCs and the inducible nitric oxide synthase expressing DCs (Geissmann, *et al*., 1998; Randolph, *et al*., 1999; Serbina, *et al*., 2003; Naik, *et al*., 2006).

The Myeloid/Lymphoid nomenclature has been shed in the light of recent new data on DC ontogeny (Shortman and Naik, 2007). Historically these two subsets were named based on the perceived notion that cDCs originated from a myeloid precursor and the pDC originated from a lymphoid progenitor. Recent evidence has revealed that a rigid dichotomy between the subsets does not exist as the development of both subsets has been shown from lymphoid as well as

myeloid precursor cells (Manz, *et al.,* 2001; Wu, *et al*., 2001; Shortman and Liu, 2002; Chicha, *et al.,* 2004; Shigematsu, *et al*., 2004). These DC subsets are plastic and can be modulated by various antigenic stimuli e.g. bacterial antigens (Bauer, *et al*., 2001).

1.1.2.2.1.1 Conventional DCs in the human

cDCs are $CD11c^{+}/CD123^{lo}/CD45^{hi}$, GM-CSF dependent for their generation from precursor cells and have a monocytoid appearance. They also exhibit class II MHC^{bright}, $CD80^{+++}$, $CD86^{+++}$, $CD83^{+}$, CD13, CD33, CD11b and a high level of HLA-DR. They express toll like receptor 2 (TLR2), TLR4 and secrete IL-12. Immature DCs express class II MHC, CD80, and CD86, and these increase with maturation. They can be divided into further distinct populations according to their expression of $BDCA1^{+}$ or $BDCA3^{+}$.

1.1.2.2.1.2 Plasmacytoid DCs in the human

pDCs prior to activation resemble plasma cells morphologically with their round or oval non dendritic structure and a well developed endoplasmic reticulum and excentered nucleus. They play an important role in antiviral immunity and autoimmunity and are found primarily in blood and lymphoid organs. They circulate in blood and are found in the steady state in the bone marrow, thymus, lymph nodes, spleen and the liver (Liu, 2005). They secrete interferon-γ (IFN-γ) when exposed to viral or bacterial antigenic stimuli and prime T lymphocytes against viral antigens which is their most important function after activation (Asselin-Parurel, *et al*., 2001; Pashenkov, *et al*., 2002; Yoneyama, *et al*., 2004). Due to the expression of the transcription

factor IRF-7, pDCs can secrete large quantities of IFN-α on exposure to viral nucleic acids (Fitzgerald-Bocarsly, *et al*., 2008).

pDCs exhibit high levels of CD123 (IL-3 receptor), CD45RA, CD4, CD62L, BDCA-2 (CD303) and BDCA-4 (CD304) and are negative for CD11c, CD33 and CD16. They do not stimulate naïve T cells, exhibit only low levels of MHC class II molecules and lack most lineage markers (Grouard, *et al.*, 1997; Liu, 2005). They also capture and process antigens onto MHC molecules less effectively when compared to cDCs (Grouard, *et al*., 1997).

1.1.2.2.2 Classification based on immunological function

DCs have a dual role and bidirectional interactions between DCs and T cells initiate either an immunogenic or a tolerogenic pathway and based on these functions DCs have been classified as Immunogenic or Tolerogenic (Steinman and Banchereau, 2007).

1.1.2.2.2.1 Immunogenic DCs

Innate immunity uses a nonclonal set of molecules for recognition and is a phylogenetically ancient non specific mechanism of host defence. The adaptive arm is clonally distributed and has immunological memory which develops on exposure to specific antigens. DCs as potent APCs are the only ones capable of activating resting T cells and initiating innate and adaptive immune responses and as part of the innate immune system DCs exhibit high phagocytic activity both in the peripheral tissues and in secondary lymphoid organs.

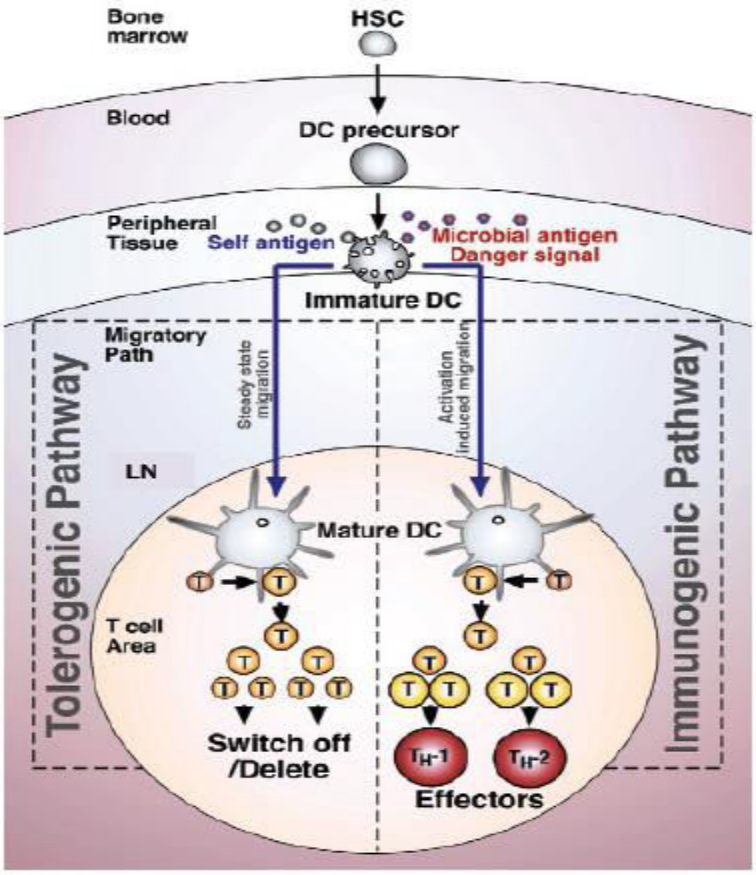


Figure 1.2 Dual role of DC in immunity and tolerance (from Quah and O'Neill. (2005). Maturation of function in dendritic cells for tolerance and immunity. J. Cell. Mol. Med. 9(3): 647)

DC precursors in blood develop from Haemopoietic stem cells (HSC) in the bone marrow. Immature DCs in the peripheral tissues develop from these DC precursors and in the steady state they take up self antigens, mature and follow a tolerogenic pathway of development. Upon exposure to infectious/inflammatory antigens, immature DCs mature and migrate to lymph nodes where antigen presentation leads to T cell differentiation

1.1.2.2.2.2 Tolerogenic DCs

Modulation of responses to self antigens and tolerance induction are functions central to maintaining a healthy immune system, which the DC coordinates effectively. DCs induce peripheral tolerance in the steady state and immature DCs in the secondary lymphoid tissues present self antigens (Scheinecker, *et al*., 2002). Immature DCs in lymphoid organs efficiently present and process self antigens to induce and maintain tolerance (Hawiger, *et al*., 2001; Bonifaz, *et al*., 2002; Lutz and Schuler, 2002). Such presentations lead to ignorance, anergy induction or deletion depending on the type of DC, affinity of the T cell receptor (TCR) and the level of costimulation on the DC (Diebold, 2008). Various pathways have been described for the induction of tolerogenic DCs; including induction of regulatory T cells by exhausted DCs, DC maturation with IL-10 or apoptotic cell uptake (De Smedt, *et al*., 1997).

1.1.2.2.3 Classification based on anatomical location

In adults, DCs have been classified based on their sites of location as Tissue DCs in nonlymphoid and lymphoid tissues and PB DCs.

1.1.2.2.3.1 DCs in nonlymphoid tissues

Non lymphoid tissue DCs can be seen distributed in internal organs such as the pancreas and the heart and DCs present in filtering sites such as the kidneys and the liver. Interface DCs are present at environmental interfaces such as the intestines, lungs and skin which form the primary

barriers to infections. Epidermal DCs also called Langherhans cells, express CD1a and play a crucial role in maintaining an impermeable barrier to infection.

1.1.2.2.3.2 DCs in lymphoid tissues

DCs on lymphoid tissues are classified as those present in the spleen, lymph nodes, thymus and those seen in the mucosa associated lymphoid tissues such as intestinal lymphoid follicles, Peyers patches and nasopharyngeal lymphoid follicles. Adaptive immune responses to pathogens are initiated in these lymphoid tissues and DCs play a critical role in delivering and presenting the antigens to T cells.

1.1.2.2.3.3 Peripheral blood DCs

Both cDC and pDC subsets as discussed above have been identified in adult PB. pDCs account for less than 1% of total peripheral blood mononuclear cells (PBMCs). The expressions of chemokine receptors on circulating blood cDCs and pDCs are similar but vary in quantity. Upon activation, pDCs can produce 100–1000 times more type I IFN than other blood cell types and are thus considered the professional type I IFN producing cells. cDC produce large amounts of IL-12, induce Th1 and cytotoxic responses and also secrete type I IFN in response to viruses, but less efficiently than pDCs (Schettini and Mukherjee, 2008).

1.1.3 DC functional characteristics

Functionally the characteristic feature of a DC is its ability to stimulate a primary lymphocyte response to presented antigen (Knight, *et al*., 1982; Knight, *et al*., 1983; Villadangos, *et al*., 2005).

1.1.3.1 Antigen surveillance and Uptake

DCs patrol the peripheral tissues where they come into contact with pathogenic antigenic material, which are recognised by germ line encoded pattern recognition receptors (PRR) on the DC. Lymphocyte activation requires the recognition of short peptides associated with MHC class I or II molecules on the DC surface. Activation signals lead to increase antigen uptake (West, *et al*., 2004) and upregulation of MHC II molecules synthesis which are preferentially delivered to endosomal compartments containing foreign antigens (Blander and Medzhitov, 2006). Large antigen fragments are broken down into 10-20 amino acid peptides, loaded onto MHC class II molecules and translocated to the cell surface (Cresswell, 1994). In MHC class I presentation ribosomal products are digested by the proteasome into small peptides that are translocated into the Endoplasmic Reticulum where they are loaded on MHC class I molecules and are transported to the cell surface (Yewdell and Nicchitta, 2006).

1.1.3.2 Antigen Processing and Presentation

DCs are the most potent APCs and one DC can stimulate 100 to 3000 T cells. Antigenic proteins can be presented by DC in association with either MHC class I molecules or MHC class II molecules on the surface of the DC. The presentation of exogenous proteins requires the antigens to be first endocytosed by pinocytosis, phagocytosis or receptor-mediated endocytosis (Wilson and Villadangos, 2005). The cytosolic pathway is more relevant where exogenous antigen is transferred from phagosomes into the cytosol for proteasomal degradation (Kovacsovics-Bankowski, 1995), while the second pathway involves peptide generation within the phagosome (Shen, *et al*., 2004).

The phenomenon of cross presentation whereby DCs present exogenous antigens on their MHC I molecules also make the DCs function as highly efficient APCs (Wilson, *et al*., 2004). Cross presentation could result in activation as well as induction of tolerance in T cells and two pathways for presentation have been described. DCs are the main cell population that can cross present *in vivo* and this ability makes them crucial in tolerance induction (Heath, *et al*., 2004).

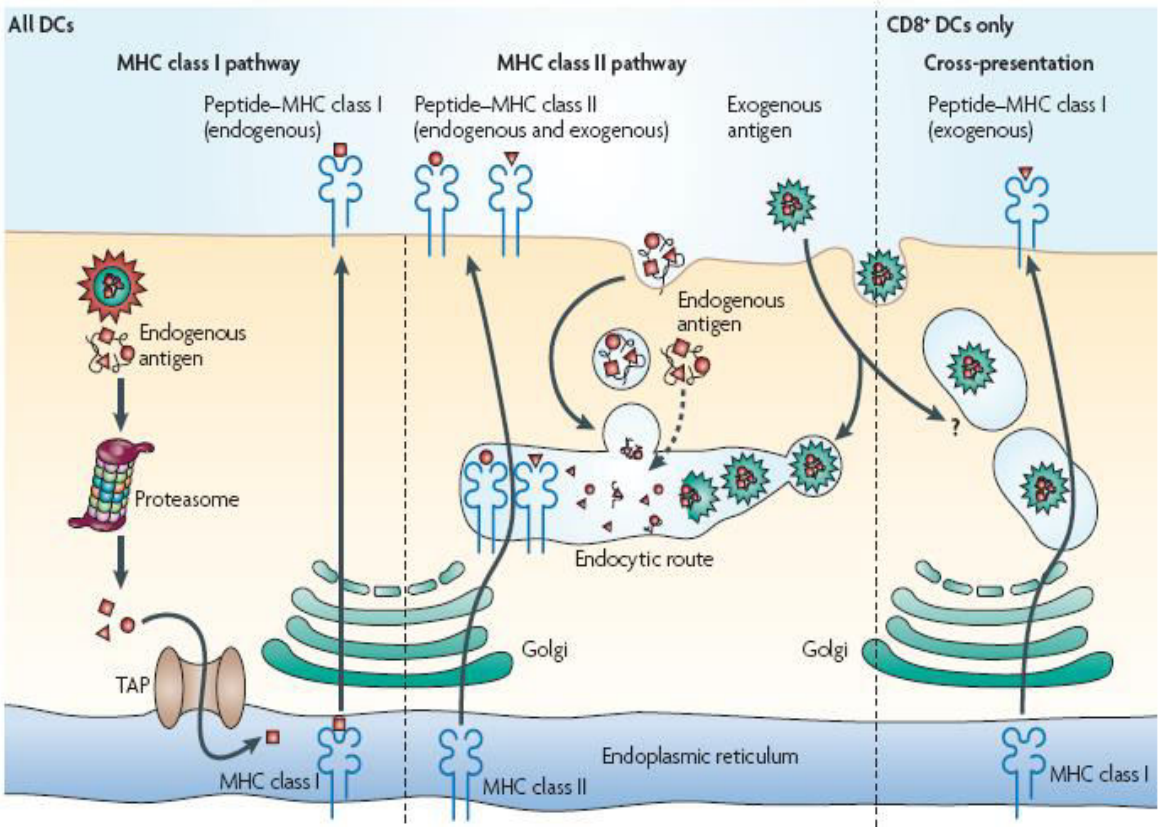


Figure 1.3 Antigen presenting pathways in DCs (from Villadangos and Schnorrer. (2007). Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets *in vivo.* Nat. Rev. Immunol.7: 548)

The two main pathways of MHC I and MHC II as well as the mechanism of cross presentation is illustrated

1.1.3.3 T Lymphocyte Activation

DCs have the cardinal ability to interact with lymphocytes and other immune cells allowing transfer of information regarding foreign antigens invading the body. On arrival in the lymph nodes activated mature DC exhibiting MHC-peptide complexes on their surface interact with

naïve T cells. The outcome of these interactions are dependent on three DC derived signals: the levels of antigen presentation delivered through the TCR by its attachment to the MHC/antigenic peptide complex (signal 1), the display of co-stimulatory molecules (e.g. CD80 and CD86) which deliver signals through counter receptors on T cells (e.g. CD28) (signal 2) and the presence of immunomodulatory factors such as cytokines (signal 3) (Diebold, 2008). After activation by DCs, T cells divide and give rise to a clone of effector T cells each specific for the same antigen–class II MHC complex. Thus DCs can direct the T cell to eliminate a specific pathogen.

1.1.4 DC life cycle

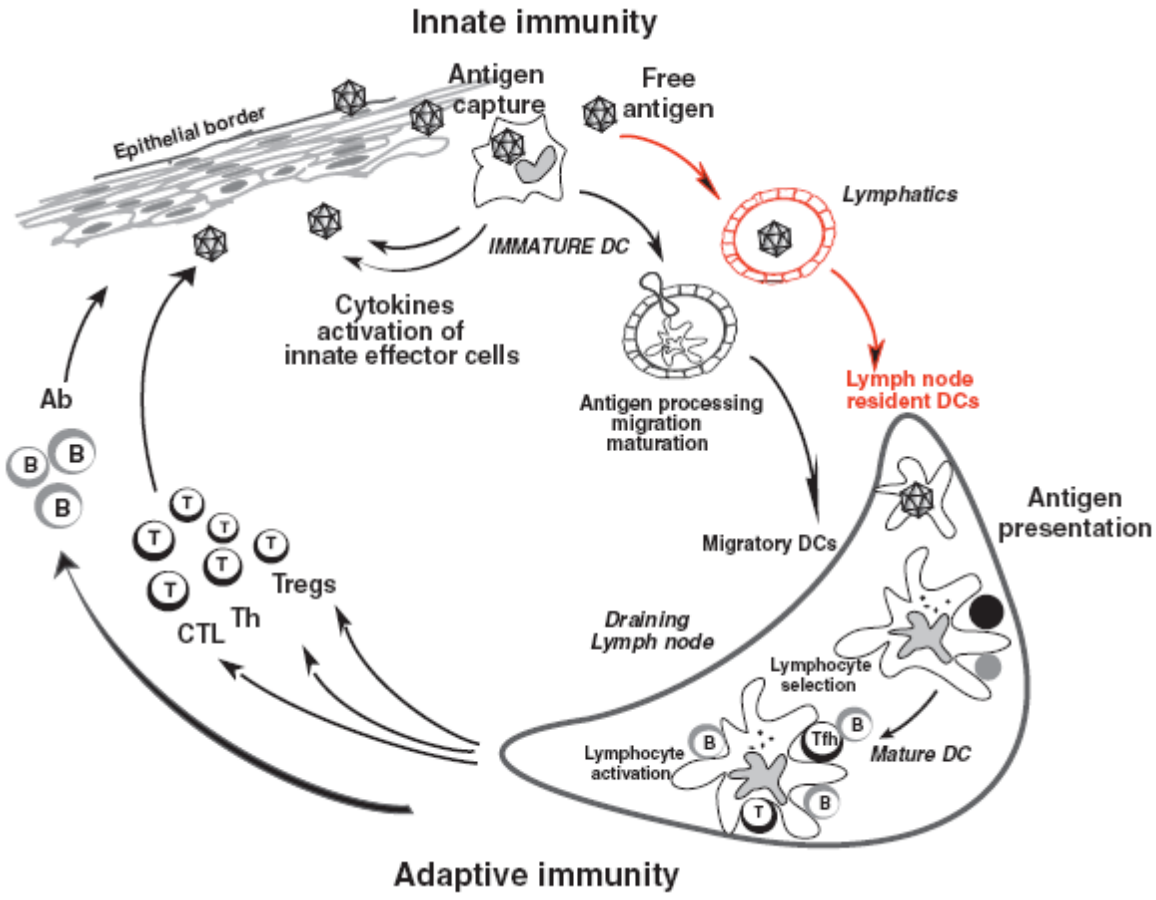


Figure 1.4 The life cycle of the DC (from Ueno, *et al.,* (2010). Harnessing human dendritic cell subsets for medicine. Immunol Rev. 234: 200)

Immature DCs in the circulation enter tissues and encounter pathogens directly or indirectly to induce cytokine secretion. Cytokines activate eosinophils, macrophages and NK cells, and these changes triggers DC migration and maturation. Mature DCs arriving at lymphoid organs display peptide-MHC complexes and recruit antigen-specific T lymphocytes

1.1.4.1 DC Ontogeny

The origins of DCs remain in relative obscurity despite extensive research. It is accepted that DCs are derived from self renewing HSC in the bone marrow giving rise to a series of downstream precursor cells that are progressively committed to particular cell lineages.

1.1.4.1.1 Early non committed progenitors

Early progenitors such as the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP) populations have been identified (Akashi, *et al*., 2000; Kondo, 2003), but both these progenitors retain developmental capacity of all DC subsets. Thus the phenotype of the DC is not diagnostic of its lineage origin. Downstream from the progenitors the capacity to develop into either DC or monocyte is governed by the cytokine milieu (Caux, *et al.,* 1996).

1.1.4.1.2 Committed/Restricted progenitors

DC committed precursors have been divided into three groups depending on their stage of restriction (Merad and Manz, 2009). Data is scarce as to whether these groups overlap and if they do so, the level of differentiation at which they overlap. They are:

- **Early DC progenitors:** These cells are present in the bone marrow and have high proliferative capacity expressing CD117 (stem cell factor receptor) with no expression of lineage markers. They include macrophage and DC progenitors (Fogg, *et al*., 2006; Waskow, *et al*., 2008) giving rise to monocytes, macrophages, and DCs; and CDPs and pre-DCs giving rise to cDCs and pDCs (Naik, *et al*., 2007). The developmental lineage has been elucidated as follows. HSC differentiate into the CLP and CMP. Both CLPs and CMPs are seen exclusively in the bone marrow.

- **Late DC progenitors:** Successive commitment steps produce macrophage/DC progenitors (MDP) which within the bone marrow differentiate into CDP. PB DC precursors which generate pDCs and precursors for cDCs (preDCs) are included in this group (del Hoyo, *et al*., 2002). MDPs also give rise to monocytes and macrophages.

- **Immediate DC progenitors:** These are precursors at a developmental stage just prior to the formation of a DC with its characteristic identifiable phenotype. These cells consist of nonproliferating monocytes with potential to immediately differentiate into DC on

exposure to inflammatory stimuli (Ginhoux, *et al*., 2006; Landsman, *et al*., 2007; Varol, *et al*., 2007).

1.1.4.2 Maturation

DCs in the steady state are immunologically 'immature' i.e. they have not acquired the capacity to prime naïve T cells (Banchereau, *et al*., 2000). The immature DC exhibits high endocytic potential with MHC class II molecule accumulation in endosomal compartments and a low T cell activation potential with low surface expression of T cell costimulatory molecules (Villadangos and Schnorrer, 2007). In the immature state DCs exhibit high levels of macropinocytosis and express endocytic receptors, such as the mannose receptors, DEC-205 and DC-SIGN. Maturation is mediated either by host-derived molecules such as CD40 ligand, Tumour necrosis factor (TNF-α), IL-1, IL-6, and IFN-α, or from microbial products stimulating TLRs (Cheng, *et al*., 2003).

On contact with an antigenic stimulation DCs exhibit characteristic phenotypic changes known as 'maturation'. The changes include upregulation of MHC class II synthesis, phagocytosis and micropinocytosis; higher surface expression of MHC molecules and changes in endosomal protease activity (Villadangos, *et al*., 2005). Physical changes take place including the development of characteristic cytoplasmic extensions or "dendrites" and cytoskeletal reorganisation. Maturation results in the upregulation of the chemokine receptor CCR7 and costimulatory CD80/86 molecules (Sallusto, *et al*., 1999) and production of IL-12. During differentiation CD34 levels are downregulated and expression of HLA-DR is increased (Banchereau and Steinman, 1998). Maturing DC down regulate their endocytic capacity, in order

to increase the stable expression of MHC II/peptide complexes at the cell surface by preventing their reabsorption and degradation (Villadangos, *et al*., 2001). The term 'maturation' currently indicates the phenotypic changes taking place on the DC and not to any specific change in its functional capacity.

1.1.4.3 DC apoptosis

Migrating DCs that reach the lymph nodes have a life expectancy of 2-3 days. The final stage in the DC life cycle is apoptosis. Cellular apoptosis is mediated either by T lymphocytes or NK cell. Apoptosis triggers signalling pathways resulting in the next wave of DC migrating from local tissues into the lymphoid tissues (Parajuli, *et al*., 1999).

1.2 THE FETAL DENDRITIC CELL

1.2.1 Introduction

The paradigm of the fetal immune system is the capacity to mount an immune response as a defence against pathogens while preserving tolerance to prevent maternal immune rejection. Fetal haemopoiesis initially occurs in the mesoderm of the yolk sac and the extraembryonic mesenchymal tissue from very early stages of gestation. Primitive pluripotent human erythroid progenitors have been detected in the yolk sac at 3-4 weeks of gestation and can be detected in the circulation from 4 weeks of gestation (Holt and Jones, 2000). The liver is the major site of haemopoiesis from 5-6 weeks of gestation until term and delivery. MHC Class II^+/$lineage^-$ cells have been identified in the lamina propria of the fetal gut from 11 to 24 wks of gestation, and $CD83^+$, putative mature DCs, were detected in lymphoid follicles from 16 wks of gestation (Jones, *et al.,* 2001). The fetal and neonatal immune systems are functionally and phenotypically different from that of the adult and studies have shown that the neonatal immune system can be deficient in its efficacy (Marchant and Newport, 2000). The relative inefficiency of fetal CBDCs in the activation of T cells has been shown to be linked to their low cell surface expression of MHC and cell adhesion molecules (Hunt, *et al.,* 1994; Petty and Hunt, 1998).

Neonatal impaired fetal Th1 type adaptive immune responses have been implicated in the neonatal increased susceptibility to infections (Klein and Remington, 2001; Adkins, *et al*., 2004). Research has shown that transfer of maternal antibodies though the placenta limits the impaired primary antibody responses of the infant to infections by antigen removal. On the contrary T cell

responses have been shown to be unaffected by maternal antibodies (Gans, *et al*., 1999; Siegrist, 2001; Glezen, 2003; Adkins, *et al*., 2004).

Neonatal impaired cytokine secretion in response to infectious stimuli have also been demonstrated; namely TNF-α (De Wit, *et al*., 2003; Levy, 2005), which could result in defective Th1 polarisation. The impaired immune activity may be partly due to increased fetal production of IL-6 which exhibit Th2 polarising functions by inhibiting neutrophil migration to sites of infection (Marchini, *et al*., 2000; Schultz, *et al*., 2002) and also leads to low levels of secretory IgA (Markel, *et al*., 2006). Levels of most cytokines have been found to be low in the neonate; IL-2, IL-6, IL-4, IL-8, IL-10, IL-12, and IFN-γ (Silver, *et al*., 2004). The overall effect is to decrease the efficiency of innate immune responses and also cell-mediated immunity.

Although of decreased efficacy on comparison with the adult immune system, the fetus can mount an innate as well as an adaptive immune response. The fetal immune system has the capacity to mount an immune response to maternal exogenous antigens; IgM responses to maternal immunisation (Gill, *et al*., 1983) and against congenital rubella (Naot, *et al*., 1981).

1.2.2 Tolerogenic neonatal immune system

Tolerogenic immune responses in pregnancy are necessary for the development of the foreign antigenic reservoir that is the fetus. Multiple evidence points to immune modulations at various levels: placental interface and the neonatal immune system. Circulating fetal antigens play a crucial role by promoting immune tolerance at the feto-maternal placental interface. Following recognition of fetally derived antigens, the immune system initiates a series of protective mechanisms. Maternal immune response is biased toward humoral immunity and deviates away

from cell-mediated immunity that could result in miscarriages. Another mechanism is by stimulation of maternal lymphocytes to develop progesterone receptors and in the presence of progesterone to produce a mediator that inhibits NK activity and protects against miscarriages. Fu *et al* found that decidual NK cells promote immune tolerance by decreasing inflammatory Th17 cells via IFN-γ secreted by NK subset. Loss of this NK cell mediated regulatory response results in a prominent Th17 response and extensive inflammation leading to recurrent spontaneous miscarriages (Fu, *et al*., 2013). Studies have suggested that persistence of paternal antigens in the maternal circulation is important for the establishment and maintenance of a paternal antigen specific Treg population which contribute to the immunological tolerance (Zenclussen, *et al*., 2010).

1.2.2.1 Materno-Placental interface

Recent studies have indicated that placental immune modulations at the materno-fetal interface down regulate the immune responses to the fetus rather than a shift in the immune system from a Th1 to a Th2 bias. Conventional concept was that during pregnancy there was a change in the balance of Th1 and Th2 cells which resulted in an increase in the production of Th2 cytokines such as IL-3, IL-4, IL-6, and IL-10 and a reduction in Th1 cytokines such as IL-2, IFN-γ, and TNF-α (Borzychowski, *et al*., 2005). Placental immune modulations depend on the stage of the pregnancy. Mor *et al* propose three immunological phases of pregnancy: A proinflammatory first trimester characterised by Th1 responses necessary for implantation and placentation, second trimester characterised by Th2 responses to mask the fetal antigens and facilitate fetal growth, and third trimester proinflammatory responses of Th1 shift to facilitate parturition (Mor, *et al.,*

2011). IL-10 is a potent immunosuppressive cytokine which protects the fetus from rejection by suppressing cellular immunity and inducing HLA-G expression in the trophoblast (Moreau, *et al*., 1999; Raghupathy, *et al*., 2001b). This raises questions about the types of immunomodulations at the materno-fetal interface and the cytokines involved, as well as the differences in these modulations between normal and abnormal pregnancies.

DCs play an active part in this tolerogenic immune modulation. Miyazaki *et al* reported that mature $CD83^{+}$ decidual DCs secrete lower levels of IL-12 when compared to PB DCs and they also induce Th2 immune activity (Miyazaki, *et al*., 2003). Tolerogenic DCs induce regulatory T cells and their activity is increased in normal pregnancy (Lutz and Schuler, 2002; Steinman and Nussenzweig, 2002). Natural Treg cells are found within the decidua and PB during the 1st and 2nd trimester (Somerset, *et al*., 2004) and they secrete IL-10 (Le and Chao, 2007) promoting differentiation of tolerogenic DCs.

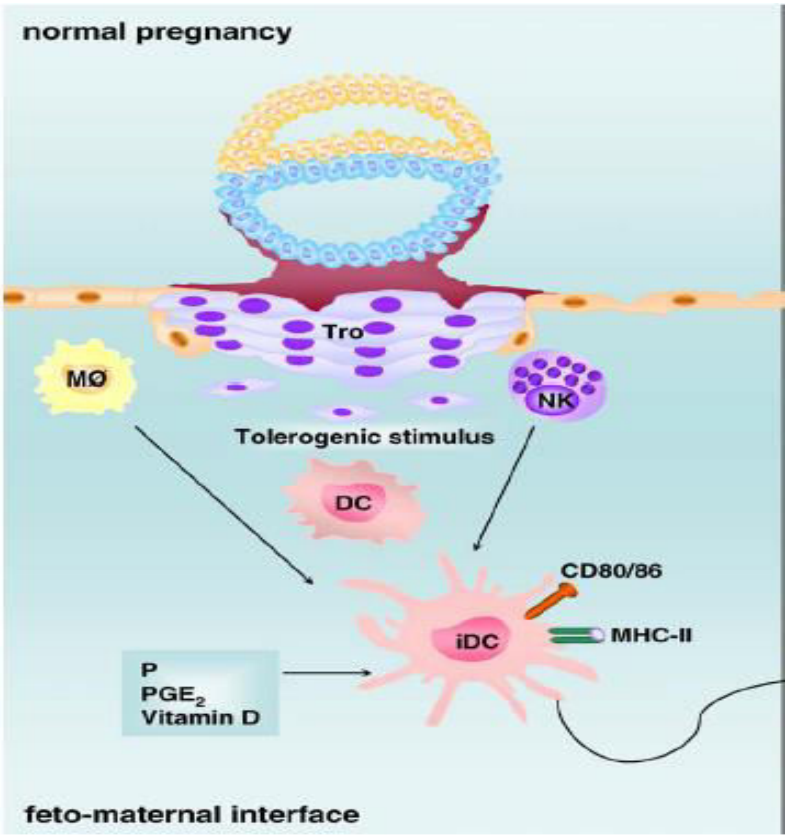


Figure 1.5 Tolerogenic activity of the DC at the feto-maternal interface (from Blois, *et al*., (2007). Dendritic cells: key to fetal tolerance? Biol Reprod. 77(4): 593)

During normal pregnancy, partial activation of the DC at the materno-fetal interface is achieved by tolerogenic stimuli. This results in the production of anti-inflammatory cytokines which promote the induction of tolerance at the fetal-maternal interface

1.2.2.2 Fetal immune system

Functional regulatory cells in the thymus and secondary lymphoid organs have been identified in the fetus from 14 to 17 weeks of gestation (Cupedo, *et al*., 2005). Immunologic self-tolerance and negative control of immune responses have been shown to be due to a subset of T regulatory cells which have the ability to inhibit T-cell proliferation (Van Parijs and Abbas, 1998; Sakaguchi, 2004). During delivery Th1 response predominates with increasing IL-12 levels as IL-12 has a potent Th1 effect (Wegmann, *et al*., 1993).

IL-12 stimulates IFN-γ production and directs T cell responses towards Th1 (Macatonia, *et al*., 1995; Goriely, *et al*., 2001). CBDCs capacity to skew the fetal immune system towards Th2 could be due to reduced production of IL-12 (Goriely, *et al*., 2001; Vanden Eijnden, *et al*., 2006). This has been shown to be associated with an increased risk of sepsis in premature infants (Lavoie, *et al.,* 2010).

1.2.3 Fetal DC characteristics

CBDCs have been identified by cell surface markers as HLA-DR$^+$ and lacking the CD3, CD14, CD16, CD19, CD34, and CD56 markers. This population represents only about 0·3% of CB mononuclear cells (Sorg, *et al*., 1999). Both lymphoid (HLA-DR$^+$ CD11c$^-$ CD33$^-$) and myeloid (HLA-DR$^+$ CD11c$^+$ CD33$^+$) DCs have been identified in CB.

1.2.3.1 Phenotypic characteristics

The majority of CBDCs shows a lymphoid phenotype resulting in an inverted CD11c$^+$/CD11c$^-$ ratio of 1:3 with predominant CD11c$^-$ DCs, when compared to adult blood DCs (3:1) where CD11c$^+$ DCs predominate (Borras, *et al*., 2001; Naderi, *et al*., 2009). This could be due to an age related, evolving antigen specific immunity as the immature CBDCs matures into DCs with adult phenotype.

Holloway *et al* reported that CBDCs exhibited higher levels of CD34, a marker of immaturity. CD34 is a marker of immaturity on precursor DCs and during differentiation DCs are known to down regulate the CD34 expression. The percentage of DCs expressing CD40, CD86 and CD54 was significantly lower than adults throughout gestation showing a reduced capacity for costimulation and the capacity increases with advancing gestation (Holloway, *et al*., 2009). These observations have been confirmed by various studies showing CBDCs to be more immature than adult PB DCs and expressing lower levels of MHC class II, ICAM-1, CD80 and CD86 (Hunt, *et al*., 1994; De Wit, *et al*., 2003; Encabo, *et al*., 2007).

1.2.3.2 Functional characteristics

Fetal blood DC are immature compared with adult (high CD34, low CD4 and CD83) and expressed lower levels of HLA-DR, CD40 and CD86 (Holloway, *et al*., 2000; Naderi, *et al*., 2009). CBDCs display a tendency to express lower levels of the gamma-chain IL-2 receptor, CD132 and of the CD86 costimulatory molecule, supporting a higher degree of immaturity as compared to adult DCs. (Crespo, *et al*., 2004). They also exhibit a lower bioactivity and reduced frequency of cytokine production as compared to adult cells (Bogunia-Kubik, 2001).

CBDCs exhibit an immature phenotype, but are still potent stimulators of allogenic CB T cells (Pacora, *et al*., 2002b). The reduced ability of cord DCs to attain a fully mature adult phenotype, and to activate naïve $CD4^+$ T cells to produce IFN-γ, suggests that they are intrinsically pre-programmed against the generation of Th1 immune responses (Langrish, *et al*., 2002). Naderi *et al* have reported on CBDCs preferential priming of näive T cells towards Th2 population. They showed CBDCs to be poor inducers of IFN-γ secretion and poor stimulators of MLR when compared to adult PB DCs and displayed decreased expression of HLA-DR and CD86 molecules. The ratio of lymphoid DCs to myeloid DCs was significantly higher in CB when compared to adult PB (Naderi, *et al*., 2009).

Regulatory T cells exert an inhibitory effect on CBDCs, the interactions of which result in the downregulation of the expression of CD80, CD86 and MHC Class II molecules on DC and also converts CBDCs into tolerogenic APCs (Cederbom, *et al*., 2000; Vendetti, *et al*., 2000). These multiple inhibitory effects on the CBDC result in their functional immaturity and also play an important role in maintaining immunological tolerance towards the fetus.

1.2.4 Fetal DCs and infection

Fetal biology has been shown to change with the onset of maternal/intrauterine infection, with a rise in IL-8 concentration in CB (Dembinski, *et al*., 2002), and also increased cytokine in gestational membranes suggesting an inflammatory process (Keelan, *et al*., 1999).

Fetal DCs have defective functions in their responses to infectious stimuli as evidenced by multiple studies, a few examples of which are given below. Research performed by Breitling and colleagues in newborn African infants exposed to Plasmodium falciparum *in utero* has shown that CB samples from those exposed to Plasmodium falciparum *in utero* reveal a Th2 bias as opposed to the Th1 bias in Plasmodium falciparum infected placentas (Breitling, *et al*., 2006). Fetal response to cytomegalovirus infection has been shown to be different to the adult where infection rarely causes a severe illness. Congenital cytomegalovirus infection leads to severe morbidity in 15-20% of infected newborns. Renneson and colleagues found that 88 of the genes regulated by cytomegalovirus were differentially regulated between neonatal and adult DC leading to lower levels of IL-12 and IFN produced by CBDCs as compared with adult DC (Renneson, *et al*., 2009). Furthermore CBDCs of fetuses with maternal chronic hepatitis B infection show lower costimulatory markers, IL-12 production and T lymphocyte stimulatory capacity when compared to CBDCs from fetuses of uninfected mothers as well as adult PBDCs (Zhang, *et al*., 2005).

Low basal expression of costimulatory molecules, altered maturation and defective production of cytokines in response to TLR or CD40 signalling and reduced endocytic activity and response to TLR agonists of CBDCs may contribute to the deficient functions (Goreily, *et al*., 2001; Langrish, *et al*., 2002; De Wit, *et al*., 2003; Crespo, *et al*., 2004; Wong, *et al*., 2005; Danis, *et al.,*

2008; Naderi, *et al*., 2009). These recent studies have mostly used DCs which are non-manipulated and freshly isolated from cord blood and thus provide robust results. Earlier studies have used DCs generated from precursors using overnight culture or stimulation using GM-CSF and IL-4. Such techniques could themselves cause DC maturation and deviate the results from those seen *in vivo*.

1.3 SPONTANEOUS PRETERM LABOUR AND DELIVERY

1.3.1 Introduction

Preterm labour is a clinical syndrome characterised by uterine contractility, cervical ripening, and/or membrane rupture occurring before 37 weeks of gestation (Romero, *et al*., 1994). It is the major cause of perinatal mortality and morbidity and infants born before the thirty-seventh week of gestation account for approximately 6% to 9% of all births, but 70% of all perinatal deaths and half of all long-term neurological morbidity (Gibbs, 2001). Compared with term infants, preterm infants have higher rates of cerebral palsy (CP), respiratory distress, hypoglycaemia, jaundice, kernicterus, seizures, periventricular leukomalacia (PVL), hospital readmissions and infant deaths (Wang, *et al*., 2004; Escobar, *et al*., 2006). At least 25% of all preterm deliveries occur in mothers with microbial invasion of the amniotic cavity and studies suggest that infection may account for 25–40% of preterm birth (Romero, *et al*., 1992; Goncalves, *et al*., 2002). Diagnostic difficulties abound as only a minority of infected pregnancies exhibit clinical signs of chorioamnionitis. Only 12.5% of women with preterm labour and intact membranes with a positive amniotic fluid culture have been found to have clinical chorioamnionitis (Romero, *et al.,* 1989b; Goncalves, *et al*., 2002). This could be a reflection of the fact that not all chorioamnionitis are infection driven. Preterm delivery may occur with intact membranes or with PROM. PROM is defined as spontaneous rupture of membranes at less than 37 weeks gestation at least one hour before the onset of contractions. One third of all patients who deliver prematurely presents with premature labour and intact membranes and one third of preterm deliveries presents with PROM. The remaining preterm deliveries are iatrogenic and due to

maternal or fetal indications (Arias and Tomich, 1982). The cause for the majority of preterm births is unknown while 30% have a definite diagnosis of infection/inflammation (Challis, 2000).

1.3.1.1 Incidence

Worldwide, there has been a dramatic increase in the incidence of preterm births, more so in the developed countries than in the developing countries in the past 20 years (Callaghan, *et al*., 2006). Since 1981 there has been a 31% increase in the preterm birth rate in the USA, of which two-thirds were late preterm births (34–36 completed weeks of gestation) (Davidoff, *et al*., 2006). The systematic review conducted by the World Health Organisation in 2010 found that the in 2005, 12.9 million births (9.6% of all births) were preterm. Africa had the highest rate of 11.9%, followed by North America at 10.6%, while Europe had the lowest rate of 6.2% (Beck, *et al*., 2010). The majority of preterm births seem to occur idiopathically (45-50%), while 30% are related to PROM.

Evidently different risk factors play different etiological roles in different parts of the world. In developing countries the major etiological factors are thought to be intrauterine infections (Beck, *et al*., 2010). In developed countries many other factors such as increased obstetric interventions, use of assisted reproduction techniques, increased rate of multiple births, increase in maternal age and a rise in iatrogenic preterm delivery rates play major roles. Changes in clinical practice such as increased use of ultrasound for gestational age estimations and changes in the definitions of stillbirth and early neonatal deaths has had an impact (Slattery and Morrison, 2002; Stanton, *et al*., 2006). Iatrogenic preterm deliveries mainly between 34-36 weeks gestation has increased due to the increase in preterm induction and preterm caesarean deliveries for fetal and maternal

indications. Such iatrogenic causes contribute to 15-20% of preterm deliveries and are on the rise mainly in developed countries (Haas, 2006; Pennell, *et al*., 2007). Norman *et al* found a significant percentage increase in crude rates of medically indicated preterm births in Scotland from 1998-2004 (Norman, *et al*., 2009). While in the United States, iatrogenic preterm birth increased from 2.2 to 3.7 per 100 live births between 1995 and 2005 (Lisonkova, *et al*., 2011).

Assisted reproductive techniques *per se* have been shown to increase the risk of preterm delivery in on analysis of singleton pregnancies alone as preterm birth rates in multiple and singleton pregnancies are different. A significantly increased risk for preterm birth in singleton pregnancies was found in pregnancies after IVF [RR of 2.04 (95% CI 1.80–2.32) by Helmerhorst *et al*; OR of 2.0 (95% CI 1.7–2.2) by Jackson *et al;* RR of 1.98 (95% CI 1.77–2.22) by McGovern *et al*; OR of 1.93 (95% CI 1.36–2.74) by McDonald *et al*] (Helmerhorst, *et al.,* 2004; Jackson, *et al.,* 2004*;* McGovern, *et al.,* 2004; McDonald, *et al.,* 2005).

1.3.1.2 Clinical and financial impact

In spite of the use of antibiotics (Svare, *et al*., 1997; King and Flenady, 2002), corticosteroids and the use of advanced drugs such as atosiban (Worldwide Atosiban study group, 2001), the incidence of preterm birth with its attendant morbidity and costs has not reduced. The short-term costs of the neonatal intensive care unit in the UK are approximately £800 a day and annual costs are approximately £340,000,000 (Petrou, 2005). The total cost of preterm birth to the public sector was estimated to be £2.946 billion (US $4.567 billion), and an inverse relationship was identified between gestational age at birth and the average public sector cost per surviving child (Mangham, *et al*., 2009).

1.3.1.3 Importance of prevention

Prematurity has significant public health impact due to its maternal, perinatal and neonatal morbidity and mortality (Iams, *et al*., 2008; Rüegger, *et al*., 2012). Preterm birth is the leading cause of neonatal mortality and morbidity accounting for one in five children with mental retardation and one in three children with visual impairments (Dammann and Leviton, 2000; Wood, *et al*., 2000; Marlow, *et al*., 2005; Vohr, *et al*., 2005). Premature infants have long term health risks such as an increased risk for cardiovascular disease and diabetes (Kajantie, *et al*., 2010; Poplawska, *et al*., 2012). These diverse sequelae, especially in the long term impose a heavy burden on the health services (Kuban and Leviton, 1994; Petrou, 2005; Kruse, *et al*., 2009; Mangham, *et al*., 2009). Hence it is important to investigate the biology of prenatal infections, which is one of the major causes of premature birth.

1.3.2 Clinical Debility

Severe morbidity, especially respiratory distress syndrome, intraventricular haemorrhage, bronchopulmonary dysplasia and necrotising enterocolitis are far more common in preterm infants than in term infants. The EPICure Study suggests that almost 50% of babies born at 23-25 wks gestation will have significant long-term handicap, with a survival of only 35% of preterm deliveries less than 25 weeks (Wood, *et al*., 2000).

1.3.2.1 Resultant Morbidity

Retinopathy of prematurity is one of the most common morbidities in very preterm infants. Rates of severe visual impairment or blindness increases with decreasing gestational age, 1–2% for at 26–27 weeks, and 4–8% at 25 weeks or below (Marlow, *et al*., 2005; Vohr, *et al*., 2005). Myopia and hypermetropia occur in at least a quarter of children born before 28 weeks gestation (O'Connor, *et al*., 2002).

Very low birth weight infants have central auditory processing difficulties, including difficulties involving auditory recognition and identifying simple speech sounds (Davis, *et al*., 2001). Hearing impairments have a negative impact on the acquisition of language skills and affects schooling.

Studies have also found that infants born with very low birth weight have lower growth attainment in weight and length than their normal birth weight counterparts during infancy and early childhood (Kitchen, *et al*., 1992; Hack, *et al*., 1996).

1.3.2.2 Resultant Mortality

In spite of the advances in medical technology the mortality associated with prematurity remain significant, as high as 42% worldwide in 2005 (Beck, *et al*., 2010). An infectious etiology of premature labour seems to be of prime importance in the resultant mortality. A link between intrauterine/placental infection and inflammation and mortality has been established. An association between genital bacterial colonisation and premature delivery and mortality has been reported and placental inflammation has been found to be an independent risk factor for preterm

birth, low birth weight, stillbirth, and perinatal death (Osman, *et al*., 1995; Mwanyumba, *et al*., 2003).

An inflammatory response by the fetus has also been evidenced as part of the pathology leading to fetal death of infectious etiology. Moyo *et al* showed that 9% of stillbirths in their series but none of the live births, had evidence of vasculitis in the chorionic plate vasculitis of the chorionic plate, which verified an inflammatory response from the infant (OR: 14, 95% CI: 2.8-72) (Moyo, *et al*., 1996).

1.3.2.3 Long Term Sequelae

1.3.2.3.1 Chronic lung disease and Bronchopulmonary dysplasia

The EPICure Study showed that the major cause of death for all infants less than 26 weeks was pulmonary insufficiency (Costeloe, *et al*., 2000). Bronchopulmonary dysplasia occurs in up to 40% of very low birth weight babies and the rate rises below a birth weight of 1500g (Darlow, *et al*., 2003). Intrauterine inflammation (Speer, 2003) and antenatal exposure to proinflammatory cytokines with amniotic fluid concentrations of IL-8 >11.5 ng/ml (nanogram/millilitre) are risk factors for the development of bronchopulmonary dysplasia (Ghezzi, *et al*., 1998). The long term prognosis of these patients is chronic pulmonary dysfunction.

1.3.2.3.2 Neurological damage

Intrauterine infection has been shown to lead to a fetal inflammatory response, which, in turn, results in adverse outcomes such as preterm labour and delivery, intraventricular haemorrhage, white matter damage, PVL and mainly CP (Dammann and Leviton, 2000). Mental retardations and sensory impairments can also present clinically in the early years.

The prevalence of CP is 1.5 to 3 cases per 1000 live births and it is the most common cause of motor disability in childhood ((SCPE) Collaborative Group, 2000; Paneth, *et al.*, 2006). The link between CP and perinatal complications was first proposed by William John Little in 1862. Increased risk for CP in intrapartum fever was first reported in 1955 by Eastman and DeLeon (Eastman and Deleon, 1955). A recent systematic review found that the proportion of CP associated with intrapartum hypoxia-ischemia is 14.5% (Graham, *et al*., 2008). Himmelman *et al* found perinatal hypoxic ischaemic encephalopathy in 71% of dyskinetic CP in children born at term (Himmelman, *et al*., 2005). In this group, multiple studies have shown clinical and histologic chorioamnionitis to be associated with increased risks of CP (Murphy, *et al*., 1995; Grether and Nelson, 1997; Alexander, *et al*., 1998; O'Shea, *et al*., 1998; Yoon, *et al*., 2000b; Wu and Colford, 2000) and white matter lesions to be associated with intrauterine inflammation and infection in preterm labour (Leviton, *et al*., 1999; Dammann, *et al*., 2002). Wu *et al* in their meta-analysis found that clinical chorioamnionitis is associated with an increased risk of both CP and white matter damage [RR 1.9 (95% CI:1.4-2.5) and 2.6 (95% CI: 1.7-3.9), respectively] (Wu and Colford, 2000).

There is a strong association between prematurity and CP; the risk of CP is 70 times greater at delivery at less than 28 weeks of gestation compared with delivery at term. Half of all cases of

CP are seen in premature infants (McCormick, 1985). Premature infants mount an exaggerated fetal systemic inflammatory response and also have but a limited ability to buffer the effect of proinflammatory cytokines (Nelson, *et al*., 1998; Grether, *et al*., 1999; Dammann, *et al*., 2005). Studies have postulated that premature infants are unable to buffer the recruitment of peripheral leukocytes as their blood-brain barrier is immature. The presence of macrophages in sites of white matter injury indicated that activated leukocytes were involved in the neurological injury processes (Dammann, *et al*., 2001; Leviton, *et al*., 2005). Furthermore Lin *et al* found increased sensitivity to LPS in children with CP leading them to suggest that inflammation could have a programming effect resulting in the altered inflammatory responses in preterm children (Lin, *et al*., 2010).

Multiple studies support a link between fetal inflammation and brain injury. Elevated concentrations of cytokines in fetal plasma (Yoon, *et al*., 1997a; Dammann and Leviton, 2000) and fetal vasculitis (Wharton, *et al*., 2004; Redline, 2005) are associated with intraventricular haemorrhage, white matter damage, and CP. Originally described by Virchow in 1867, PVL are cerebral lesions characterised by foci of necrosis in the white matter near the lateral ventricles. PVL is the most common form of brain injury in preterm infants (Inder, *et al*., 1999). Clinical (Leviton and Paneth, 1990; Verma, *et al*., 1997) and experimental evidence (Hagberg, *et al*., 2002; Debillon, *et al*., 2003; Mallard, *et al*., 2003) indicates that intrauterine infection results in PVL and neuronal lesions. A meta-analysis examining the relationship between chorioamnionitis and PVL demonstrated that PVL was significantly associated with both clinical (RR, 3.0; 95% CI, 2.2–4.0) and histological chorioamnionitis (RR, 2.1; 95% CI, 1.5–2.9) (Wu and Colford, 2000).

Inflammatory cytokines such as TNF-α induce fetal hypotension and brain ischemia, release tissue factor and platelet activating factor which leads to coagulation necrosis of white matter (Leviton, 1993; Kadhim, *et al*., 2003). TNF-α also has a direct cytotoxic effect on oligodendrocytes and myelin (Leviton, 1993). Deguchi *et al* has shown increased expression of TNF-α in the microglial cells in PVL lesions (Deguchi, *et al*., 1996). Elevated IL-6 levels are associated with an increased risk for PVL in infants born at less than 32 weeks gestation (Goepfert, *et al*., 2004). Recently experimental evidence points to TLRs as having a crucial role in white matter injury. Administration of bacterial LPS through the systemic activation of TLR-4 has been shown to have toxic effects on the immature central nervous system (Mallard, *et al*., 2003). A strong body of evidence implicates damage to immature oligodendrocytes as the pathology underlying PVL (Takashima, *et al*., 1995; Back, *et al*., 2001; Johnston, *et al*., 2001).

1.3.2.3.3 Behavioural sequelae

Prematurity leads to developmental and behavioural impairments which have long term effects and can persist into adulthood. Huddy *et al* found in their study that up to a third of 7-year-old children born at 32–35 weeks gestation had difficulties in motor, educational and behavioural skills (Huddy, *et al*., 2001). Children born prematurely also exhibit high rates of executive dysfunction and global impairment in cognitive functions such as attention, visual processing and academic progress compared with their normal birth weight peers. (Anderson, *et al*., 2004). The cognitive and educational problems associated with prematurity are hypothesised to be caused by brain injury. PVL, which is related to ischemia and results in focal or diffused lesions has been implicated in long term cognitive dysfunction (Volpe, 1998). Long-term follow-up

reveals that many of the above difficulties persist into adolescence and early adulthood, all of which have significant implications for educational services and costs.

1.3.2.4 Fetal Inflammatory Response Syndrome

The fetal inflammatory response syndrome (FIRS) is a condition characterised by systemic activation of the fetal innate immune system and was originally defined by an elevation of fetal plasma IL-6 concentration of >11pg/ml in fetuses with preterm labour and PROM (Gomez, *et al*., 1998b). The multisystemic organ involvement is evidenced by increased concentrations of fetal plasma matrix metalloproteinases (MMP-9) (Romero, *et al*., 1998). Prevalence of severe neonatal morbidity (defined as the presence of respiratory distress syndrome, suspected or proved neonatal sepsis, pneumonia, bronchopulmonary dysplasia. intraventricular haemorrhage, PVL, or necrotising enterocolitis) was higher in neonates with FIRS (Gomez, *et al*., 1998b). Funisitis and chorionic vasculitis are the histopathologic hallmarks of FIRS (Pacora, *et al*., 2002a) and the endothelial activation associated with funisitis leads to organ damage and increased risk of sepsis (Yoon, *et al*., 2000a), bronchopulmonary dysplasia (Yoon, *et al*., 1999b) and CP (Yoon, *et al*., 2000b).

Two thirds of fetuses with FIRS have neutrophilia and a higher median nucleated red blood cell (RBC) count (Gomez, *et al*., 1998a) and show evidence of monocyte and neutrophil activation with higher expressions of CD11c, CD13, CD15 and CD67 (Berry, *et al.,* 1995).

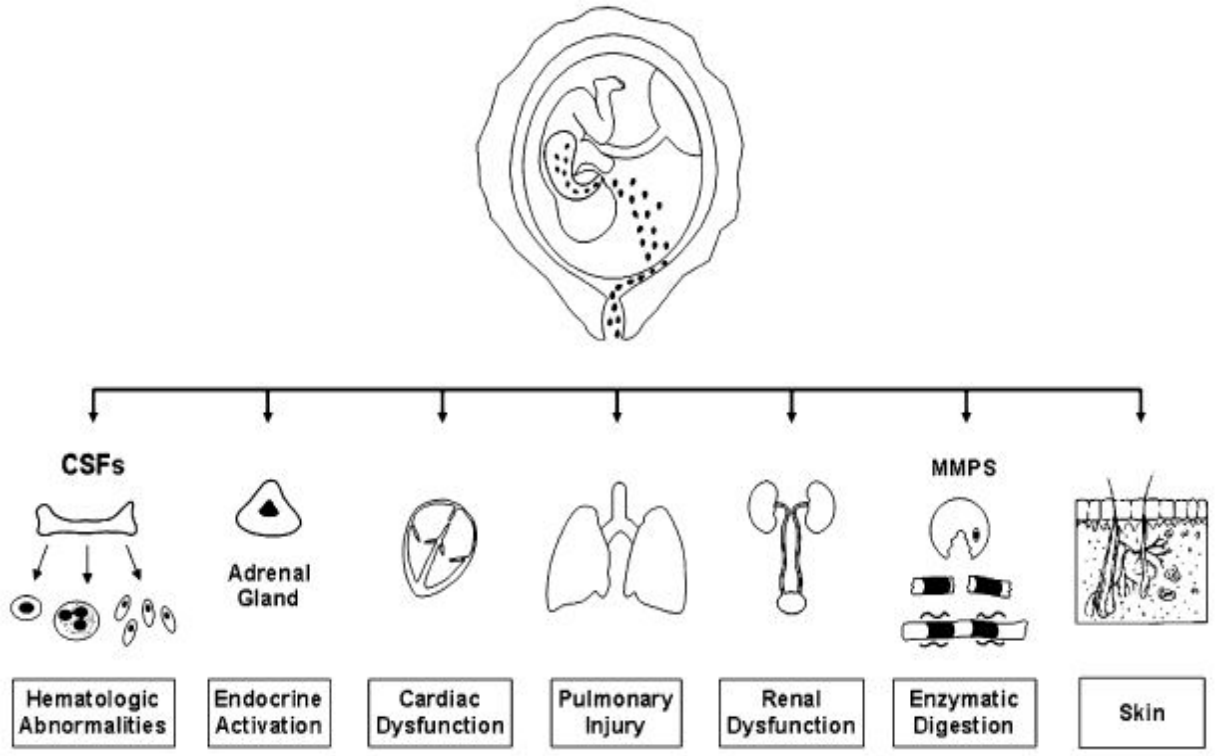


Figure 1.6 Multiple fetal organs targeted during FIRS

(from Romero, et al., (2006). Inflammation in preterm and term labour and delivery. Semin Fetal Neonatal Med. 11(5): 322)

Fetal organs targeted during the fetal inflammatory response syndrome (FIRS) are the brain, haematopoietic system, adrenals, heart, lungs, kidneys and skin

1.3.3 Molecular events in Spontaneous Preterm Labour

The process of labour in preterm as well as in normal term gestation involves three physiologically interdependent processes: the initiation of rhythmic uterine contractions, remodelling of the cervical tissue to allow it to soften and stretch, and weakening and rupture of

the membranes. Microbial stimuli and stress related proteins act through TLRs and result in the release of cytokines and chemokines. These stimuli also activate the innate immune system within the pregnant uterus leading to release of MMPs, PGs and increase the collagenase activity resulting in rupture of the membranes (Romero, *et al.,* 2007). Actions of the proinflammatory cytokines are key factors in the initiation of labour and they also act on the downstream PGs and tissue proteinases resulting in the recruitment of inflammatory neutrophils and macrophages (Patni, *et al*., 2007).

1.3.3.1 Cervical Softening and Dilatation

Liggins in the early 1980s described cervical ripening as an inflammatory process with an influx of leukocytes (Liggins, 1980). The proinflammatory cytokines; IL-8, IL-1β, IL-6 and TNF-α increase at the onset of labour and act on downstream PGs to increase the production of tissue proteinases (Osman, *et al*., 2003). IL-1β and TNF-α increase the production of MMP and cathepsins, and IL-1β downregulates the expression of an endogenous inhibitor of MMP (Watari, *et al*., 1999). Cervical dilatation is also induced by cyclo-oxygenase (COX)-2 and PGE2 which is increased by IL-1β and IL-1a. PGE2 in turn increases the production of proteinases and the permeability of blood vessels for leukocyte recruitment, thus initiating and promoting labour (Kelly, 2002).

1.3.3.2 Onset of Myometrial Contractions

The sequence of proinflammatory cytokine induced actions described above also occurs in the myometrium and facilitates onset of myometrial contractions. IL-1β, TNF-α and IL-6 are mainly associated with labour (Young, *et al*., 2002; Osman, *et al*., 2003). IL-1β and TNF-α increase the production of PGE2 and COX-2 by the myometrium (Pollard and Mitchell, 1996; Todd, *et al*., 1996). PGE2 in turn increases the intracellular calcium concentrations in myometrial cells leading to increased contractility of the myometrial muscle cell and uterine contractions (Thornton, *et al*., 1992). IL-6 increases the expression of oxytocin receptors on myometrial cells and also increases its secretion by myometrial cells (Friebe-Hoffmann, *et al*., 2001; Rauk, *et al*., 2001). Oxytocin has a similar action to PGE2 as outlined above and leads to uterine contractions.

1.3.3.3 Rupture of membranes

The molecular events leading to cervical dilatation also facilitate rupture of membranes. Increased production of proinflammatory cytokines from infiltrating leukocytes, decidual cells and amniochorionic cells lead to the increase in the production of PGs and MMP (Laham, *et al*., 1999; Maymon, *et al*., 2000; Young, *et al*., 2002). There is also a significant upregulation of IL-1β and IL-6 seen in labour (Norman, *et al.,* 2007). During labour at term the collagenase acts on the area of the fetal membranes overlying the cervix, altering its morphology and integrity and degrade the extracellular matrix resulting in rupture (Moore, *et al*., 2006). During preterm labour the degradation of the extracellular matrix is seen diffusely and is not isolated to the area of the fetal membranes overlying the cervix. The proinflammatory cytokines also promote interactions

between the fetal membranes and myometrium resulting in myometrial contractions (McLaren, *et al*., 1999; Osman, *et al*., 2006).

1.3.4 Immunology of Spontaneous Preterm Labour

Immunomodulations at the materno-fetal interface serve to protect fetal tissues and prevent rejection of the fetal allograft. Infections and other factors have been postulated to disrupt the delicate balance between pro and anti inflammatory modulations as discussed below, resulting in premature labour. There is strong evidence for the infectious aetiology and many studies have shown that bacteria can cross intact chorioamniotic membranes (Galask, *et al*., 1984; Romero, *et al*., 1987; Romero, *et al*., 1989b).

1.3.4.1 Role of cellular mediators

The innate immune system also recognises micro organisms through PRRs e.g. acute phase proteins and TLRs, which are crucial for the recognition of microorganisms. PRRs bind to patterns of molecular structures present on the surfaces of microorganisms. TLR-2 is involved in the response to products of gram-positive bacteria, mycoplasmas, and yeast and TLR-4 in the response to LPS (Akira, *et al*., 2001). To date eleven different TLRs have been recognised in humans and they are key upstream mediators of inflammation (Zhang, *et al*., 2004; Janeway, *et al*., 2005). Defective signalling through TLRs will impair defences against bacteria-induced preterm labour and there is experimental evidence that TLRs are crucial in the aetiology of preterm labour (Wang and Hirsch, 2003).

Studies have provided evidence for an association between raised markers of inflammation and subsequent preterm delivery. Raised levels of amniotic fluid MMP-8 (Yoon, *et al*., 2001) and angiogenin (Spong, *et al*., 1997) are some of the markers of inflammation implicated in the aetiology. Intravascular maternal inflammation in preterm labour of infectious aetiology leads to upregulation of maternal monocytic and granulocytic markers e.g. CD11b, CD15 and CD66 on granulocytes, and CD11b and CD15 on monocytes in preterm labour with intact membranes (Gervasi, *et al*., 2001). Similarly, PROM is associated with upregulation of CD11b, CD14, CD64 and CD66b on granulocytes and CD11b on monocytes (Gervasi, *et al*., 2001).

1.3.4.2 Role of cytokines

Cytokines are small proteins in the extracellular environment that interact with specific receptors on target cells and facilitate communication between cell types (Peltier, 2003). Both pro as well as anti inflammatory cytokines play a central role in the aetiology of preterm parturition. Decidual cells produce the proinflammatory cytokines IL-1 and TNF-α in response to bacterial products such as LPS (Casey, *et al*., 1989; Romero, *et al*., 1989a). They increase in concentration and bioactivity in the amniotic fluid of women with preterm labour (Casey, *et al*., 1989; Romero, *et al*., 1989a). IL-1 is produced by activated monocytes and macrophages and stimulates myometrial contractions through the production of PGE2 and/or F2α resulting in an increase in COX-2 synthesis and activity (Romero, *et al*., 1989c; Mitchell, *et al*., 1990).

Other proinflammatory cytokines such as IL-6, IL-16, IL-18, colony stimulating factors, and chemokines such as IL-8 and monocyte chemotactic protein-1 are also implicated in the aetiology of preterm labour (Agrawal and Hirsch, 2011). Abrahams *et al* have shown that a

Gram-positive bacterial infection, through TLRs 1 and 2, promote trophoblast cell apoptosis (Abrahams, *et al*., 2008). Trophoblast cells secrete IL-6 and IL-8, which stimulate neutrophil and T cell chemotaxis and activation leading to apoptosis. The authors have postulated that may be one of the underlying mechanisms of preterm delivery.

Anti-inflammatory cytokines contribute to the onset of preterm labour by their downregulation or cessation of activity. IL-10 is the key anti inflammatory cytokine for the maintenance of pregnancy, its downregulation is a central event prior to the onset of normal labour (Hanna, *et al*., 2000) and it is reduced in preterm labour (Blanco-Quiros, *et al*., 2000; Hanna, *et al*., 2006). IL-10 inhibits the activation of T cells, monocytes and macrophages and has potent immunosuppressive activity by inhibiting both IL-12 and IFN-γ synthesis thus maintaining the pregnancy (Moore, *et al*., 1993).

1.3.5 Infections and Spontaneous Preterm Labour

Microorganisms invade the uterine milieu through different routes such as retrograde from abdominal cavity and haematogeneously through placenta, but ascending infection through the vagina and cervix is the most common (Romero and Mazor, 1988). Ascending infection can reach the decidua and stimulate the production of proinflammatory cytokines and inflammatory cellular mediators. Transplacental infection and retrograde seeding through the fallopian tubes have been reported (Goldenberg, *et al*., 2000). Microorganisms cross the membranes into the amniotic cavity and they can also cause fetal infection resulting in a fetal inflammatory response syndrome (Gomez, *et al*., 1997a).

1.3.5.1 Infections and cytokines

Studies have provided evidence suggesting that a chronic intra-amniotic inflammatory process is associated with spontaneous preterm labour and preterm delivery. The Preterm Prediction Study found that an elevation of cervicovaginal fluid IL-6 concentration above the 90th percentile was a risk factor for preterm delivery (Goepfert, *et al*., 2001).

1.3.5.2 Infectious aetiology of Spontaneous Preterm Labour

The relationship between genital tract infection and preterm delivery has been established on the basis of biochemical, microbiological and clinical evidence (Hay, *et al*., 1994; McGregor, *et al.,* 1994). It is the only pathological process for which a causal link with preterm birth has been established, especially in the preterm delivery at less than 30 weeks gestation (Gomez, *et al*., 1997b).

This evidence includes the following: (Romero, *et al*., 2007).

(1) patients with preterm labour have been found to have higher levels of inflammatory cytokines and microbial colonisation than preterm patients not in labour and term patients in labour (Romero, *et al*., 1989b).

(2) even subclinical intrauterine infections have been shown to be associated with preterm delivery (Gomez, *et al*., 1995).

(3) intrauterine infection/inflammation in midtrimester has been shown to be a risk factor for subsequent preterm delivery (Gray, *et al*., 1992; Wenstrom, *et al*., 1998; Yoon, *et al*., 2001).

(4) extrauterine maternal infections such as malaria, pyelonephritis, pneumonia and periodontal disease have been associated with premature parturition (Benedetti, *et al*., 1982; Fan, *et al*., 1987; Xiong, *et al*., 2006).

(5) animal studies have shown that intrauterine or systemic administration of microbes or microbial products to pregnant animals can result in preterm labour and delivery (Schlafer, *et al*., 1994; Bennet, *et al*., 2000; Ilievski and Hirsch, 2010).

Additionally women with PROM have a rate of positive amniotic fluid cultures of 32.4% (Goncalves, *et al*., 2002). This rate falls to 22% in women with preterm labour and intact membranes who deliver prematurely and is less than 1% in women not in labour at term (Goncalves, *et al*., 2002). Preterm labour with intact membranes is the final result of a multifactorial inflammatory pathway leading to initiation of parturition. Inflammatory processes triggered by infection along with a secondary component of protease activity leading to weakening of the membranes results in PPROM. Thus amniotic fluid cultures are higher in PPROM. A strong correlation between positive amniotic fluid cultures and histological chorioamnionitis has been shown (Romero, *et al*., 1992; Hillier, *et al*., 1993). The review by Romero *et al* found an association between preterm birth and the occurrence of acute chorioamnionitis (Romero, *et al*., 1993a). A positive correlation with the frequency of histological chorioamnionitis and lower gestational age at birth was also shown by Hillier *et al* (Hillier, *et al*., 1988).

The most common micro organisms involved in intrauterine infections are *Ureaplasma urealyticum, Fusobacterium species* and *Mycoplasma hominis* (Goncalves, *et al*., 2002). Other microorganisms found in the amniotic cavity include *Streptococcus agalactiae, Escherichia coli, Gardnerella vaginalis, Petostreptococcus species, Staphylococcus aureus, Streptococcus*

viridans and *Bacterioides spp.* (Romero, *et al*., 1989b). *Lactobacillus species, Enterococcus faecalis* and *Neisseria gonorrhea* have been identified occasionally and *Haemophilus influenzae,* and *Clostridium species* are seen rarely (Hitti, *et al*., 1997; Alanen, 1998). *Bacterial vaginosis* during pregnancy is associated with a statistically significant increased risk for adverse outcomes (McGregor, 1994; Flynn, *et al*., 1999) as is genitourinary *Chlamydia trachomatis* infection (Andrews, *et al*., 2000) and *Ureaplasma urealyticum* (Abele-Horn, *et al*., 2000). *Bacterial vaginosis* is characterised by a change in the microbial ecosystem of the vagina and is a risk factor for spontaneous preterm delivery (Eschenbach, 1993; Gibbs, 1993; Hay, *et al*; 1994) and PROM (Gravett, *et al*., 1986; Lamont, 2003).

Fetal infection takes place in approximately 10% of pregnancies with intra-amniotic infection, and is the most serious culmination of ascending infection. Although the fetus is capable of mounting an inflammatory response from the mid-trimester of pregnancy the mortality rate for congenital sepsis ranges from 25 to 90% (Gerdes, 1991; Thompson, *et al*., 1992).

1.4 MARKERS OF EXPOSURE TO INFECTION

The cells of the immune system, including DCs, neutrophils and macrophages mediate and effect their immunological effects through cellular peptides and glycoprotein molecules.

1.4.1 Cytokines and Chemokines

Cytokines are soluble peptides or glycoproteins, which are produced by leukocytes, and whose primary function is intercellular communication. The primary role of cytokines is in cell-to-cell communications and they exert their effects by binding to specific receptors with high affinity. This receptor binding results in the expression of other cytokines and a network of activation is set into motion. Cytokines are categorised as: (1) interferons, (2) interleukins, (3) TNF and related molecules, (4) transforming growth factors, (5) haematopoietic growth factors, and (6) chemokines. Cytokines are also classified by their cellular origin into Th1 and Th2 cytokines. Th1 cytokines (i.e., IFN, IL-2, and TNF) mainly promote cell-mediated immunity for protection against intracellular bacteria and viruses and Th2 cytokines (i.e., IL-4, IL-6, IL-10, and IL-13) mainly promote humoral immunity for protection against extracellular pathogens (Gotsch, *et al*., 2007). Cytokines can have proinflammatory (IFN-γ, IL-1, IL-12, and TNF-α) as well as anti inflammatory (IL-4, IL-10, IL-11, IL-13) actions.

Chemokines are substances that attract cells to migrate in a particular direction. DCs can recruit pathogen specific T cell subsets through their specific production of chemokines. DCs produce several chemokines specifically involved in DC maturation and migration to lymphnodes for T

cell stimulation (Sallusto, *et al*., 1999; Villadangos and Schnorrer, 2007). These include CCL17/TARC and CCR7. CCR7 is key to DC migration to the lymphoid tissues.

1.4.1.1 Interleukin-12

IL-12 is a proinflammatory cytokine that induces the differentiation of Th1 cells and forms a link between innate and adaptive immunity (Kobayashi, *et al*., 1989; Hsieh, *et al*., 1993). IL-12 acts on pre-activated T cells and NK cells through IFN-γ leading to their proliferation (Perussia, *et al*., 1992). It acts to enhance the generation of CTLs, augments the cytotoxic activity of CTLs and NK cells (Trinchieri, 1998) and induces T cells and NK cells to produce cytokines: GM-CSF, TNF and IFN-γ (Chan, *et al*., 1991; Kubin, *et al*., 1994). It acts on B cells and increases the activation and production of Th1-associated classes of Ig. DCs produce IL-12, independent of IFN-γ and of signals from T cells, during interactions with T cells (Gazzinelli, *et al*., 1994; Macatonia, *et al*., 1995; Scharton-Kersten, *et al*., 1996). The above ability of IL-12 to promote an early nonspecific response via the innate system through NK activation and IFNγ production and a late specific response adaptive immune reaction via Th1 differentiation allows it to link innate and adaptive immune mechanisms.

It is a heterodimer and induces the production of mainly IFN-γ. IL-12 is produced mainly from activated DCs and phagocytes (monocytes/macrophages and neutrophils). Production is triggered by activation through the Toll pathway as a response to pathogens; in response to signals from activated T cells and NK cells and also in response to inflammatory extracellular components. The production of IL-12 is regulated by positive and negative regulatory mechanisms. IFN-γ, IL-4 and IL-13 are potent stimulators of IL-12 production (D'Andrea, *et al*., 1995). Negative

regulation is mainly by the anti inflammatory cytokine IL-10 and acts by blocking transcription of both of its encoding genes (Aste-Amezaga, *et al*., 1998). IL-12 has been shown to increase not only in preterm labour and infections, but also in exposure of the fetoplacental unit to stressor stimuli such as in severe pre eclampsia (Dudley, *et al*., 1996). The ability of IL-12 to stimulate innate resistance as well as its action as a potent inducer of Th1 responses through its proinflammatory functions portrays IL-12 as a crucial link between the innate and adaptive arms of the immune system (Hsieh, *et al*., 1993; Manetti, *et al*., 1993).

1.4.2 Costimulatory markers

The term ‘‘costimulation’’ is used to describe the additional signals required to initiate T lymphocyte activation apart from an adhesion interaction (Schwartz, 1990). Activation of the Toll pathway induces upregulation of the surface costimulatory molecules which lead to induction of adaptive immune responses. The upregulated costimulatory molecules are presented by MHC class II proteins in DCs and initiation of the adaptive immune system takes place with activation of naïve $CD4^{+}$T cells. Mature DCs exhibit class I and class II MHC and costimulatory molecules such as CD40, CD80 and CD86 (Almeida, *et al*., 1999; Almeida, *et al*., 2001). Immature DCs exhibit decreased expression of the costimulatory molecules (Banchereau, *et al*., 2000).

1.4.2.1 CD40

The CD40 molecule was first identified in human tonsil DC (Hart and McKenzie, 1988). It is a member of the TNF receptor family and its ligand CD40L is expressed on activated T lymphocytes (Fanslow, *et al*., 1994). CD40/CD40L is a major signalling pathway for DC maturation and enhanced stimulatory ability. DCs are activated by the ligation of CD40 by CD40L expressed on the surface of T lymphocytes (Schoenberger, *et al*., 1998). Ligation triggers production of high levels of IL-12 and activates DCs thus leading to T cell stimulation (Cella, *et al*., 1996). Ligation can also occur directly. The recognition of antigen loaded MHC class II molecules on the surface of DCs by the TCR on $CD4^+$ T cells results in the upregulation of CD40L on the T cell surface and subsequent ligation to the DC CD40. This binding of CD40/CD40L upregulates costimulatory molecules and enables the DC to efficiently activate T lymphocytes (Ridge, *et al*., 1998).

1.4.2.2 CD86

CD86 is a member of the Ig superfamily. The costimulatory molecules CD80 and CD86 deliver signals which modulate DC function (Zheng, *et al*., 2004). The MHC-peptide complexes formed in the DC is transported to the cell surface in vesicles together with CD86. At the DC surface the complexes along with the CD86 molecule are deposited as stable clusters (Inaba, *et al*., 2000). They bind on to the CD28 molecules on T cells delivering a costimulatory signal without which antigen triggering of T lymphocytes could lead to anergy (Schwartz, 1990). CD86 molecules are not found on resting blood DC and are upregulated on DC activation (McLellan, *et al*., 1995) and

trigger a Th2 response preferentially. CD86 induction results in the subsequent secretion of IFN-γ (Logue and Sha, 2004).

1.4.3 CD11b on neutrophils

Neutrophils arrive at sites of inflammation before any other immunologically active cell and thus are key to the inflammatory process. Neutrophils are primed by proinflammatory cytokines and inflammatory mediators such as LPS (Hallett and Lloyds, 1995; Svanborg, *et al*., 1999). CD11b (Mac-1) is a β2 integrin; an adhesion glycoprotein involved in neutrophil adherence. It is a cell surface antigen, stored in intracellular granules and translocated to the neutrophil surface upon neutrophil activation and is expressed at a low level on the cell surface of nonactivated neutrophils (Weirich, *et al*., 1998). CD11b also activates other key adhesion and defence receptors (Springer, 1995; Plow and Zhang, 1997). CD11b adhesion results in attachment of the neutrophil to the endothelium, leading to transendothelial migration of the neutrophil to sites of inflammation/infection (Anderson, *et al*., 1984; Kishimoto, 1989; Jones, *et al*., 1990). Effective adhesion is complement receptor 3 (CR3/CD11b) dependent, upregulation of which is effected by the rapid degranulation of intracellular granules (Elghetany, 2002). The upregulation leads to phagocytosis and intracellular killing of opsonised infective agents (Lehrer, 2004). After neutrophil stimulation the number as well as the affinity of the CD11b receptors increases (Vedder and Harlan, 1988). Studies have shown that the average expression levels of CD11b on PB neutrophils can be over three and two-fold higher in bacterial infections compared with viral infections and controls (Nuutila, *et al*., 2006; Nuutila, *et al*., 2009). Thus, neutrophil surface density of the CD11b marker has been used to identify sepsis in term infants (Weirich, *et al*.,

1998; Nupponen, *et al*., 2001; Cui, *et al*., 2003) and adults (Lin, *et al*., 1993; Kylanpaa-Back, *et al*., 2001). The overall reported incidence of neonatal sepsis is 1 to 10 per 1000 live births, the mortality rate is 4.2-26% and it is associated with 10% of cases of neonatal death (Stoll, *et al*., 1998). But in infants with birth weight less than 1000g, the incidence is 16-21% and correlates inversely with birth weight (Stoll, *et al*., 2002b). Adib *et al* in their study to determine CD11b sensitivity and specificity for early detection of neonatal sepsis found that the sensitivity and specificity were 75% and 100% respectively. The positive and negative predictive values were 100% and 86% respectively (Adib, *et al*., 2007). Thus measurement of neutrophil surface markers can be useful for diagnosis of neonatal sepsis in the early phases. FIRS is also associated with a higher expression of markers of monocyte and neutrophil activation (Berry, *et al*., 1995).

Currently there is no reliable and accurate test to detect early onset of sepsis in the neonate. The clinical picture can be variable and contributes to the difficulty in early detection. Low serum levels of CRP do not exclude the possibility of onset of sepsis as *de novo* protein synthesis is necessary to elevate CRP levels (Benitz, *et al*., 1998; Ronnestad, *et al*., 1999). On the contrary, CD11b upregulation does not need *de novo* protein synthesis and occurs promptly with exposure to infectious stimuli (Calafat, *et al*., 1993). Additionally CD11b levels can be obtained with a very small volume of blood, 25 microlitre (µl), which makes it ideal for testing in premature infants. There are no associations between birth weight nor gestational age and the levels of CD11b thus increasing the accuracy of the test (Nupponen, *et al*., 2001). Increased usage in clinical setting is facilitated by the rapid availability of the results within 30-60 minutes after sampling, unlike cultures which usually take 3-5 days.

1.5 NEED FOR RELIABLE TEST TO IDENTIFY AT RISK NEONATES

Microbial infections are responsible for more than 2 million annual deaths between the ages of 1–6 months (Siegrist, 2007). Despite the development of advanced pharmacotherapeutics and life support treatment, infection in the neonate accounts for approximately 10% of cases of neonatal death (Stoll, *et al*., 1998). Pathologic examination has been the gold standard for the diagnosis of inflammation. However, chemotactic signals must be present for the white blood cells to migrate to the site of injury or infection.

The innate immune system is activated within 12 hours of initial microbial exposure. Phagocytic cells such as neutrophils and macrophages and NK cells are activated. During this phase no symptomatology is present. This initiation of the inflammatory cascade results in the activation adaptive immune system with lymphocyte activation and antibody secretion, 12 hours to 5 days after exposure to microbial antigens (Abbas and Lichtman, 2003). This results in microbiological evidence by culture which is seen as the 'gold standard' for diagnosis of sepsis. Thus there is a window of time in which infection/inflammation is present before histologic/microbiological evidence is observed and this results in false negative results.

The following clinical criteria were used in the diagnosis of intra amniotic infection associated with microbial invasion of the amniotic cavity by Gibbs *et al*. Premature rupture of membranes, fever (~37.8 C), maternal tachycardia (pulse rate, >100/min), leukocytosis (~15,000 cells/rnm), fetal tachycardia (pulse rate, ~160/min), uterine tenderness, fetal distress and foul smelling amniotic fluid (Gibbs, *et al*., 1982). Most cases of intra amniotic infections are subclinical in nature. Romero *et al* found that clinical signs were present in only 12.5% of the patients with positive amniotic fluid cultures. (Romero, *et al*., 1989b). Additionally the majority of women

delivering before 30 weeks have clinically silent chorioamnionitis (Goldenberg, *et al*., 2000). Chorioamnionitis has been proven to be frequent, but only 2% of extremely preterm infants have positive blood cultures at birth (Stoll, *et al*., 2002a). Tests with such low sensitivity result in late diagnosis of neonates with infection and delay in instituting appropriate timely treatment which can result in fatalities. Treatment of asymptomatic women has been shown not to be of value. Guise *et al* has shown that routine screening and treatment of asymptomatic mothers for microbes associated with an increased frequency of preterm birth did not reduce adverse pregnancy outcome and preterm delivery (Guise, *et al*., 2001).

To compound the above difficulties, current tests to detect neonatal infection are not accurate; reference ranges for neonatal neutrophil counts has been shown to have a sensitivity of only 77% and has a poor predictive value of the total cell count and differential (Manroe, *et al*., 1979). The frequently used CRP levels have a sensitivity and specificity of 80% and 92% respectively (Berger, *et al*., 1995). The gold standard for diagnosis of bacterial sepsis is blood cultures, but it has certain drawbacks in the neonate; false negatives are common in newborns, serial blood samples may be contraindicated especially in a preterm newborn and cultures may be negative as only very small quantities of blood can be used (Isaacman, *et al*., 1996).

The early clinical signs of neonatal sepsis are variable and non specific and hence the diagnosis is difficult as routine laboratory markers, such as CRP is of limited value. Early diagnosis is imperative as prompt treatment with antibiotics improves outcomes. In early onset GBS the infection is acquired by the fetus *in utero* and hence neonatally symptoms of infection manifest within 3 days of birth and usually within the first 12 hours. Studies have shown with clarity that immediate introduction of antimicrobials is essential as most deaths associated with late-onset (occurring after 3 days of age) infections in low birth weight infants occur during the first few

days after sampling for blood culture (Stoll, *et al*., 2002b). On the contrary, indiscriminate use of antibiotics with no objective test to prove or disprove the presence of infection in the neonate is suboptimal. Screening for maternal infection has its down falls as exemplified by the maternal Group B streptococcus screening programmes in the UK and USA. Lin et al found approximately 10% of antenatally Group B Streptococcus negative women showed intrapartum carriage and missed antibiotic prophylaxis, whereas approximately 50% of antenatally Group B Streptococcus positive women were negative intrapartum and received intrapartum antibiotics (Lin, *et al*., 2011). A retrospective review by Puopolo et al found that the majority of cases of early onset Group B Steptococcal disease occurred in neonates whose mothers were screen negative (Puopolo, *et al*., 2005).

Early detection of infection to facilitate start of antimicrobials is hampered by the lack of a reliable diagnostic test. Furthermore lack of a reliable diagnostic test makes the decision about antimicrobial therapy inaccurate (Krediet, *et al*., 1992; Benitz, *et al*., 1998; Ronnestad, *et al*., 1999). It is therefore accepted common practice that antimicrobial therapy is started on the basis of clinical signs. However, this indiscriminate use results in exposure of premature and low birth weight infants to side effects of the drugs and also results in multi drug resistant strains, increases risk of invasive fungal infections as well as increased hospital costs (Murray, 1994).

Antibiotics have often been shown to be ineffective in treating preterm labour (Van Den Broek, *et al.,* 2009). Additionally evidence has accumulated on the adverse fetal effects of antibiotics to treat preterm labour with or without rupture membranes (Shennan and Chandiramani, 2008). The ORACLE I randomised controlled trial found that co-amoxiclav usage in PPROM is associated with neonatal necrotising enterocolitis. (Kenyon, *et al.,* 2001). Follow-up of participants in the found ORACLE trial an increased risk of CP at 7 years in the children of women with intact

membranes who received antibiotics for spontaneous preterm labour [odds ratio 1.93 (95% confidence interval 1.21 to 3.09) for erythromycin and 1.69 (1.07 to 2.67) for co-amoxiclav] (Kenyon, *et al.,* 2008). Increased bacterial lysis effected by the antibiotic could amplify the inflammatory response mounted by the innate immune system, thereby increasing the fetal exposure to inflammatory mediators with subsequent multisystem organ damage (Holzheimer, 2001).

Furthermore very little is known about the response of CBDCs to maternal/intrauterine infections and the factors modulating the responses. Research investigating the fetal responses to infectious stimuli prior to birth as evidenced in the CB changes is essential to our understanding of the molecular basis of the clinical effects which in turn is essential for instigating efficient curative and preventative strategies.

Thus, it is recognised that novel and reliable infection markers are needed to improve the prompt diagnosis of sepsis in premature infants. Tests based on immunological changes in response to infection have recently been proven to be advantageous over traditional tests. Premature infants are significantly more susceptible to infections. As discussed above the overall reported incidence of neonatal sepsis leading to neonatal death is 10% (Stoll, *et al*., 1998), but in infants with birth weight less than 1000g, the incidence is 16-21% and correlates inversely with birth weight (Stoll, *et al*., 2002b). Hence immunological tests have an advantage as they can discriminate between changes due to prematurity and infection and can identify the premature infant at risk. Testing with IL-8 levels was found to reduce unnecessary antibiotic therapy for newborn bacterial infants in a cost-effective manner by 73% (Franz, *et al*., 1999). Additionally no phenotypic changes consistent with inflammation have been detected in maternal immune cells during FIRS, while inflammatory changes have been detected consistently in fetal

granulocytes and monocytes (Kim, *et al*., 2009). Naccasha *et al* has shown that pregnant women with acute infection upregulate granulocytic and monocytic markers (Naccasha, *et al*., 2001). Thus FIRS does not seem to trigger a proinflammatory maternal immune response similar to the immune response triggered by a maternal infection. Hence analysis of CB would give an accurate depiction of the fetal immune status as opposed to analysing maternal blood.

Cell surface markers, chemokines and cytokines and adhesion molecules have an advantage over traditional blood counts and cultures as their levels change early in the infective process, and thus they are promising diagnostic markers. Until recently their usefulness was limited to their assessment by ELISA performed in batches leading to delays in obtaining results. Advances in flow cytometric technology have resulted in quick and reliable detection of cell surface antigens on cells. Tests can be done on an ad-hoc basis and within a turnaround time of 4 hours, thus preventing compromise in the clinical care of the neonate (Ng and Lam, 2006). An additional major advantage is the requirement of only a minimal volume of blood sample (0.05ml of whole blood) enabling CB samples to be analysed, which provides information on the in utero infective status of the fetus and is the earliest test available other than analyses of cordocentesis samples. Thus developing future tests to analyse the cellular changes in CB in response to infection seem to be the logical solution to the above problems.

1.6 HYPOTHESIS AND AIMS

Hypothesis:

Fetal cord blood contains phenotypically and functionally distinct populations of dendritic cells, which mature with increasing gestational age and on exposure to intrauterine stressor stimuli.

Specific aims:

- To identify and characterise the phenotype and functions of the dendritic cell populations in cord blood.
- To evaluate the measurement of neutrophil activation markers in conjunction with clinical parameters for the identification of cord bloods exposed to stressors and thus validate the clinical classification of cord bloods exposed to stressors.
- To characterise the change in numbers of cord blood dendritic cell populations occurring with advancing gestational age and on exposure to intrauterine stressor stimuli.
- To analyse the expressions of the costimulatory markers CD40 and CD86 and production of Interleukin-12 on cord blood dendritic cell populations occurring with advancing gestational age and on exposure to intrauterine stressor stimuli.

1.7 EXPANDED HYPOTHESIS

- It was postulated that CB contained different populations of DC, the identification and phenotyping of which would serve to further our understanding of fetal immunology. The identification and phenotyping investigations were carried out on whole blood using monoclonal antibody labelling and multi colour flow cytometry. The functional studies included assessment of endocytic uptake of FITC labelled Dextran particles and assessment of lymphocytic stimulation by MLR. The results are presented in **chapter 3**.

- It was postulated that CBDC subpopulations changed with advancing age and exposure to stressors. Analysis was performed to assess the change in the expression of the maturation markers, CD45 and HLA-DR and the change in numbers of CBDC in the subpopulations with advancing age and exposure to intrauterine stressors. Analysis was done on whole blood using labelling with monoclonal antibodies and flow cytometry acquisition. The results are presented in **chapter 5**.

- It was postulated that the CBDC subsets changed their regulation of the costimulatory markers, CD40 and CD86 with advancing age as well as on exposure to stressors. It was also postulated that the production of the intracellular cytokine, IL-12 changed with advancing age as well as exposure to stressors. Analyses of whole blood with monoclonal antibody labelling and flow cytometer acquisition were performed on all the CB samples. The results are presented in **chapter 6**.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

Materials are listed under the headings of Buffers and Media, Reagents, Cytokines, Plastics and Antibodies. Where materials have been prepared in the laboratory, details of protocol and individual constituents are described.

2.1.1 Buffers and Media

Complete medium:

Complete medium used for dilution of blood consist of RPMI -1640 medium (Dutch Modification) (Sigma Chem Co, Poole, England) with added antibiotics (penicillin and streptomycin) (Sigma Aldrich Co, Poole, England) and L-glutamine (Sigma Aldrich Co, Poole, England). Antibiotics (0.25ml) and 0.25ml of L-glutamine were added to 23ml of the RPMI medium to make up a solution with 100μ/ml penicillin, 100μ/ml streptomycin and 20mM L-glutamine.

For cell culture: 10% w/v (10 ml) heat inactivated Fetal calf serum (FCS) (Tissue culture system, Berks, England) was added when the medium was used for cell culture.

For FACS (Fluorescence Activated Cell Sorting) cell sorting: 5ml of 10% w/v FCS was added when the medium was used for cell sorting in FACS.

Dulbecco's Phosphate Buffered Saline (PBS):

A balanced salt solution used in cell culture, it serves as an irrigating and diluting fluid while maintaining intra and extracellular osmotic balance of cells *in vitro* and also provides a

buffering system to maintain the medium within the physiological pH range (7.4-7.5) (Sigma Aldrich Co, Poole, England).

Ficoll-Paque^{TM} PLUS:

Ficoll-Paque PLUS is a sterile density gradient centrifugation medium for separation of PBMCs (peripheral blood mononuclear cell) from peripheral whole blood (Pharmacia Biotech AB, Uppsala, Sweden). The density of Ficoll-paque solution is 1.076-1.078, it is stored at 4 ^{0}C and warmed to room temperature prior to use.

Flow cytometry (FACS) buffer:

Cells prepared for flow cytometry were washed and re-suspended in FACS buffer.

PBS containing : 2% FCS

: 0.02% Sodium Azide (NaN_3) (BDH, Poole, England)

: 1mM EDTA (Sigma Aldrich Co, Dorset, England)

10ml FCS, 0.1gram (gm) NaN_3 and 0.18gm EDTA were added to 500ml volume of PBS to make up the FACS buffer.

Separation medium:

PBS containing 5% FCS and 1mM EDTA.

Metrizamide:

DCs were enriched from cord and peripheral whole blood over a 14.5% w/v metrizamide gradient (Sigma, Dorset, England). Metrizamide (7.25gm) is weighed out in a 50ml falcon tube

(Becton Dickinson, UK) and 40ml of HEPES-buffered RPMI-1640 at room temperature was added in 15ml stages, till all metrizamide is dissolved. Once dissolved, the volume was made up to 45ml with the RPMI medium at room temperature, divided into 2ml aliquots and stored at -20 ^{0}C. FCS (5ml) was added to the above solution when used for culture and the suspension was filter sterilised with a 0.2 (micrometer) µm filter (Nalgene, Rochester, NY, USA).

MiniMACS Buffer:

PBS containing 4% Bovine Serum Albumin (BSA) (Equitech Bio, USA) and 2mM EDTA was used as a buffer for heterogeneous cell suspensions during positive selection of human cells.

For 50ml of MiniMACS buffer, 0.25gm BSA and 0.04gm of EDTA were added to PBS and the volume made up to 50ml. The suspension was filter sterilised with a 0.2µm Nalgene filter, stored at 2^0 C – 8^0 C, and reused after filtering for up to one month.

Leucoperm A and B:

Reagent A: Fixation medium (contains Formaldehyde 10%)

Reagent B: Permeabilisation medium (contains Saponin)

Leucoperm reagents were used for fixing cells in suspension with Reagent A and then permeabilising the cells with Reagent B during intracellular cytokine staining (Serotec, UK). This procedure gives antibodies access to intracellular structures leaving the morphological scatter characteristics of the cell surface intact.

RPMI -1640 medium (Dutch Modification):

Tissue culture medium containing sodium salt buffers (Sigma Chem Co).

2.1.2 Reagents

Antibiotics:

Anti microbial supplements of Penicillin and Streptomycin (Sigma Aldrich Co) was used to ensure sterility in cell culture media. Sterile filtered and stored in aliquots at -20^0 C.

Bovine Serum Albumin (BSA):

BSA is also known as "Fraction V" and is a serum albumin protein used as a nutrient in cell culture (Equitech Bio).

CaliBRITE^{TM} beads:

CaliBRITE beads simulate unstained cells and cells that have been labelled with fluorochrome conjugated antibodies and they are used to adjust the instrument settings before cell samples are run on the flow cytometer (BD Biosciences, Becton Dickinson, USA).

EDTA:

Ethylenediaminetetraacetic acid chelates metal ions to prevent clustering and was used as a component in FACS buffer and stored at room temperature (Sigma Aldrich Co).

Fetal Calf Serum (FCS):

Fetal calf serum was used as a supplement for cell culture media as a source of nutrition. It blocks non specific binding during antibody labelling. Storage was done in aliquots at -80^0 C (Tissue culture system).

FITC-dextran:

Fluorescein-isothiocyanato-dextran is the polysaccharide, dextran labelled with fluorescent dye and used to assay the endocytic activity of DC (Sigma, Poole, England). It was used in concentrations of 1mg/ml.

Flow-Count™ Fluorospheres:

Flow-Count™ Fluorospheres are a suspension of fluorescent beads used to determine absolute counts on the flow cytometer (Beckman Coulter, USA). Each bead contains a dye which has a fluorescent emission range of 525nm to 700nm.

L-glutamine:

Essential amino acid used as a nutritional supplement in cell culture media (Sigma Aldrich Co). Storage was done in aliquots at -20^0 C.

Monensin:

Monensin is a protein transport inhibitor used to enhance intracellular cytokine staining signals by blocking transport processes during cell activation leading to the accumulation of most cytokines at the Golgi Complex/Endoplasmic Reticulum (Sigma, MO, USA). The stock concentration of monensin has a concentration of 41.57µg/5ml and is stored at 4° C. To 1ml of complete medium 7.5µl of the above monensin stock solution is added. 100µl of the above dilution is added to 900µl of medium for a working concentration of 1 in 10 dilution of which 50 µl is added to each tube for intracellular cytokine staining.

Optilyse C:

OptiLyse C is an erythrolytic reagent used for lysing human RBCs following direct immunofluorescence staining of whole blood for flow cytometry (Immunotech, Marseille).

Paraformaldehyde (1%):

Paraformaldehyde (1%) is a fixative for surface immunophenotyping. Paraformaldehyde (1 gm) (BDH chemicals, Poole, England) was added to 100ml of PBS and heated at 70^0 C in a fume hood, till the solution was clear. A pH of 7.4 was achieved with 0.1M Sodium hydroxide (NaOH) or 0.1M Hydrogen chloride (HCL) as required. Storage was at 4^0 C in a foil wrapped bottle for no more than one month.

Sodium Azide:

Sodium azide is a conservant for preservimg antibodies and prevents microbial contamination. It is added to an antibody preparation to a final concentration of 0.02% w/v (BDH).

[H^3]-Thymidine:

[H^3]-Thymidine (concentration of 1µg/ml) (specific activity 2 Ci/mmol, Amersham International, Buckinghamshire, UK) is a precursor of DNA and is incorporated into new DNA in proliferating cells. Metabolic incorporation of tritrated thymidine into cellular DNA is used to monitor rates of DNA synthesis and cell proliferation and used in MLR to assess stimulatory capacity of DC.

Trichloroacetic acid 5%

5% trichloroacetic acid was used in MLR (Fisons scientific equipment, Loughborough, UK). The mat filters were washed with 5% trichloroacetic acid to precipitate the cellular DNA prior to imaging.

2.1.3 Anti-cytokine antibodies

Anti-IL-12:

PE conjugated Mouse Anti-Human IL-12 p70 heterodimer (BD Pharmingen, San Diego, USA) (Clone: C11.5, Isotype: Mouse IgG1).

Biologically active IL-12 is secreted by activated B lymphocytes and macrophages as a 70 kD heterodimeric glycoprotein comprised of disulfide-bonded 35 kD (p35) and 40 kD (p40) subunits. It was used to assay intracellular DC levels of IL-12. Storage was done undiluted at 4° C and protected from prolonged exposure to light.

2.1.4 Plastics

The following commercially available plastics were used during experiments:

Vacutainer^{TM} tubes containing Heparin – Becton Dickinson, UK.

FACS tubes – 5 ml polystyrene round bottom test tubes - Becton Dickinson, UK.

Separation tubes – 100 x 16 mm conical based screw top tubes – Sterilin.

Tube for Ficoll separation – 50 ml Falcon screw top tube - Becton Dickinson, UK.

Syringe Filter – Nalgene, 0.2µm, 25 mm surfactant free, cellulose acetate membrane – (Cat no-190-2520. Rochester, NY, USA).

T25 flasks – Canted neck tissue culture flasks - Becton Dickinson, UK.

Terasaki plates – Tissue culture plates with 60 wells for hanging drop cultures – Nunc, Life technologies, Scotland.

Mat filters – for harvesting Thymidine pulsed cells - Skafron instruments, Suffolk, UK.

2.1.5 Antibodies

The antibody clones used, their fluorochrome conjugates, isotypes and suppliers for surface marker staining are listed in Table 2.I.

The fluorochrome conjugates used were: FITC, PE (Phycoerythrin), PE-indotricarbocyanine (PE-Cy5), Allophycocyanin (APC) and phycoerythrin-cyanine5 conjugate (Cy-Chrome or PC5).

Table 2.I Monoclonal antibody list

Surface Marker	Clone	Conjugate	Isotype	Supplier
BDCA2	AC 144	FITC	Mouse IgG1	Miltenyi Biotec
CD11b	ICRF44 (44)	PE	Mouse IgG1	Becton Dickinson
CD11c	KB90	FITC	Mouse IgG1	Dako
CD11c	S-HCL	PE	Mouse IgG2b	Becton Dickinson
CD11c	Bly6	Cychrome	Mouse IgG1	Pharmingen
CD123	7G3	PE	Mouse IgG1	Becton Dickinson
CD14	TUK4	Cy5	Mouse IgG1	Serotec
CD14	RMO52	PC5	Mouse IgG2a	Immunotech
CD16	NKP15	FITC	Mouse IgG1	Becton Dickinson
CD16	3G8	Cychrome	Mouse IgG1	Pharmingen
CD16	3G8	PC5	Mouse IgG1	Immunotech
CD19	SJ25-C1	Cy5	Mouse IgG1	Serotec
CD19	J4.119	PC5	Mouse IgG1	Immunotech
CD3	UCHT1	FITC	Mouse IgG1	Pharmingen
CD3	UCHT1	PE	Mouse IgG1	Pharmingen
CD3	UCHT1	Cychrome	Mouse IgG1	Pharmingen
CD3	UCHT1	PC5	Mouse IgG1	Immunotech
CD3	SK7	APC	Mouse IgG1	Pharmingen
CD34	581	Cychrome	Mouse IgG1	Pharmingen
CD4	SK3	FITC	Mouse IgG1	Becton Dickinson
CD4	RPA-T4	PE	Mouse IgG1	Becton Dickinson
CD40	1590F	FITC	Mouse IgG1	Serotec
CD45	2D1	FITC	Mouse IgG1	Becton Dickinson
CD45	H130	PE	Mouse IgG1	Pharmingen
CD45RA	HI 100	FITC	Mouse IgG1	Becton Dickinson
CD45RO	UCHL1	PE	Mouse IgG2ak	Pharmingen
CD56	B159	Cychrome	Mouse IgG1	Pharmingen
CD56	N901 (NKH1)	PC5	Mouse IgG1	Immunotech
CD8	SK1	FITC	Mouse IgG1	Becton Dickinson
CD8	SK1	PE	Mouse IgG1	Becton Dickinson
CD8	HIT8A	Cychrome	Mouse IgG1	Pharmingen
CD8	B9.11	PC5	Mouse IgG1	Immunotech
CD8	SK1	APC	Mouse IgG1	Becton Dickinson
CD80	L307.4	FITC	Mouse IgG1	Pharmingen
CD83	HB15e	FITC	Mouse IgG1	Ancell
CD86	1118F	FITC	Mouse IgG1	Serotec
HLA-DR	G46-6	APC	Mouse IgG2a	Pharmingen
γ1	X40	FITC	Mouse IgG1	Becton Dickinson
γ1	X40	PE	Mouse IgG1	Becton Dickinson
γ1	MOPC-21	Cychrome	Mouse IgG1	Pharmingen
γ1	679.1Mc7	PC5	Mouse IgG1	Immunotech
γ2a	G155-178	PE	Mouse IgG2a	Pharmingen
γ2a	X39	Cychrome	Mouse IgG2a	Pharmingen
γ2a	U7.27	PC5	Mouse IgG2a	Immunotech
γ2a	X39	APC	Mouse IgG1	Becton Dickinson
γ2b	27-35	PE	Mouse IgG2b	Pharmingen

CD (cluster of differentiation) antigens are used to designate the complex pattern of surface antigens found on immunologically active cells as they develop from precursor cells to functionally mature forms. The above antibodies bind to designated antigens facilitating identification of the antigen.

2.2 METHODS

2.2.1 Density gradient separation of DCs

2.2.1.1 Ficoll separation of PBMCs

Ficoll-paque technique: A density-gradient centrifugation technique to separate lymphocytes from other cellular elements in the blood.

PB was drawn into heparinised, green top vacuum tubes and diluted 1:2 with complete medium and layered over Ficoll-paque gradient in a 50ml Falcon tube. Five ml of Ficoll-paque/10ml of diluted blood was used. The above suspension was centrifuged for 30 minutes at 1700 rpm (650G) in a bench top centrifuge (Mistral 3000i). RBC and granulocytes pass through the Ficoll leaving the mononuclear cells as a band at the interface between the serum and the Ficoll (Figure 2.1). RBC and polymorphs sediment at the bottom. PBMCs were removed from the interface with a pipette, washed three times with complete medium (1500 rpm for 5 minutes) and resuspended in 5ml of complete medium. Following the final wash the supernatant was discarded and the cell pellet was resuspended in 5ml of complete medium. The total number of mononuclear cells in suspension was calculated by Trypan blue viability count.

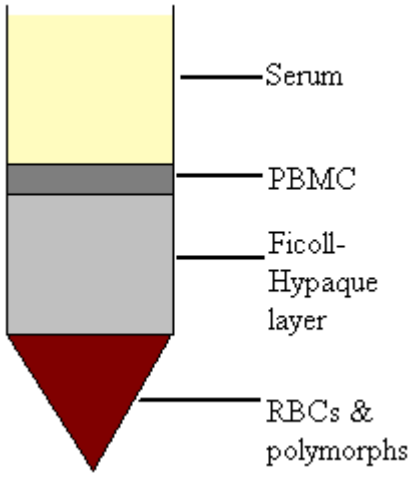


Figure 2.1 Ficoll density gradient separation of PBMCs

The PBMCs form a dark layer between the serum and the Ficoll layer facilitating harvesting of cells

2.2.1.2 Metrizamide enrichment of DCs

The PBMC cell suspension obtained by the above separation was made up to the required volume with complete medium (with added FCS) and added to T25 flasks (5ml in each flask) using sterile techniques. The flasks were left in the Carbon di oxide (CO_2) incubator overnight with loosened caps. After 12 hours of incubation the base of the flasks were washed with complete medium and the cultured PBMCs were pipetted out. One aliquot of metrizamide each was poured into test tubes and 5 ml of cultured PBMC suspension was layered gently onto the metrizamide. The tubes were centrifuged for 10 minutes at 1700 rpm (650G) in a bench top centrifuge (Mistral 3000i). The DCs form a dark coloured layer at the interface between the culture medium and metrizamide and the lymphocytes sediment at the bottom of the tubes

(Figure 2.2). The DC layer was pipetted out, washed twice with complete medium and resuspended in 1 ml of complete medium.

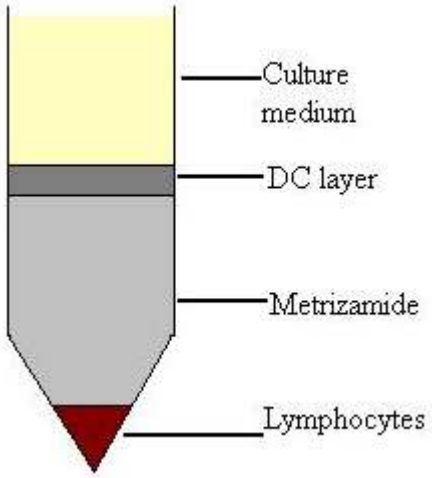


Figure 2.2 Metrizamide density gradient separation of DCs

The DCs form the dark layer between the culture medium and the metrizamide thus providing an enriched layer for harvesting

2.2.2 Labelling of DCs

2.2.2.1 Labelling of Whole blood DCs

Fetal venous CB and adult peripheral venous blood samples were obtained anti-coagulated in heparinised tubes. Both adult blood and CB samples were labelled in parallel to minimise inter experiment variations. Previous work had developed and validated a technique which permits the use of the small volume of whole blood available from preterm neonates (Mason, *et al*., 2005). Analysis of whole blood eliminates the loss of cell populations associated with cell separation

procedures, and provides data which accurately reflect the *in vivo* situation. Undiluted blood was used for staining of DC due to the inherent paucity of events.

Whole blood (100µl) was added to round bottomed tubes along with the appropriate volumes of flourochrome conjugated monoclonal antibodies as required (5-10µl). The contents of the tubes were mixed gently and incubated in the dark at room temperature for 15 minutes. Red cells were lysed by adding 500µl Optilyse C, and the cell suspension was incubated in the dark at room temperature for a further 20 minutes. Optilyse C (500µl) was added to adult and term fetal CB, but 700µl was added to preterm CB. The increased volumes were necessary as term CB RBCs are more resistant to lysis than adult RBCs, and preterm CB RBCs more resistant to lysis than term CB RBCs (Serrani, *et al*., 1989; Bautista, *et al*., 2003).

Subsequent to the lysis, the tubes were topped up with FACS buffer solution and centrifuged at 300g (1190 rpm) for 5 minutes. After centrifuging, the supernatant was poured off, the cells resuspended in FACS buffer, centrifuged for a further 5 minutes, drained and resuspended. Paraformaldehyde 1% was used to fix the cells, and 0.3ml was added to each tube. The tubes were covered with foil and stored in the refrigerator at 4 ^{0}C, until they were acquired on the flow cytometer. The tubes were covered in foil to slow down the photo-bleaching of the fluorophore colours or in other words to slow down the fading process of the fluorescent colours.

2.2.2.1.1 Labelling for DC phenotype

Table 2.II Protocol for DC phenotype labelling

BLOOD	ADULT	FL1 [FITC]	FL2 [PE]	FL3 [PC5]	FL4 [APC]	FETUS	BLOOD
	1	CD11c	CD45	LIN-CK	HLA-DR	9	
A	2	γ_1	γ_1	LIN-CK	HLA-DR	10	**F**
D	3	γ_1	γ_1	γ_1-γ_{2a}	γ_{2a}	11	**E**
U	4	CD8	—	—	—	12	**T**
L	5	—	CD8	—	—	13	**U**
T	6	—	—	CD8	—	14	**S**
	7	—	—	—	CD8	15	
	8	—	—	—	—	16	

Antibody labelling for DC phenotyping and identification performed with parallel tubes of adult and fetal blood samples

Dilutions (see **section 2.3.4** Determination of antibody dilutions)

HLA-DR =1:4 = 10µl Ab to 30µl medium and add 5µl into each tube.

Linear Cocktail (LIN-CK) = CD3 + CD14 + CD16 [1:25 DIL] + CD19 + CD34 + CD56 [5µl of each Ab or 30µl of cocktail mixture into each tube]

FITC = 10µl into each tube.

γ_1 PC5 = 25µl into each tube. γ_{2a} PC5 = 5µl into each tube.

All other Ab's = 5µl into each tube.

The paraformaldehyde fixed cell suspension was acquired in the flow cytometer (FACScalibur, Becton-Dickinson) using the four colour acquisition as given in the table above.

Lineage cocktail conjugated with PC5 consisted of CD3 to label T cells, CD14 to label monocytes, CD19 to label B cells, CD16 to label granulocytes, CD56 to label NK cells and CD34 for the stem cells. Five µl of each antibody was added to each tube. Isotype controls were used in parallel tubes to establish the limits of cell subset regions and CD8 labelled tubes with all the above fluorescent dyes were acquired in the respective channels for compensation. A single tube without any antibodies was also labelled to ensure changes seen were due to the phenotype and not due to any other intrinsic factor such as the low level of fluorescence inherent in all living cells known as autofluorescence.

To assess whether the DC subpopulation of interest has a plasmacytoid or myeloid phenotype, blood was labelled with FITC conjugated CD45RA, BDCA2, CD33 and CD13 and acquired in FL-1 channel and PE conjugated CD123 was acquired in the FL-2 channel.

2.2.2.1.2 Labelling for Costimulatory markers

Table 2.III Protocol for costimulatory marker labelling

BLOOD	ADULT	FL1 [FITC]	FL2 [PE]	FL3 [PC5]	FL4 [APC]	FETUS	BLOOD
	1	CD11c	CD45	LIN-CK	HLA-DR	16	
	2	CD11c	CD45	LIN-CK + CD11c	HLA-DR	17	
A	3	—	—	LIN-CK	γ2a	18	**F**
	4	—	—	γ1-γ2a	HLA-DR	19	
D	5	CD8	—	—	—	20	**E**
	6	—	CD8	—	—	21	
U	7	—	—	CD8	—	22	**T**
	8	—	—	—	CD8	23	
	9	—	—	—	—	24	
L	10	CD40	CD11c	LIN-CK	HLA-DR	25	**U**
	11	γ1	CD11c	LIN-CK	HLA-DR	26	
	12	CD40	CD45	LIN-CK + CD11c	HLA-DR	27	
T	13	γ1	CD45	LIN-CK + CD11c	HLA-DR	28	**S**
	14	CD86	CD11c	LIN-CK	HLA-DR	29	
	15	CD86	CD45	LIN-CK + CD11c	HLA-DR	30	

Labelling with antibodies for analyses of CD40 and CD86 markers. Antibody dilutions were as described above

The paraformaldehyde fixed cell suspension was acquired in the flow cytometer in the four colour channels as given in the table above.

To free a fluorescence channel on the four colour flow cytometer in order to enable a detailed analysis of the $CD11c^-$ subset, the anti CD11c antibody was included in the lineage cocktail.

Labelling was performed with CD40 and CD86 in parallel tubes. Isotype controls and the unlabelled tube were used as described above.

2.2.2.1.3 Labelling for intracellular cytokines

Table 2.IV Protocol for intracellular cytokine labelling

BLOOD	**ADULT**		**FL1 [FITC]**	**FL2 [PE]**	**FL3 [PC5]**	**FL4 [APC]**	**FETUS**		**BLOOD**
	No Monensin	With Monensin					No Monensin	With Monensin	
A	1	3	CD11c	IL-12	LIN-CK	HLA-DR	14	16	**F**
	2	4	CD45	IL-12	LIN-CK +CD11c	HLA-DR	15	17	
D	5		CD11c	IL-12	LIN-CK +CD11c	HLA-DR	18		**E**
	6		γ1	—	LIN-CK	HLA-DR	19		
U	7		—	—	LIN-CK	γ2a	20		**T**
	8		—	—	γ1-γ2a	HLA-DR	21		
L	9		CD8	—	—	—	22		**U**
	10		—	CD8	—	—	23		
T	11		—	—	CD8	—	24		**S**
	12		—	—	—	CD8	25		
	13		—	—	—	—	26		

Labelling with antibodies for analyses of intracellular cytokine, IL-12. Antibody dilutions were as described above

Whole blood (50µl) was added to round bottomed tubes, in two parallel sets of adult PB and fetal CB. RPMI medium (50µl) was added to all tubes except the isotype and compensation labelling tubes to which 100µl of the medium was added. 25µl of the 1 in 10 dilution of monensin was added to alternating tubes, while the rest of the tubes, including the isotypes and compensation controls were incubated without monensin. All the tubes were incubated for 4 hours at 37 ^{o}C and 5% CO_2. After the 4 hour incubation period surface labelling was done by adding the required antibodies and labelling was done in two sets of parallel tubes as described above. After incubation with the antibodies for 15 minutes in the CO_2 incubator, Optilyse C (500µl) was added to lyse the RBCs. The tubes were mixed with the vortex and incubated for 20 minutes in the dark. Subsequently they were washed with FACS buffer and 100µl of Leukoperm A was added to each tube to fix the cells. After 15 minutes incubation with Leukoperm A, the cells were washed again, resuspended and 100µl of Leukoperm B was added to each tube. Leukoperm B permeabilises the cell membrane, so as to allow the antibodies to penetrate and stain the intracellular cytokines. Along with Leukoperm B, 5µl of IL-12 was added to the required tubes and incubated again for another 30 minutes. After centrifuging, the supernatant was poured off and the cells resuspended in FACS buffer, centrifuged for a further 5 minutes, drained and resuspended. Paraformaldehyde 1% was used to fix the cells, and 0.3ml was added to each tube. The tubes were covered with foil and stored in the refrigerator, until they were acquired on the flow cytometer in the four channels as described in the table above.

Isotype controls and the unlabelled tube were used as described above.

2.2.2.1.4 Labelling for Neutrophilic CD11b and CD16

Table 2.V Protocol for Neutrophilic CD11b and CD16 labelling

BLOOD	ADULT	FL1 [FITC]	FL2 [PE]	FL3 [PC5]	FL4 [APC]	FETUS	BLOOD
	1	CD16	CD11b	LIN-CK	HLA-DR	9	
A	2	CD16	γ2a	LIN-CK	HLA-DR	10	**F**
D	3	γ1	γ1	γ1-γ2a	γ2a	11	**E**
U	4	CD8	—	—	—	12	**T**
L	5	—	CD8	—	—	13	**U**
T	6	—	—	CD8	—	14	**S**
	7	—	—	—	CD8	15	
	8	—	—	—	—	16	

Labelling with antibodies for analysis of the neutrophilic marker, CD11b and CD16. Antibody dilutions were as described above

The paraformaldehyde fixed cell suspension was acquired in the flow cytometer in the four colour channels as given in the table above. Isotype controls and the unlabelled tube were used as described above.

2.2.2.2 Labelling of density gradient separated DCs

The density gradient separated DCs from CB were used in functional assays. The functional capacity was assayed by labelling with FITC-dextran (see section **2.3.2.1** for protocol) and also by culturing as stimulators in MLR after sorting on the flow cytometer (see section **2.3.2.2** for protocol).

2.2.3 Flow Cytometry

The Becton-Dickinson FACSCalibur is a benchtop flow cytometer that can simultaneously analyse four fluorescent parameters using a dual argon and diode laser system (488nm argon laser and a 635nm diode laser). The detailed phenotypic properties of each cell can be established by using monoclonal antibodies to specific cell surface and intracellular antigens **(Table 2.I)**. The antibodies were conjugated to a fluorescent dye that emits light of a known wavelength when excited by laser energy. The four fluorochromes used were FITC, PE, Cy-Chrome or PC5 and APC. The peak emission of FITC is approximately 515nm, it absorbs at 488nm (argon laser) and is detected in the FL-1 channel. The peak emission of PE is approximately 580nm, it absorbs at 488nm (argon laser) and is detected in the FL-2 channel. PC5 is an energy transfer dye which consists of the indotricarbocyanine dye, Cy5 coupled to PE. Its peak emission is approximately 630nm, it absorbs at 488nm (argon laser) and is detected in the FL-3 channel. APC emits at about 680nm, and can be excited by either a dye laser (595-605nm), or the Helium Neon laser at 633nm and is detected by the FL-4 channel. The emission

intensity detected in each channel is proportional to the amount of antibody labelling on that cell thus quantifying the levels of expression of the target antigen on that cell.

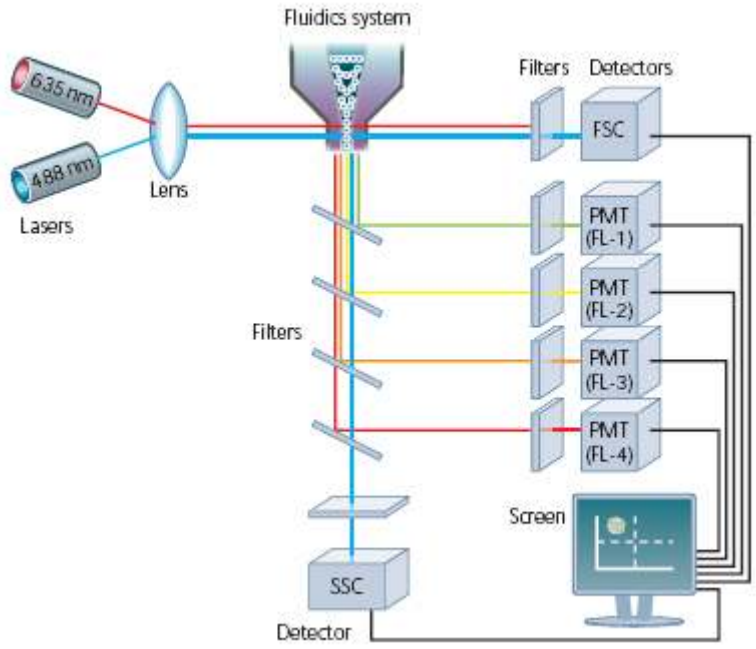


Figure 2.3 Schematic overview of a flow cytometric setup (Rahman, M. (2006). An introduction to flow cytometry. Serotec Ltd. Oxford. UK.)

The main components of the flow cytometer are shown: the laser system, the fluidics system and the filters with the detectors

2.2.3.1 Data Acquisition

The paraformaldehyde fixed cell suspension was acquired in the flow cytometer. The nozzle tip probe draws up the cell sample and the cells are passed singly through the path of the laser beam which takes measurements on cell size, granularity and complexity. This process is called

hydrodynamic focussing. For rare cell types, such as the DC a large number of events at a relatively low acquisition rate are used in order to improve accuracy. Prior to acquisition, the Flow Check™ flourospheres were run through to ensure the laser was aligned and the fluidics system working correctly. Data acquisition used the CellQuest software.

2.2.3.1.1 Calculation of cell numbers

Prior to acquisition in the flow cytometer 20µl of a prenumbered bead solution of a known concentration (Beckman Coulter flow count™ flourospheres) was added to each sample of cell suspension. Enumerating the bead events enabled accurate calculation of cell numbers in each cell subset, per µl of blood.

2.2.3.1.2 DC sorting

The BD FACSCalibur cell sorting option allows identification and isolation of a population of interest. Initially a sort gate was created with plots drawn around the required regions. After the population of interest was gated, as the cells passes through the laser the cytometer electronics will establish if it is a cell of interest based on the sort gate characteristics. During acquisition once the cell is established as a target cell a unique catcher tube mechanism moves in and out of the sample core stream at a rate of roughly 300 times per second to capture target cells and direct them to a collection tube for further processing. The sort settings used in this study were low flow rate and 2000 events/sec. Exclusion mode was used as it yields high purity sample. After

the sorting, the collection tubes were centrifuged at 300g for 5 minutes to concentrate the cells and the cell pellet was resuspended in 0.5 ml of complete medium on ice.

2.2.3.1.3 Compensation

In a four colour flow cytometer, the emission profiles of the different fluorochromes coincide and overlap. Thus measurements of the true fluorescence emitted by each fluorochrome can be inaccurate. Fluorescence compensation calculates how much interference a fluorochrome will have in a channel that was not assigned specifically to measure it. The interference is calculated in percentages and corrected. List mode data from all events were collected with online spectral compensation. Lymphocytes stained with CD8 antibody for each of the four fluorochromes (FITC, PE, PC5 and APC) were used as the spectral standards to compensate for each flourochrome and were included in all experiments and in all labelling protocols. Spectral compensation was achieved by data transfer off line and analysed using the Color Compensation Toolbox using the WinList flow cytometry analysis software (WinList Version 5.0, Verity, Topsham, ME).

2.2.3.2 Data Analysis

Data from the flow cytometer was analysed using Winlist^{TM} 5.0 software (Verity Software House, Topsham, ME, USA) and performed offline.

2.2.3.2.1 Gating strategy

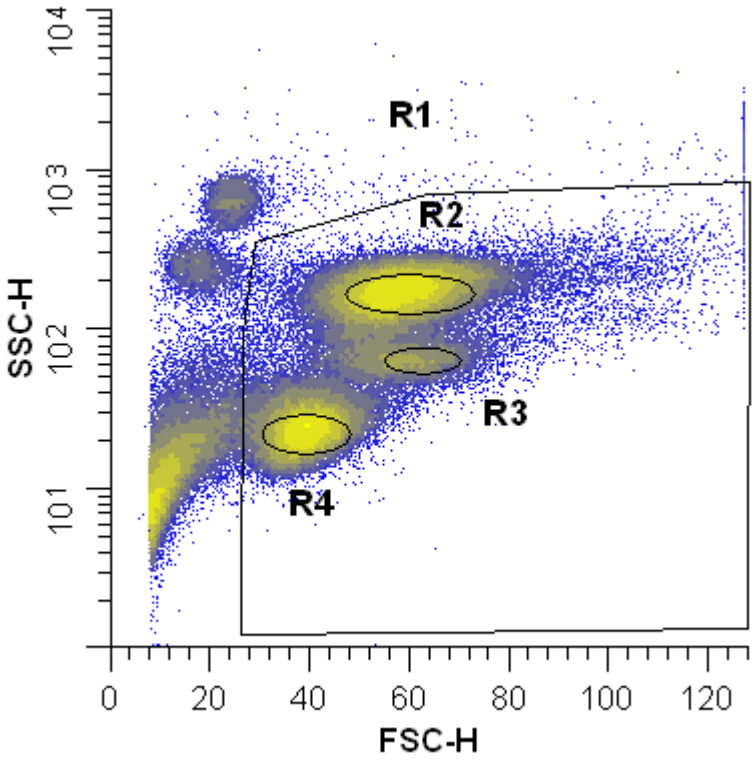


Figure 2.4 Viable cell region is identified on the side scatter and forward scatter histogram (R1 region)

The viable cells are identified in the following regions: R2 region – Granulocytes; R3 region – Monocytes; R4 region – Lymphocytes. Representative histogram of 40 experiments shown

Light that is scattered in the forward direction is collected by a lens known as the forward scatter channel (FSC) and consists of refracted laser light collected at low angles between 1^0 and 10^0. The intensity of the scattering denotes the particle's size. Light that is scattered at a 90^o angle to the excitation line is called side scatter is collected by a lens known as the side scatter channel

(SSC). Side scatter denotes the granular content and complexity within a particle. Both FSC and SSC are unique for every particle, and a combination of the two is used to differentiate different cell types e.g. lymphocytes have a low SSC (R4 region in Figure 2.4) and granulocytes have a high SSC (R2 region in Figure 2.4). These parameters are also used to distinguish between cellular debris and living cells as dead cells have lower FSC and higher SSC than living cells.

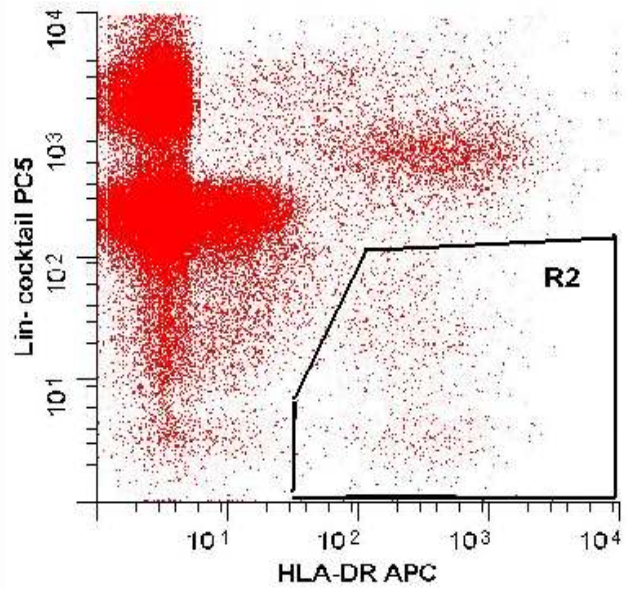


Figure 2.5 DC gating

The two parameter histogram shows the DCs as the HLA-DR positive cell population in the right lower quadrant (R2, Region 2). Representative histogram of 40 experiments shown

Initially the total viable cell region was delineated based on the FSC and SSC properties as described above. Identification of the DC region is done by drawing a region around the cell population positive for HLA-DR and negative/dim for LIN-CK and gated on the viable cell region (R2 in Figure 2.5).

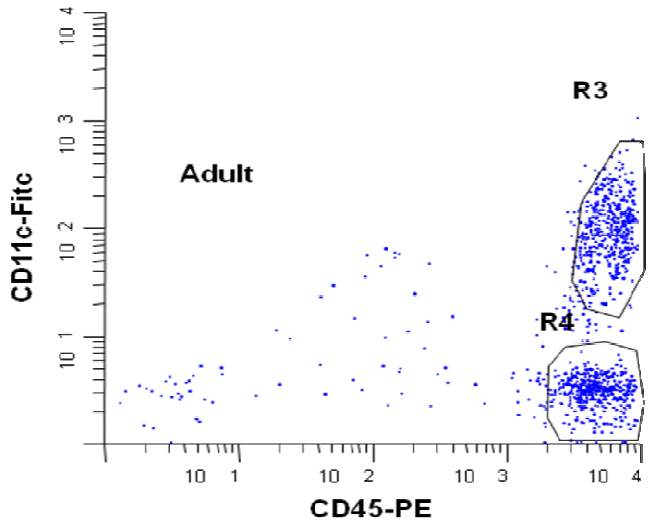


Figure 2.6 DC subsets were identified on the basis of CD11c and CD45 expression

DC subsets in adult blood were identified as $CD11c^{+}CD45^{+}$ (R3 region) and $CD11c^{-}$ $CD45^{+}$ (R4 region). Representative histograms of 40 experiments shown

Two populations of DCs can be seen in adult PB – the myeloid $CD11c^{+}CD45^{+}$ population (R3) and the plasmacytoid $CD11c^{-}$ $CD45^{+}$ (R4) population. As discussed in chapter 1, pDCs in humans express the surface markers CD123, BDCA-2 and BDCA-4, but do not express high levels of CD11c, which distinguishes them from conventional (myeloid) dendritic cells. As our flow cytometer had only four channels it was not possible to identify the pDCs by positive selection using CD123. Presence of CD11c positively identified the cDCs and their absence was used to identify the pDCs, thus keeping the usage of channels to the minimum to allow further detailed analysis of the DC subsets.

2.2.3.2.2 Enhanced normalised subtraction

The level of expression of a marker was determined by subtracting staining with an isotype-matched control antibody from staining with the specific reagent (Winlist user guide, 2001). This was achieved using the super-enhanced Dmax normalized subtraction (SED) method of Winlist 5.0. Also named as the KS statistic, it measures the maximum absolute difference or Dmax between two cumulative probability distributions. The data acquired by the flow cytometer is displayed in the form of a histogram. The frequency histogram is a graphical representation of the number of events occurring for each channel. The median values from both frequency histogram (test and control) distributions were linearised and the positive linearised median was divided by the control median. The intensity of the positive staining, termed the positive control intensity (PCI) ratio measures the ratio of linearised positive median to the linearised control median. Thus subtraction estimates the number of positive cells for the measured marker, in the test histogram.

The Kolmogorov-Smirnov (K-S) algorithm is used to determine the confidence interval with which one can make the assertion that two flow cytometric univariate histograms are different. The significance of a SED result can be tested by calculating the critical D value (D_{crit}) using the following equation: $D_{crit} = D_{max}/\sqrt{[(n1+n2)/(n1 \times n2)]}$ and validated using Kolmogov-Smirnov statistical tables. D_{max} is the maximum value between the test and control samples after the two histograms have been converted into cumulative normalised histograms, n1 is the number of events in the test sample, and n2 is the number of events in the control sample. Only positive values which give a D_{crit} with a $p < 0.05$ were accepted. Reversed histograms or p values >0.05 were taken as having zero expression.

Table 2.VI Kolmogorov-Smirnov statistic table

(D_{CRIT})	P-VALUE
1.0727	0.200
1.2238	0.100
1.3581	0.050
1.5174	0.020
1.6276	0.010
1.7317	0.005
1.8585	0.002
1.9526	0.001

The table gives the p-values to calculate the critical D_{max} (D_{crit}) (Watson, 1992)

2.2.3.2.3 Analysis for costimulatory marker assay

The percentage of DC expressing CD80 and CD86 was determined by subtracting staining with an isotype-matched control antibody from staining with the specific reagent. To free a fluorescence channel on the four colour flow cytometer in order to enable a detailed analysis of the $CD11c^-$ subset, the anti CD11c antibody was included in the lineage cocktail. This removed the $CD11c^+$ DCs from the DC gate staining, thus freeing up a channel. Freeing of one channel enabled the further delineation of $CD11c^-$ subsets with respect to their expression of CD40 and CD86 costimulatory markers (see section **2.3.2**).

2.2.3.2.4 Analysis for neutrophilic CD11b expression

Neutrophils were identified on their light scattering properties and the positive expression of CD16 antigen gated on all the viable cells. The expression of CD11b on the CD16 positive neutrophils was calculated by subtracting the isotype histogram from the histogram delineating the CD11b expression. As CD16 is a PIG-A anchored glycoprotein any deficiency of cell surface proteins as seen in paroxysmal nocturnal hemoglobinuria could result in the absence of CD16. Of the 29 samples analysed in this study none were negative for CD16.

2.2.3.2.5 Analysis for FITC-dextran endocytosis

Histograms of FITC-dextran were plotted for all the DC subsets and the mean value was subtracted from the mean value of the negative control tubes. FITC-dextran uptake was measured at 4^0C and at 37^0C. The uptake at 4^0C was subtracted from that measured at 37^0C to obtain the net mean fluorescence intensity (MFI) which represented the active endocytosis. FITC Dextran shows an increase with uptake which is temperature dependant. Thus at 4^0 C binding takes place and uptake is mostly inhibited. With rising temperatures internalisation increases and is optimal at 37^0 C. Additionally, as more time elapses the amount of internalised particles increases and thus the MFI is higher at 30 minutes compared to 5 minutes. Thus we measured uptake at 4 and 37^0C and at 5 and 30 minutes time.

2.2.4 Functional Assays

2.2.4.1 FITC-dextran endocytosis assay

Table 2.VII Protocol for FITC-dextran endocytosis assay

ADULT		**FL1 [FITC]**	**FL2 [PE]**	**FL3 [PC5]**	**FL4 [APC]**	**FETUS**	
37 ^{0}C	**4 ^{0}C**	**5 MINUTES**				**37 ^{0}C**	**4 ^{0}C**
1	**14**	FITC-dextran	CD11c	LIN-CK	HLA-DR	**27**	**40**
2	**15**	—	CD11c	LIN-CK	HLA-DR	**28**	**41**
3	**16**	FITC-dextran	CD45	LIN-CK+CD11c	HLA-DR	**29**	**42**
4	**17**	—	CD45	LIN-CK+CD11c	HLA-DR	**30**	**43**
30 MINUTES							
5	**18**	FITC-dextran	CD11c	LIN-CK	HLA-DR	**31**	**44**
6	**19**	—	CD11c	LIN-CK	HLA-DR	**32**	**45**
7	**20**	FITC-dextran	CD45	LIN-CK+CD11c	HLA-DR	**33**	**46**
8	**21**	—	CD45	LIN-CK+CD11c	HLA-DR	**34**	**47**
9	**22**	CD8	—	—	—	**35**	**48**
10	**23**	—	CD8	—	—	**36**	**49**
11	**24**	—	—	CD8	—	**37**	**50**
12	**25**	—	—	—	CD8	**38**	**51**
13	**26**	—	—	—	—	**39**	**52**

Labelling with antibodies (dilutions as before) for analysis of Dextran endocytosis

Adult and CB samples were labelled in parallel to minimise inter experiment variations. Whole blood (20ml) was Ficoll separated and the PBMCs resuspended with 100µl of complete medium in each tube. Half the tubes were incubated in a water bath at 37 ^{0}C and the other half at 4 ^{0}C on ice for 30 minutes to delineate the temperature dependant uptake. FITC-dextran solution (5µl of a final concentration of 1mg/ml) was added at 5 and 30 minutes. A parallel set of tubes were labelled without FITC-dextran and used as controls. All the tubes were washed twice and resuspended with cold FACS buffer and kept on ice to halt the endocytosis process. All the other conjugated antibodies were added as above and the tubes were incubated in the dark on ice for 15 minutes. The cell suspension was fixed with 300µl of 1% paraformaldehyde prior to acquisition on the flow cytometer. Isotype controls and the unlabelled tube were used as described above.

2.2.4.2 DC sorting and MLR

The stimulatory capacity of fetal DCs was assessed in an allogenic MLR using naïve T cells as responders. Heparinised tubes were used to collect the blood as the proliferative capacity of the lymphocytes is preserved in heparin (Weinberg**,** *et al.,* 1998).

2.2.4.2.1 Stimulator cells

To obtain stimulator DCs, PBMCs in fetal venous CB were Ficoll separated and resuspended in 5 ml of complete medium (see section **2.2.1.1** for protocol). The total number of PBMCs in suspension per ml was calculated by counting on the haemocytometer. The cell suspension was

diluted with 40 ml of complete medium and cultured overnight in T25 flasks. After the culture period, the low density cells (LDC) were separated out over a metrizamide gradient and resuspended in 1ml of complete medium (see section **2.2.1.2** for protocol). The total number of LDCs in suspension per ml was calculated by counting on the haemocytometer. One third of the cell suspension was removed to provide unlabelled DCs and serially diluted with complete medium to obtain four decreasing concentrations (3000 cells/10µl, 1000 cells/10µl, 300 cells/10µl and 100 cells/10µl). The rest of the DC cell suspension was washed with MiniMacs buffer and labelled on ice to prevent DC activation.

Table 2.VIII Protocol for labelling DC prior to sorting

Tube	FL2 [PE]	FL3[PC5]	Cells
1	HLA-DR	LIN-CK	All LDCs
2	—	—	Fetal lymphocytes
3	CD8	—	Fetal lymphocytes
4	—	CD8	Fetal lymphocytes

Labelling with antibodies for identification of the DC populations. Antibody dilutions were as described above

The cells were resuspended in 500µl of MiniMacs buffer. After labelling, a small volume (25µl) was removed to plate as labelled DCs in wells of a Terasaki plate. The volume removed was added to 500µl of complete medium and serially diluted to obtain four decreasing concentrations (3000 cells/10µl, 1000 cells/10µl, 300 cells/10µl and 100 cells/10µl). The rest of the volume of labelled DC cell suspension was sorted for CBDCs through the flow cytometer. The sorted DCs were spun in cold centrifuge and resuspended in 500µl of complete medium and serially diluted

to obtain four decreasing concentrations (3000 cells/10µl, 1000 cells/10µl, 300 cells/10µl and 100 cells/10µl).

2.2.4.2.2 Responder cells

Responder PBMCs were obtained from adult PB as described above. The total number of PBMCs in suspension per ml was calculated by counting on the haemocytometer. The cell suspension was serially diluted to obtain decreasing concentrations of 100,000, 50,000, 25,000 and 12,500 PBMCs per 10µl of cell suspension.

2.2.4.2.3 Coculture

The responder cells were plated with the decreasing concentrations as above (10µl aliquots in each well) of unlabelled DCs, labelled DCs and DCs sorted after labelling. Four Terasaki plates were plated; with concentrations of 100,000, 50,000, 25,000 and 12,500 PBMCs per 10µl of cell suspension. Due to the small volumes involved additions to the plate were made using electronic dispensing pipette (Anachem) and drying out and pH changes were avoided. The plates were inverted over normotonic saline to form hanging droplet cultures. They were cultured for 4 days in a 37 ^{0}C humidifier incubator with 5% CO_2, placed on a fenestrated stand without the lid to ensure adequate gas exchange.

2.2.4.2.4 Harvest

After 4 days of culture, each well of the four Terasaki plates were pulsed with 1 µl of [H^3]-thymidine (concentration of 1 µg/ml) (specific activity 2 Ci/mmol, Amersham International, Buckinghamshire, UK) and incubated for two hours. During pulsing, care was taken not to disturb the cell pellet at the meniscus of the droplet and to keep the plates inverted. The plates were harvested onto mat filters using a vacuum pump and ensuring even suction across the harvesting head. The filters were washed with saline to clear the debris, with 5% trichloroacetic acid to precipitate the DNA and with methanol to dry the filter. Subsequently the filters were left to dry for two days and photoimaged on a benchtop liquid scintillation analyser for 4 hours (Packard, Berks, UK) and counted.

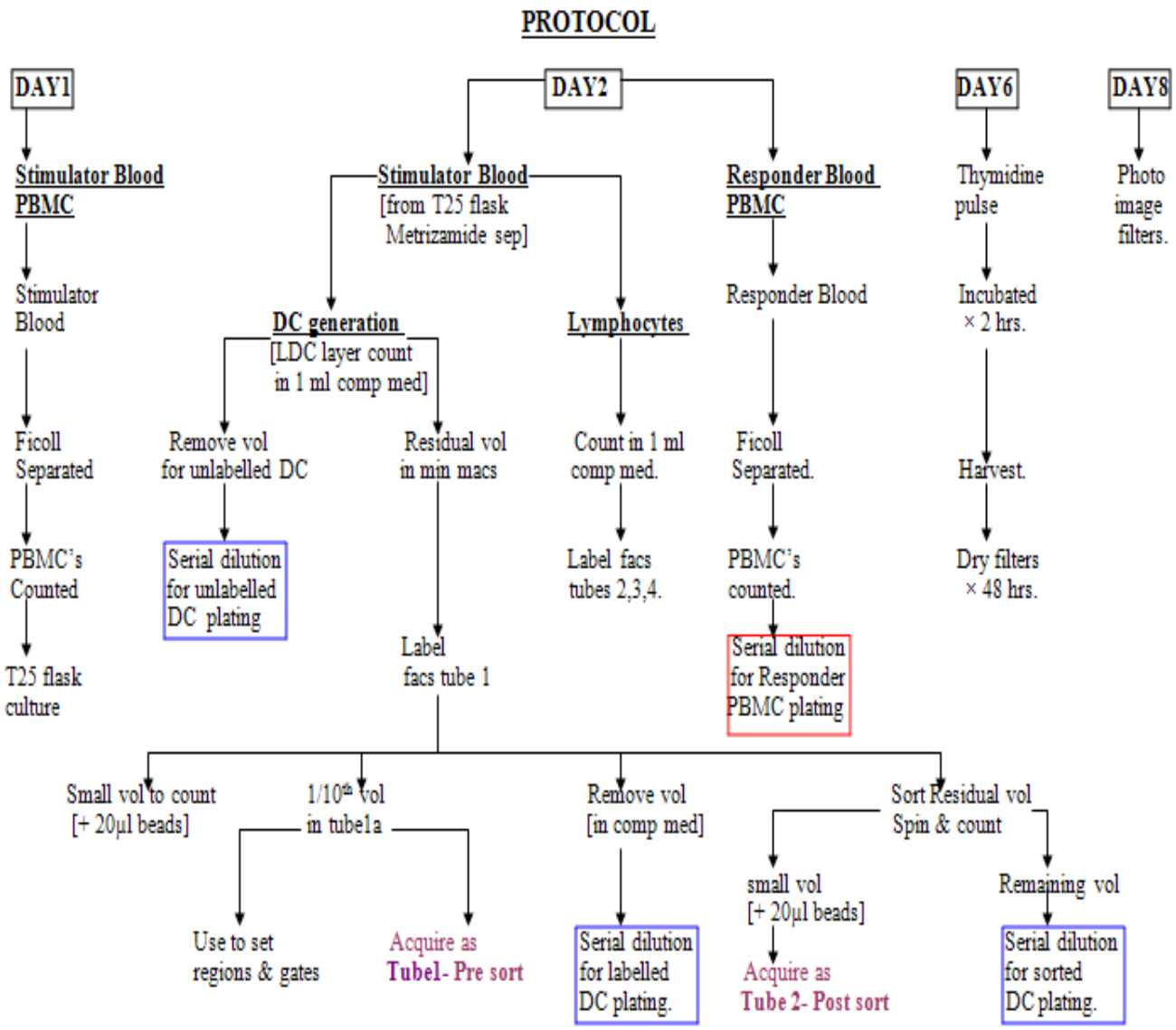


Figure 2.7 Protocol for DC sorting and labelling

The above protocol shows the temporal sequence of PBMC separation, DC generation and coculture with responder cells and thymidine pulsing

2.3 DEVELOPMENT OF METHODS

2.3.1 Freeing of an extra flow cytometer channel

The flow cytometer used had only four channels. In order to enable a detailed analysis of the CD 11c$^-$ subset an extra channel was required. To free a fluorescence channel the CD11c$^+$ subset was included within the cells stained by the lineage cocktail. To this purpose, cychrome conjugated CD11c was added to the lineage cocktail in the third channel which removed the CD11c$^+$ DCs from the DC gate staining. Freeing of one channel enabled the further delineation of CD11c$^-$ subsets with respect to their expression of CD40 and CD86 costimulatory markers. Concurrent cell samples were stained with CD123 and CD45RA to ensure no loss of CD11c$^-$ DCs occurred due to the addition of CD11c to the lineage cocktail.

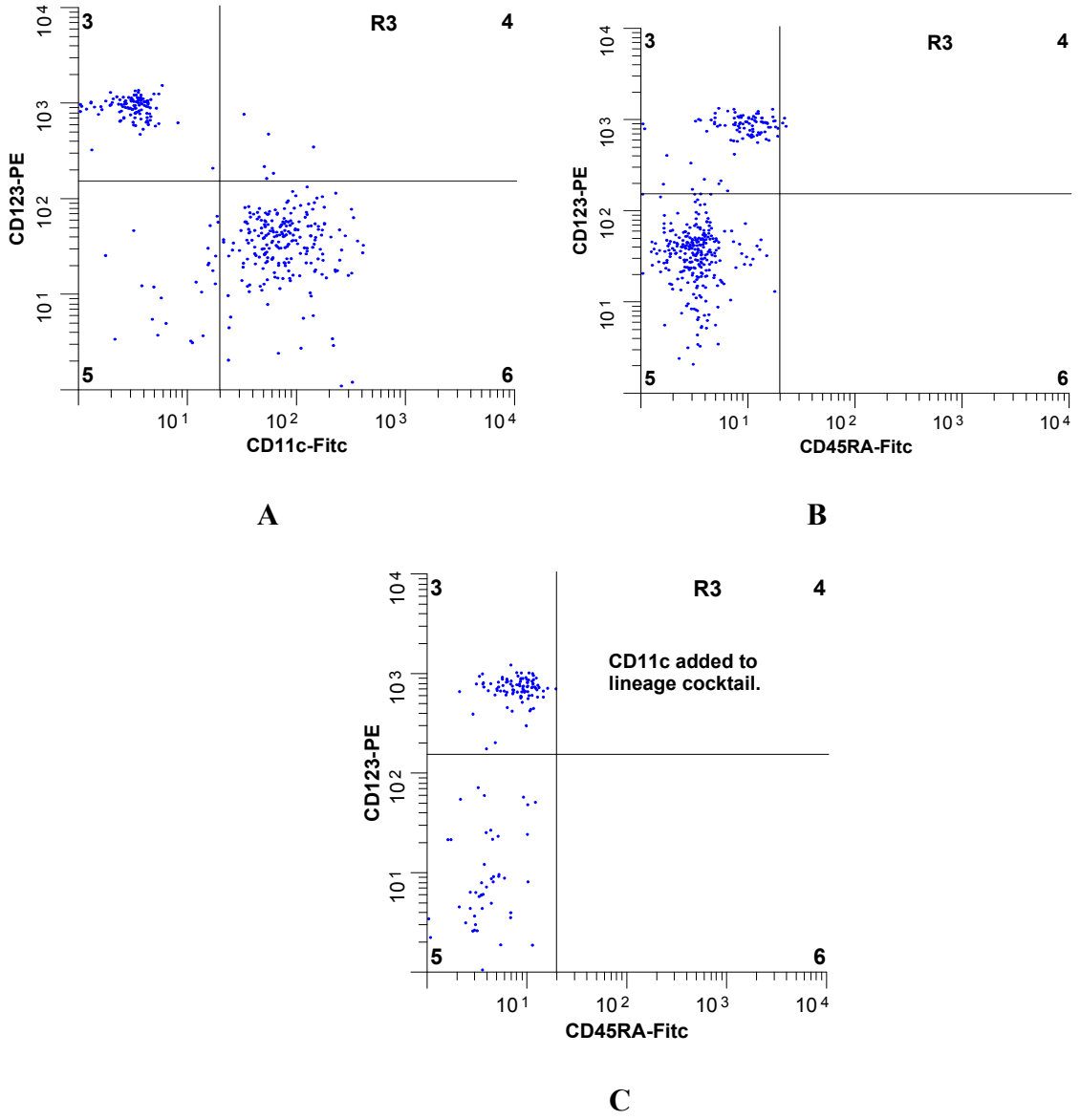


Figure 2.8 Labelling to check potential loss of CD11c$^-$ DCs

Antibody staining showing no loss of CD11c$^-$ DCs due to the addition of CD11c antibody to the lineage cocktail. Results from a single experiment

Effective disappearance of the CD11c$^+$ subset was confirmed by the above labelling. In Figure 2.8A, the CD123^{low} subset expresses CD11c (quadrant 6). This CD11c$^+$ subset could be seen to disappear with the addition of CD11c to the cocktail (compare quadrant 5 in Figure 2.8B with

quadrant 5 in Figure 2.8C). This also demonstrates that there is no loss of the $CD11c^{-}/CD123^{+}$ subset with the addition of CD11c to the cocktail (compare quadrant 3 in Figure 2.8A with quadrant 3 in Figure 2.8C).

2.3.2 Integrity of DC sorting and potential DC loss with metrizamide separation

2.3.2.1 Integrity of DC sorting

DC sorting and labelling experiment was conducted to check the integrity of flow cytometer sorting. Both adult and fetal samples were labelled and the protocol for DC sorting and labelling was followed as discussed above (see **Figure 2.7**).

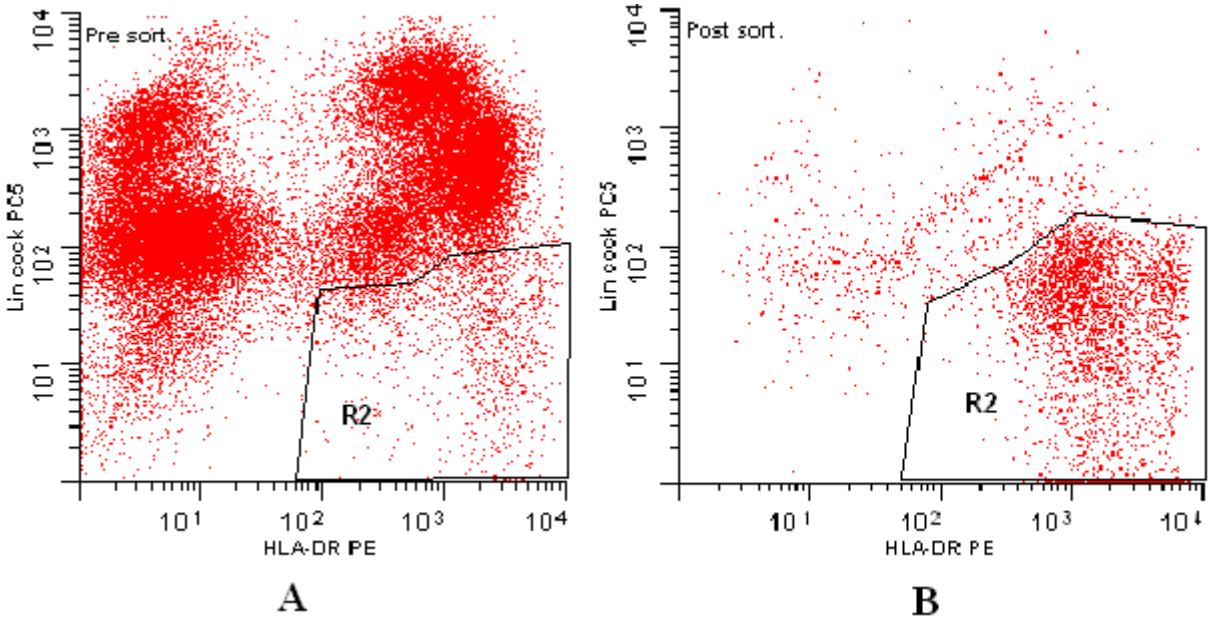


Figure 2.9 Integrity of flow cytometry sorting

Pre and post sorting two parameter histograms showing DC populations. Results from a single experiment

The purity of the sorted population after sorting was confirmed (compare region R2 in figure 2.9A with region R2 in figure 2.9B). Effective disappearance of cell populations other than DCs was also confirmed.

2.3.2.2 To check loss of DC subpopulations

DC sorting and labelling experiment was conducted to ascertain if subpopulations of DC, especially the novel fetal subpopulation of interest, which was identified on whole blood labelling (see **chapter 3**) could be identified without any loss after metrizamide labelling.

Both adult and fetal samples were labelled and the protocol for DC sorting and labelling was followed as discussed above (see **Figure 2.7**).

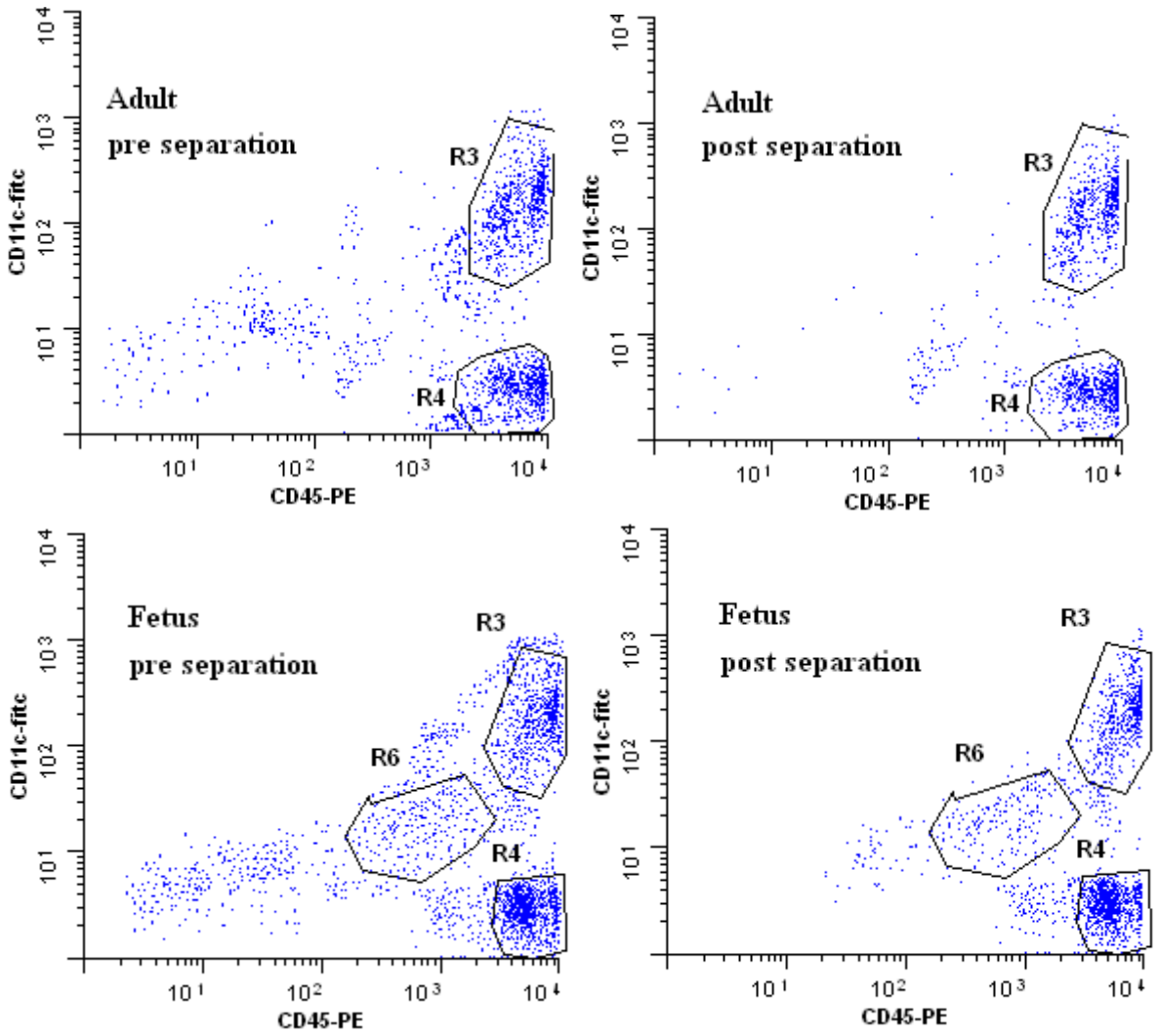


Figure 2.10 Pre and post separation labelling of Adult and fetal DCs

Pre and post separation two parameter histograms after metrizamide separation showing DC populations in adult PB and fetal CB. Results from a single experiment

No significant loss of DC populations was seen after metrizamide separation both in adult and fetal CB.

2.3.3 Whole blood labelling

A validated technique which permits usage of the small volumes of whole blood available from preterm neonates was used (Mason, *et al.,* 2005). All the experiments for DC phenotyping, costimulatory marker and intracellular cytokine analysis used whole blood. Analysis of whole blood precluded the usage of antibiotics in cell culture media which was important as this study assessed the effects of infections on DCs. Antibiotics in the media was used only in the functional studies which did not assess the effects of intrauterine infections. Additionally whole blood analysis eliminates any cell separation procedures with its potential loss of cell populations, and provides data which accurately reflect the *in vivo* state.

2.3.4 Determination of antibody dilutions

Optimal dilutions of each antibody used were determined based on the fluorescent intensity. Experiment was conducted to delineate staining of individual antibodies in the cocktail and 5µl of each antibody was added. The results showed that CD16 stained very brightly while CD19, CD34 and CD56 hardly stained. CD14 and CD3 flourescence was optimal for detection. A repeat experiment was performed with serial dilutions of CD16, double dilutions of CD19, CD34 and CD56. The optimal concentrations arrived at were as follows and were used in all the experiments:

HLA-DR =1:4 = 10µl Ab to 30µl medium and add 5µl into each tube.

Linear Cocktail (LIN-CK) = CD3 + CD14 + CD16 [1:25 DIL] + CD19 + CD34 + CD56 [5µl of each Ab or 30µl of cocktail mixture into each tube]

FITC = 10µl into each tube.

γ_1 PC5 = 25µl into each tube. γ_{2a} PC5 = 5µl into each tube.

All other Ab's = 5µl into each tube.

2.4 SUBJECTS AND SAMPLES

2.4.1 Ethical Approval

Ethical approval for the study was obtained from the Harrow Research Ethics Committee (ethics number-3244).

2.4.2 Subjects

Samples were obtained from CB of fetuses delivered at Northwick Park Hospital NHS Trust, UK and from personnel working in the above Trust who volunteered. Informed consent was obtained from all mothers who donated the CB towards this study and also from all adult volunteers who donated PB. Adult PB were taken from both male and female donors and were not gender compatible with the fetal sample. The researchers did not find any evidence to date of gender related differences in CBDC.

2.4.3 Samples

All the blood samples taken were antibody labelled within 1-2 hours of delivery of the fetus, to eliminate changes in immunological activity occurring with time. To eliminate systematic variations over time we have analysed each fetal sample with an adult sample with the labelling done contemporaneously. All the labelling and analyses were done by the MD candidate.

2.4.3.1 Fetal umbilical CB

Umbilical CB was obtained by venupuncture of the placental vein near the insertion of the umbilical cord into the placental base. Between 5 and 10 ml of CB was drawn into heparinised tubes immediately following the delivery of the baby.

A total number of 43 CB samples (26 preterm and 17 term) were analysed for the identification of CBDC subsets. Their expression of CD45 and HLA-DR was analysed in 40 CB samples (26 preterm and 14 term). Blood from the umbilical cord of 25 preterm neonates (less than 37 completed weeks of gestation) and 12 term neonates (more than 37 completed weeks of gestation) was analysed for their CBDC change in numbers with age and exposure to stressors. Neutrophilic activation markers were analysed in a subsection of the above 37 samples (term-18; preterm-11). To obtain more detailed information on the functional aspects of the DC subsets, a subset of the preterm and term CB collected were analysed for costimulatory markers (CB from 14 preterm and 7 term neonates) and IL-12 production (CB from 8 preterm and 10 term neonates). CB samples from complicated and uncomplicated pregnancies as well as vaginal and caesarean births were analysed to incorporate all the parameters which influence changes *in vivo*.

As infections and its sequelae are retrospective diagnoses when the CB for analysis is taken (at the time of birth), we have used various factors to differentiate those neonates considered to have been ‘stressed’ or exposed to intrauterine infectious/stressor stimuli and those neonates who were ‘non-stressed’. The following criteria were employed to classify the neonates into the two groups of stressed or non-stressed. The first criteria used to classify babies as stressed were clinical signs and symptoms of infection with or without support by laboratory tests. Neonates with prolonged, complicated labours or other clinical findings indicating a higher infection risk, intrauterine

growth restriction and non-reassuring patterns on the fetal heart rate monitor indicating fetal hypoxia or acidosis which required urgent delivery were also classified as stressed. Other obstetric complications such as severe pre eclampsia requiring delivery were also classified as stressed. Preterm neonates delivered for maternal indications or acute events not affecting the fetus such as APH and vaginal deliveries with no signs of infection were classified in the non stressed group. Term neonates with an uncomplicated intrapartum period and a normal antenatal period were classified in the non-stressed group. Equal numbers of babies in the stressed and non stressed groups had antenatal steroids (two doses for prematurity). No fetuses were exposed to long term steroid or anti inflammatory therapy for maternal indications. (See **chapter 4** for validation of the classification).

2.4.3.2 Adult PB

Forty three samples of adult blood for analysis were obtained after informed consent from non-pregnant healthy volunteers between the age groups of 22 to 56 years, by venupuncture of the ante-cubital vein.

Table 2.IX Patient Demographics

FETAL CORD BLOOD					
PRETERM			**TERM**		
Gestation	Delivery	Group	Gestation	Delivery	Group
21/40	Twin2, IU infection, PROM, twin1 Intra uterine death	S	38+/40	Twin1, Elective LSCS	NS
27/40	Preterm labour, Vaginal Delivery	S	38+/4	Twin2, Elective LSCS	NS
29+6/40	Emergency LSCS, fulminating PET	S	39/40,	Elective LSCS, Breech	NS
32/40	LSCS, maternal indication	NS	39/40	Spontaneous vaginal delivery	NS
30+2/40	Emergency LSCS, APH	NS	39/40	Elective LSCS, previous LSCS	NS
32/40	LSCS, maternal indication	NS	39+/40	Elective LSCS, previous LSCS	NS
33/40	Severe maternal PET	S	40/40	Elective LSCS, previous LSCS	NS
34/40	Twin1,Elective LSCS, severe IUGR	S	40/40	Emergency LSCS	NS
34/40	Twin2,Elective LSCS, severe IUGR	S	40/40	Emergency LSCS, Elective in labour	NS
34+4/40	twin1, Elective LSCS, severe IUGR	S	40/40	Emergency LSCS, Elective in labour	NS
34+4/40	twin2, Elective LSCS, severe IUGR	S	40/40	Emergency LSCS, Elective in labour	NS
35/40	Elective LSCS, fulminating maternal PET	S	40/40	Twin1, Elective LSCS	NS
35/40	Vaginal Delivery, IOL, maternal PET	S	40/40	Ventouse, failure to progress	S
35+1/40	Emergency LSCS, APH	NS	40/40	Ventouse, failure to progress	S
35+1/40	Twin1, Spontaneous labour, Vaginal delivery - infection	S	40/40	Ventouse, failure to progress	S
35+1/40	Twin2, Spontaneous labour, Vaginal delivery - infection	S	40/40	Ventouse, failure to progress	S
36/40	Spontaneous Vaginal Delivery	NS	40/40	Emergency LSCS, failure to progress	S
36/40	Spontaneous Vaginal Delivery, infection clinically	S			
36/40	Twin1,LSCS, inf malpresentation, GDM	S			

36/40	Twin2,LSCS, inf malpresentation, GDM	S			
36/40	twin1, Emergency LSCS, in labour, booked elective	NS			
36/40	twin2, Emergency LSCS, in labour, booked elective	NS			
36+/40	Emergency LSCS, in labour, booked elective	NS			
36+1/40	Spontaneous labour, Vaginal delivery	NS			
36+3/40	Twin1, Emergency LSCS, PROM	S			
36+3/40	Twin2, Emergency LSCS, PROM	S			

Abbreviations used:

S	Exposed to stressors
NS	Not exposed to stressors
IU infection	Intra uterine infection
PROM	Premature rupture of membranes
PET	Pre eclamptic Toxaemia
IUGR	Intra uterine growth restriction
GDM	Gestational Diabetes mellitus
APH	Antepartum haemorrhage
IOL	Induction of labour
LSCS	Lower Segment Cesarean section

Clinical parameters and the gestational age of the CB samples are given above

2.5 Statistical analyses

Statistical analysis was performed using the SigmaStat 3.0 software programme (SPSS Inc. Chicago). Pooled data were expressed as mean values +/- standard deviation. The number and proportions of the different cellular groups were compared between fetus and adult and also between preterm and term births. One Way Analysis of Variance (ANOVA) was used to compare multiple groups in all the analyses and corrections for multiple comparisons were included if appropriate. Where data were non parametric pair-wise comparisons were made using the Mann-Whitney Rank Sum Test. A p value of $<0{\cdot}05$ was considered significant.

CHAPTER 3

RESULTS I

CHARACTERISATION, PHENOTYPE AND FUNCTION OF CORD BLOOD DENDRITIC CELL POPULATIONS

3.1 SUMMARY

Significant exposure of the unborn fetus to infection is clinically and microbiologically poorly defined and difficult to identify. DCs as potent APCs are at the forefront of immunological responses in the defence against infections and their responses to infection occur rapidly. It was postulated that identifying DC subsets in CB and their phenotypic changes in response to an infectious stimuli could further the understanding of the fetal response to infectious and stressor stimuli.

The identification and phenotyping investigations were carried out on whole blood using monoclonal antibody labelling and multi colour flow cytometry. The functional studies included assessment of endocytic uptake of FITC labelled Dextran particles and assessment of lymphocytic stimulation by MLR.

CB contained distinct populations of DCs, and this study has successfully identified both the plasmacytoid (HLA-$DR^{+}CD11c^{-}$) and myeloid (HLA-$DR^{+}CD11c^{+}$) DC populations in CB as seen in adult PB. In addition to the above two populations, this study has also identified an immature putative DC subset with a HLA-$DR^{+}CD11c^{-}CD45^{intermediate(inm)}$ phenotype in CB and present only as a trace population in adult PB. This population was positive for HLA-DR, expressed a lower level of CD45 compared with the plasmacytoid and myeloid CBDCs, and did not express CD123, BDCA2 or CD45RA showing it to be phenotypically distinct from the plasmacytoid DC subset. These putative DCs were also negative for the myeloid lineage markers of CD33 and CD13. The above three CBDC populations were identified consistently in all the CB samples analysed for the purposes of this study (*n*=43).

The functional studies revealed that all three subsets exhibited the canonical feature of DCs which was macropinocytosis and was measured by endocytic uptake of Dextran particles. Gradient separated unlabelled CBDCs exhibited lymphocytic stimulatory capacity, but this stimulatory function diminished with antibody labelling and cell sorting in the flow cytometer.

Taken together, these data indicate that CB contains the known plasmacytoid (HLA-$DR^{+}CD11c^{-}$) and myeloid (HLA-$DR^{+}CD11c^{+}$) DC populations as seen in adult PB. In addition to the above two populations this study has identified a hitherto unreported putative subset of CBDC with an immature phenotype, exhibiting endocytic activity and phenotypically distinct from plasmacytoid DCs.

3.2 INTRODUCTION

In adults two distinct pathways of DC development have been identified historically, as myeloid ($CD11c^+/CD123^-/CD45^{hi}$) and plasmacytoid ($CD11c^-/CD123^+/CD45^{hi}$) (Robinson, *et al*., 1999). The Myeloid/plasmacytoid nomenclature has been shed in the light of recent new data on DC ontogeny and the two major subsets of DC have been named as conventional/myeloid (cDC) and the plasmacytoid/lymphoid (pDC) (Shortman and Liu, 2002; Randolph, *et al*., 2005; Shortman and Naik, 2007; Wu and Liu, 2007; Hochrein and O'Keeffe, 2008).

The fetal and neonatal immune systems are functionally and phenotypically different from that of the adult. Little is known about the development or activation of the immune system during the development of the human fetus and the role of APCs in the maturation of fetal immunity (Hayward, 1981). The few studies conducted on human fetal tissue show that monocytes/macrophages are the first cells to appear in the fetal circulation (Linch, *et al.,* 1982) and T cells are detected in the fetal lamina propria from as early as 12 weeks gestation (Spencer, *et al.,* 1986). Both plasmacytoid ($HLA\text{-}DR^+CD11c^-$) and myeloid ($HLA\text{-}DR^+CD11c^+$) DCs have been identified in CB. CBDCs have been identified by the presence of the cell surface marker HLA-DR, and lack of the CD3, CD14, CD16, CD19, CD34, and CD56 markers. This population represents only about 0.3% of CB mononuclear cells (Sorg, *et al*., 1999). The majority of CBDCs show a plasmacytoid phenotype resulting in an inverted $CD11c^+/CD11c^-$ ratio with predominant $CD11c^-$ DCs, when compared with adult peripheral blood DCs; in adults $CD11c^+$ DCs dominate with a predominant myeloid phenotype (Borras, *et al*., 2001; Drohan, *et al*., 2004). CBDCs are immature compared with adult DCs; showing high levels of CD34, low CD4, low CD83, and lower levels of HLA-DR, CD40 and CD86 (Holloway, *et al*., 2000). They have a

reduced ability to attain a fully mature adult phenotype and to activate $CD4^+$ T cells to produce IFN-α (Langrish, *et al*., 2002).

Preterm birth is the major cause of perinatal mortality and morbidity in the developed world (Magowan, *et al*., 1998; Wood, *et al*., 2000). Approximately 65% of babies born between 22 and 26 weeks gestation will die before discharge from hospital and in the survivors at 30 months of age, half will have some form of disability and in half of these that disability will be severe (Wood, *et al*., 2000). The aetiology of preterm birth is multifactorial, but there is overwhelming evidence that infection is responsible in up to 40% of cases, particularly in very early preterm birth (Seo, *et al*., 1992; Lettieri, *et al*., 1993). In the amniotic fluid of babies born preterm due to infection, there is a significantly increased concentration of proinflammatory cytokines and these babies are more likely to suffer long term lung and brain tissue damage leading to bronchopulmonary dysplasia, PVL and CP (Yoon, *et al*., 1996; Romero, *et al*., 1997; Yoon, *et al*., 1997b; Yoon, *et al*., 2000b).

The aim of this study was to elucidate the different DC subsets present in CB and adult PB, in terms of their phenotypic and functional characteristics. The present study characterised the different CBDC subsets as well as analysed the differences seen with the adult DC subsets. This is important in understanding the function of the immune system as a whole as each subset seems to have a different role in the activation of the immune system (Thomas and Lipsky, 1994; Reid, *et al.,* 2000). Understanding the differential role of the DC subsets in the fetal circulation would serve to illuminate the immunological basis of the neonatal response to infection.

3.3 SUBJECTS

Blood from the umbilical cord of 26 preterm babies and 17 term babies was analysed for identification of the DC subsets. Blood from the umbilical cord of 25 preterm babies and 12 term babies was analysed for numbers of the DC subsets. CB samples from complicated and uncomplicated pregnancies as well as vaginal and caesarean deliveries were analysed to incorporate all the parameters which influence changes *in vivo*. Forty three samples of adult peripheral venous blood from healthy non-pregnant volunteers were obtained for analysis.

3.4 RESULTS

3.4.1 Identification of populations in CB and adult PB

As discussed above the validated technique which permits usage of the small volumes of whole blood available from preterm neonates was used (Mason, *et al.,* 2005). Analysis of whole blood eliminates any cell separation procedures with its potential loss of cell populations, and provides data which accurately reflect the *in vivo* state. All the CB samples were analysed within 1-2 hours after delivery to obtain information as close to the *in vivo* state as possible and adult PB DCs were labelled and analysed contemporaneously as controls.

3.4.1.1 Myeloid and plasmacytoid DC populations identified in CB and adult PB

The two major subsets of DCs namely; $CD11c^{+}CD45^{hi}$ (myeloid) and $CD11c^{-}CD45^{hi}$ (plasmacytoid) were identified in both CB and adult PB.

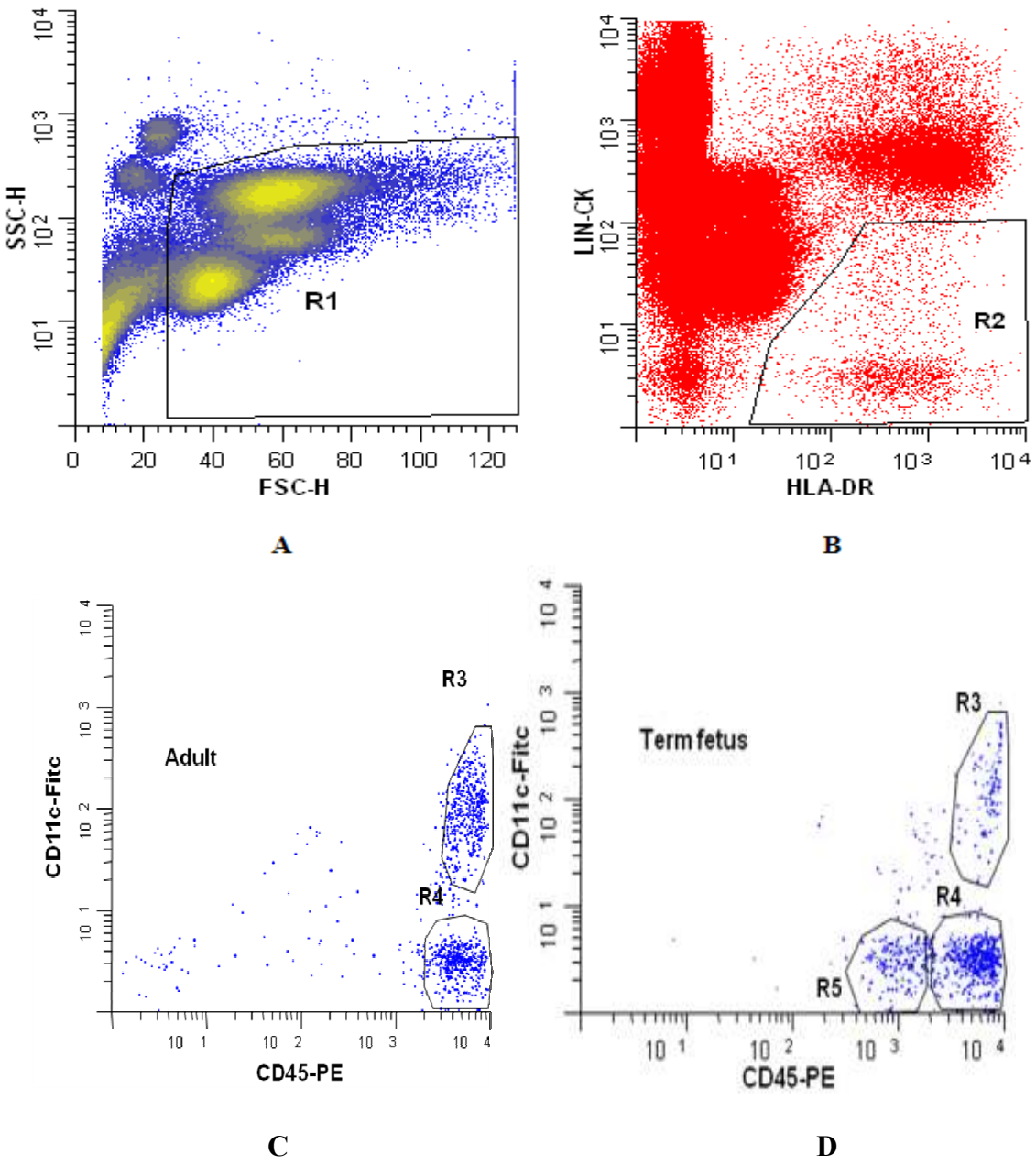


Figure 3.1 Identification of DC populations in CB and adult PB

DCs were identified by expression of HLA-DR and LIN-CK. The viable cell region (R1) was identified on the SSC and FSC histogram (Figure 3.1A). The DC region (R2) was positive for HLA-DR and negative/dim for LIN-CK and gated on the viable cell region, R1 (Figure 3.1B). The DC subsets were delineated by their expression of CD45 and CD11c (Figure 3.1C and 3.1D). Representative histograms from 43 experiments shown

Initially the total viable cell region was delineated based on the FSC and SSC properties. DCs were identified as HLA-DR^{+} and LIN-CK^{neg} (Almeida, *et al*., 1999) (Figure 3.1A). The lineage cocktail consisted of antibodies to markers of other leukocyte populations; namely CD3 (T cells), CD14 (monocytes), CD34 (stem cells), CD16 (granulocytes), CD19 (B cells) and CD56 (NK cells). DCs in both CB and adult PB were defined as a population positive for HLA-DR and negative/dim for LIN-CK (i.e. expressing little or no CD3, CD14, CD34, CD16, CD19 and CD56) (Figure 3.1B). DC subsets were further delineated based on their expression of CD11c and CD45 (Figure 3.1C and 3.1D).

As seen above the two major subsets of DC, namely CD11c^{+} (myeloid) and CD11c^{-} (plasmacytoid) DCs were present in adult (Region (R3) and Region (R4) respectively in Figure 3.1C) as well as in CB (Region (R3) and Region (R4) respectively in Figure 3.1D). Both subsets expressed high levels of CD45. Both the myeloid and the plasmacytoid DC populations were identified consistently in all the CB (n=43) and adult PB samples (n=43). Region 5 in Figure 3.1D represented a CD45 intermediate subset of DC in the CB samples which is delineated and discussed further below.

3.4.1.1.1 Backgating: Adult myeloid and plasmacytoid DC populations

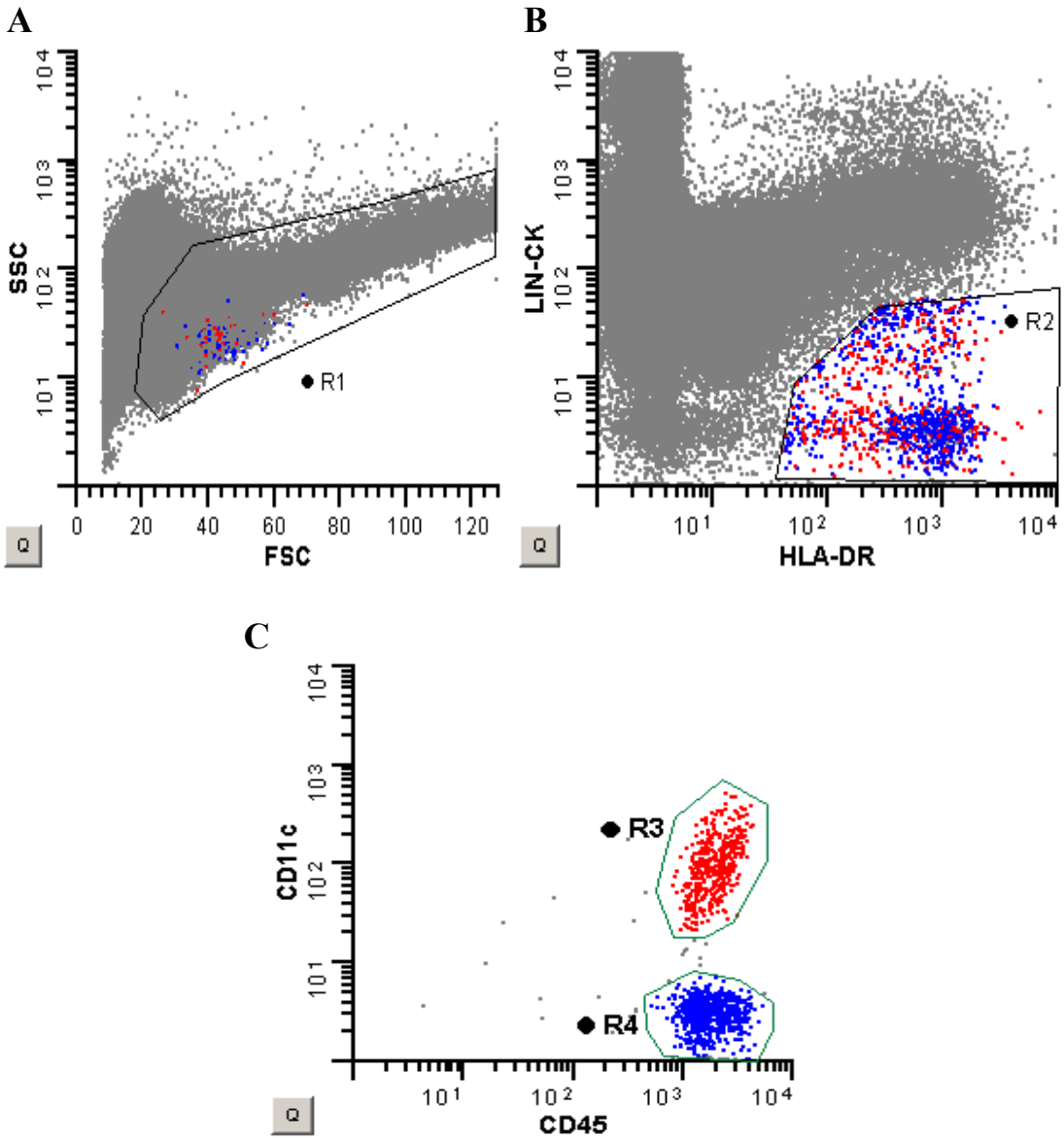


Figure 3.2 Backgated histograms of the CD11c^{+}CD45^{hi} and the CD11c^{-}CD45^{hi} DC populations in adult PB

The two parameter histograms above delineates the myeloid and plasmacytoid DC subsets in adult PB (R3: CD11c^{+}CD45^{hi}, R4: CD11c^{-}CD45^{hi}) (Figure 3.2C) and their position on the HLA-DR^{+}/Lineage cocktail^{neg} gating (Figure 3.2B) and the gating on total viable cells (Figure 3.2A). Representative histograms shown from 43 experiments shown

The adult myeloid and plasmacytoid DC populations can be identified as discrete cell populations in the HLA-DR/LIN-CK plot and within the region of total viable cells. This establishes that DC populations identified on the CD11c/CD45 phenotype can be traced back as discrete cell populations.

3.4.1.1.2 Backgating: Myeloid and plasmacytoid CBDC populations

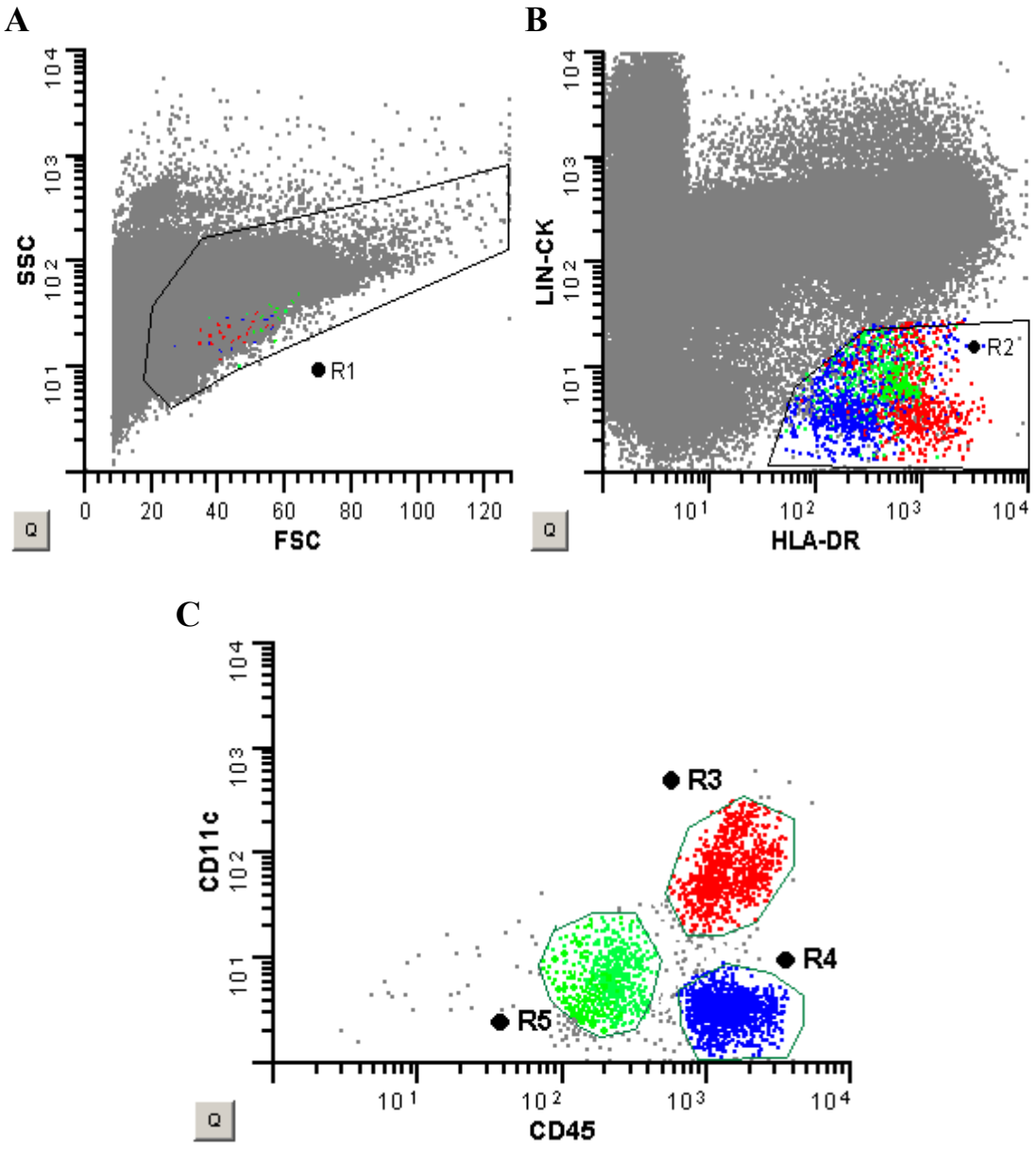


Figure 3.3 Backgated histograms of the DC populations in CB

The two parameter histograms above delineates the myeloid and plasmacytoid DC subsets in CB (R3: $CD11c^{+}CD45^{hi}$, R4: $CD11c^{-}CD45^{hi}$) (Figure 3.3C) and their position on the $HLA\text{-}DR^{+}$/Lineage $cocktail^{neg}$ gating (Figure 3.3B) and the gating on total viable cells (Figure 3.3A). Representative histograms shown from 43 experiments shown

The fetal myeloid and plasmacytoid DC populations can be identified as discrete cell populations in the HLA-DR/LIN-CK plot and within the region of total viable cells. This establishes that CBDC populations identified on the CD11c/CD45 phenotype can be traced back as discrete cell populations. The CBDC populations lie in the same area of the total viable cell histogram as the adult PB DCs (compare Figure 3.3A with Figure 3.2A).

3.4.1.1.3 Numbers of DCs

The absolute numbers of DC per µl of blood are given below (see **chapter 5** for further elucidation).

Table 3.I Absolute numbers of DC in each subset

	Adult (*n*=25)	**Fetus** (*n*=37)
$CD11c^{+}CD45^{hi}$ DC	20.88+/-12.22	24.92+/-31.34
$CD11c^{-}CD45^{hi}$	18.56+/-14.26	37.43+/-50.13

Mean values and the numbers analysed in each subset are given

3.4.1.2 A novel population of CD11c^{-}CD45^{inm} putative DC identified in CB

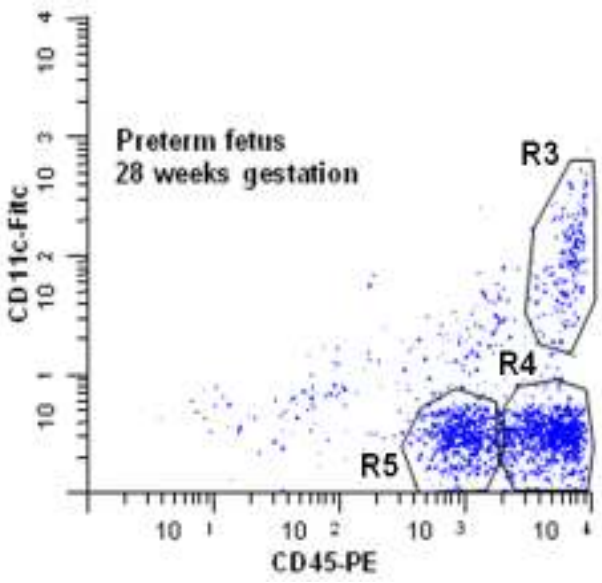


Figure 3.4 Identification of the novel CD11c^{-}CD45^{inm} putative CBDC population

A third population of DC was identified in CB. These DCs did not express CD11c and expressed intermediate levels of CD45 (Regions (R5) in Figure 3.1D and in Figure 3.4). Representative histograms from 43 experiments shown

In addition to the myeloid DCs (CD11c^{+}CD45^{hi}) Region R3 in Figure 3.4 and the plasmacytoid DCs (CD11c^{-}CD45^{hi}) Region R4 in Figure 3.4, CB contained a third population of putative DCs comprising of HLA-DR^{+}/lin^{-} cells which had a CD11c^{-}CD45^{inm} phenotype (Regions R5 in Figure 3.1D and 3.4). In Figure 3.4, the region marked R5 was this subset which appeared almost absent in the adult sample (compare with Figure 3.1C). This subset was developmentally immature as seen by the lower intensity of expression of CD45 (CD45^{inm} subset). On comparison between term and preterm CB, this subset was seen to be more predominant in the preterm fetus (compare Regions R5 in Figure 3.1D and in Figure 3.4). This CB population was identified consistently in all the CB samples (*n*=43).

3.4.1.2.1 Backgating: Fetal $CD11c^{-}CD45^{inm}$ CBDC population

Refer Figure 3.3

The histograms in Figure 3.3 delineates the $CD11c^{-}CD45^{inm}$ CBDC subset (Region R5 in Figure 3.3C) and its position on the $HLA\text{-}DR^{+}/LIN\text{-}CK^{neg}$ gating (Figure 3.3B) and the gating on total viable cells (Figure 3.3A). Thus this CBDC population can be identified as a discrete cell population in the HLA-DR/LIN-CK plot and within the region of total viable cells. It lies in the same area of the total viable cell histogram as the adult PB DCs (compare Figure 3.3A with Figure 3.2A).

3.4.1.2.2 Numbers of DC

The absolute numbers of DC per µl of blood are given below (see **chapter 5** for further elucidation).

Table 3.II Absolute numbers of DC in the $CD11c^{+}CD45^{inm}$ subset

	Adult (*n*=25)	**Fetus** (*n*=37)
$CD11c^{+}CD45^{inm}$ DC	0.64+/-1.02	25.22+/-42.26

Mean values and the numbers analysed in each subset are given

3.4.2 Phenotypic characterisation of the DC populations in CB and adult PB

The $CD11c^{+}CD45^{hi}$ and the $CD11c^{-}CD45^{hi}$ DC subsets in CB and adult PB, and additionally the $CD11c^{-}CD45^{inm}$ DC subset in CB were analysed for their phenotypic characters.

3.4.2.1 Phenotypic characterisation of the $CD11c^{+}CD45^{hi}$ and the $CD11c^{-}CD45^{hi}$ CBDCs

3.4.2.1.1 Expression of plasmacytoid markers

To explore the potential relationship between $CD11c^{-}$ DC populations expressing high or intermediate levels of CD45, expression of CD123, BDCA2 and CD45RA, markers characteristic of the plasmacytoid DC, was examined. The shaded histograms represent the proportion that was positive, i.e. the DCs exhibiting the marker. The CBDC subsets of $CD11c^{+}CD45^{hi}$ and $CD11c^{-}CD45^{hi}$ were analysed for their expression of the plasmacytoid phenotypic markers: CD123, CD45RA and BDCA2. Both the subsets exhibited differential levels of these markers.

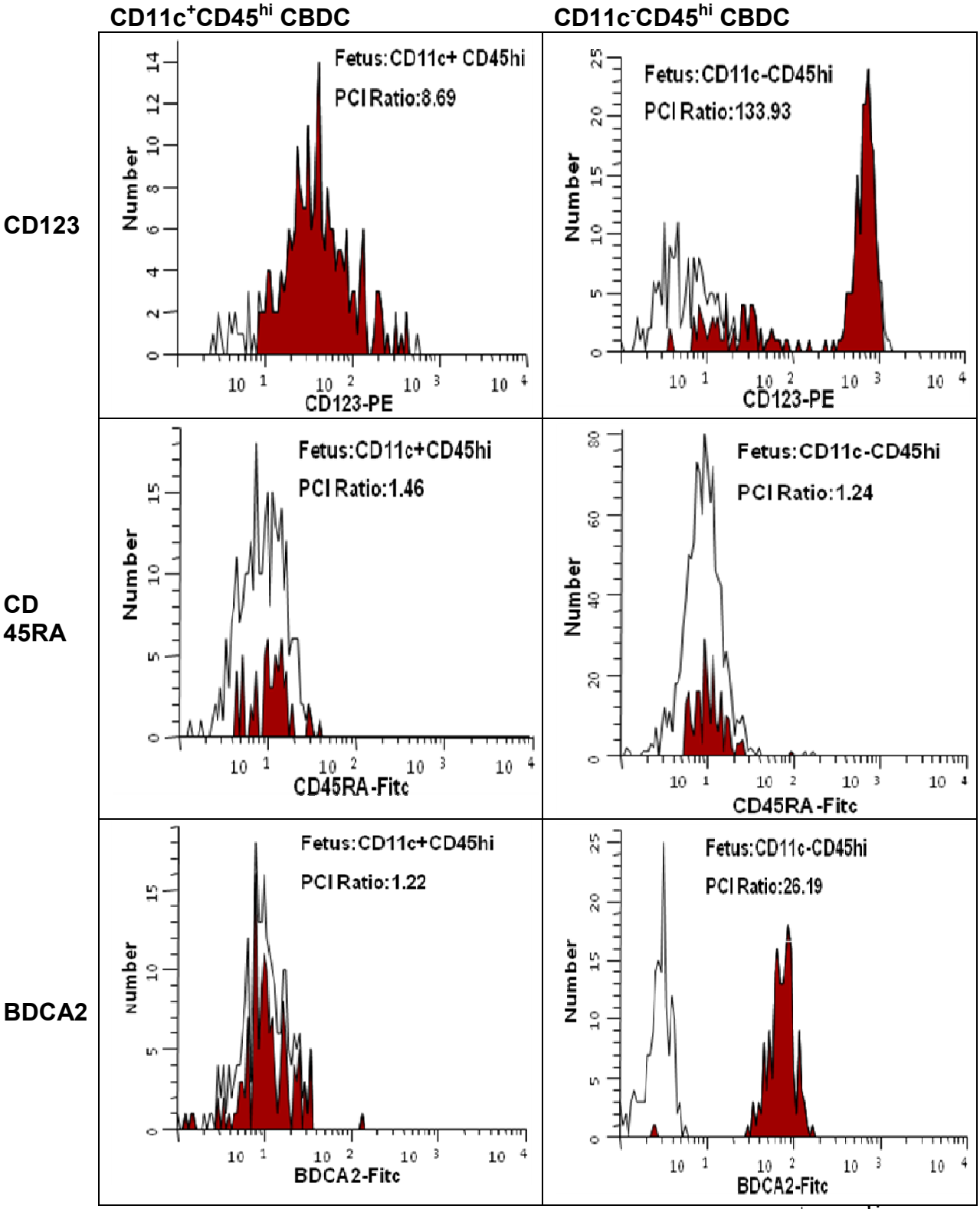


Figure 3.5 Expression of plasmacytoid markers on the $CD11c^{+}CD45^{hi}$ and the $CD11c^{-}CD45^{hi}$ CBDCs

Representative histograms of three experiments are shown above. The PCI ratio for CD123 in the $CD11c^{+}CD45^{hi}$ subset was low/negative (average PCI ratio of 15.43) and the PCI ratio for CD123 in the $CD11c^{-}CD45^{hi}$ subset was high/positive at (average PCI ratio of 131.30). The PCI ratios for CD45RA in the $CD11c^{+}CD45^{hi}$ and the $CD11c^{-}CD45^{hi}$ subset were low/negative (average PCI ratios of 1.82 and 2.08 respectively). The PCI ratio for BDCA2 in the $CD11c^{+}CD45^{hi}$ subset was low/negative (average PCI ratio of 1.23) and the PCI ratio for BDCA2 in the $CD11c^{-}CD45^{hi}$ subset was high/positive (average PCI ratio of 24.30)

The $CD11c^{+}$ subset was negative for and the $CD11c^{-}$ subset was positive for the expression of CD123 and BDCA2. Both the subsets were negative for the expression of CD45RA.

3.4.2.1.2 Expression of myeloid markers

To explore the potential lineage of the DC subsets, the expression of the myeloid markers CD33 and CD13 was examined. The shaded histograms represent the proportion that was positive, i.e. the DCs exhibiting the marker.

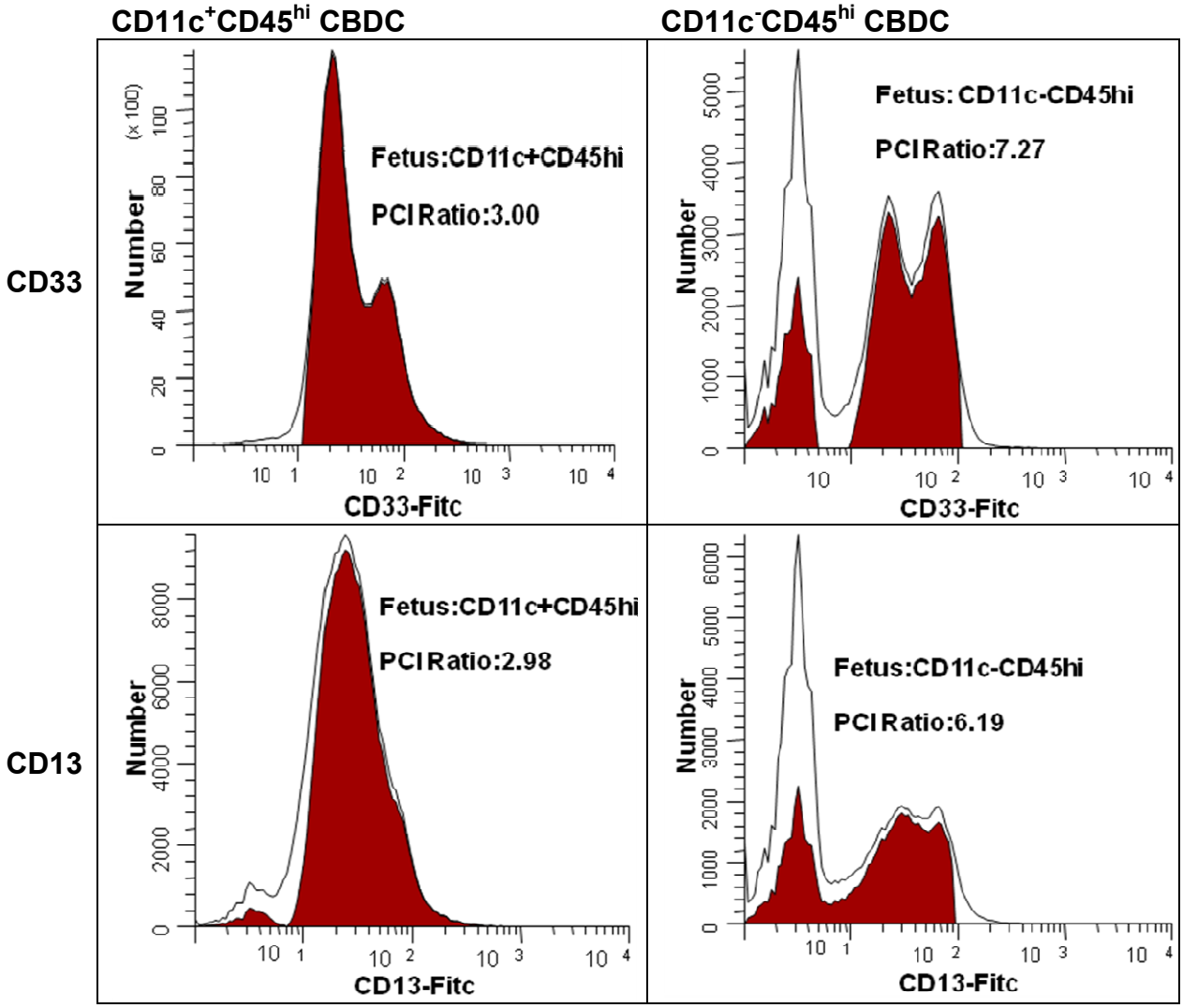


Figure 3.6 Expression of myeloid markers on the $CD11c^{+}CD45^{hi}$ and the $CD11c^{-}CD45^{hi}$ CBDCs

Representative histograms of three experiments are shown above. The PCI ratios for CD33 in the $CD11c^{+}CD45^{hi}$ and the $CD11c^{-}CD45^{hi}$ subset were low/negative at 3.00 and 7.27 respectively

The $CD11c^{+}$ subset and the $CD11c^{-}$ subset were negative for the expression of CD33 and CD13.

3.4.2.2 Phenotypic characterisation of the $CD11c^{+}CD45^{hi}$ and $CD11c^{-}CD45^{hi}$ adult DC subsets

3.4.2.2.1 Expression of plasmacytoid markers

Adult DC subsets were analysed for their expression of the plasmacytoid phenotypic markers: CD123, CD45RA and BDCA2.

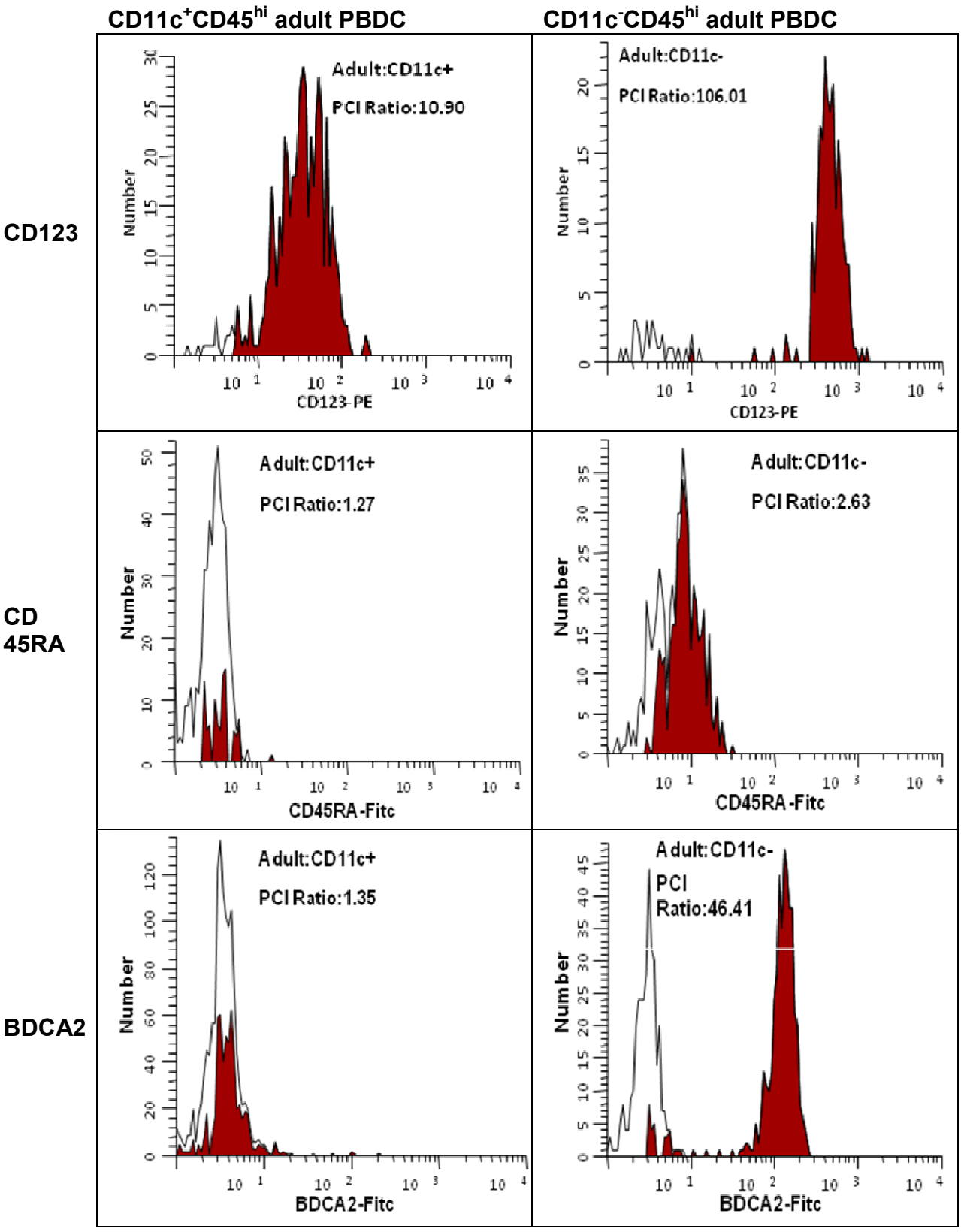


Figure 3.7 Expression of plasmacytoid markers on the adult PB DC subsets

Representative histograms of three experiments are shown above. The PCI ratio for CD123 in the $CD11c^+$ subset was low/negative (average PCI ratio of 27.77) and the PCI ratio for CD123 in the $CD11c^-$ subset was high/positive (average PCI ratio of 169.11). The PCI ratios for CD45RA in the $CD11c^+$ subset and the $CD11c^-$ subset were negative (average PCI ratio of 1.82 and 2.60 respectively). The PCI ratio for BDCA2 in the $CD11c^+$ subset was low/negative (average PCI ratio of 1.40) and the PCI ratio for BDCA2 in the $CD11c^-$ subset was high/positive (average PCI ratio of 49.50)

The $CD11c^+$ subset was negative for and the $CD11c^-$ subset was positive for the expression of CD123 and BDCA2 markers. The $CD11c^+$ and the $CD11c^-$ subsets were negative for the expression of CD45RA.

3.4.2.2.2 Expression of myeloid markers

To explore the potential lineage of the DC subsets, the expression of the myeloid markers CD33 and CD13 was examined. The shaded histograms represent the proportion that was positive, i.e. the DCs exhibiting the marker.

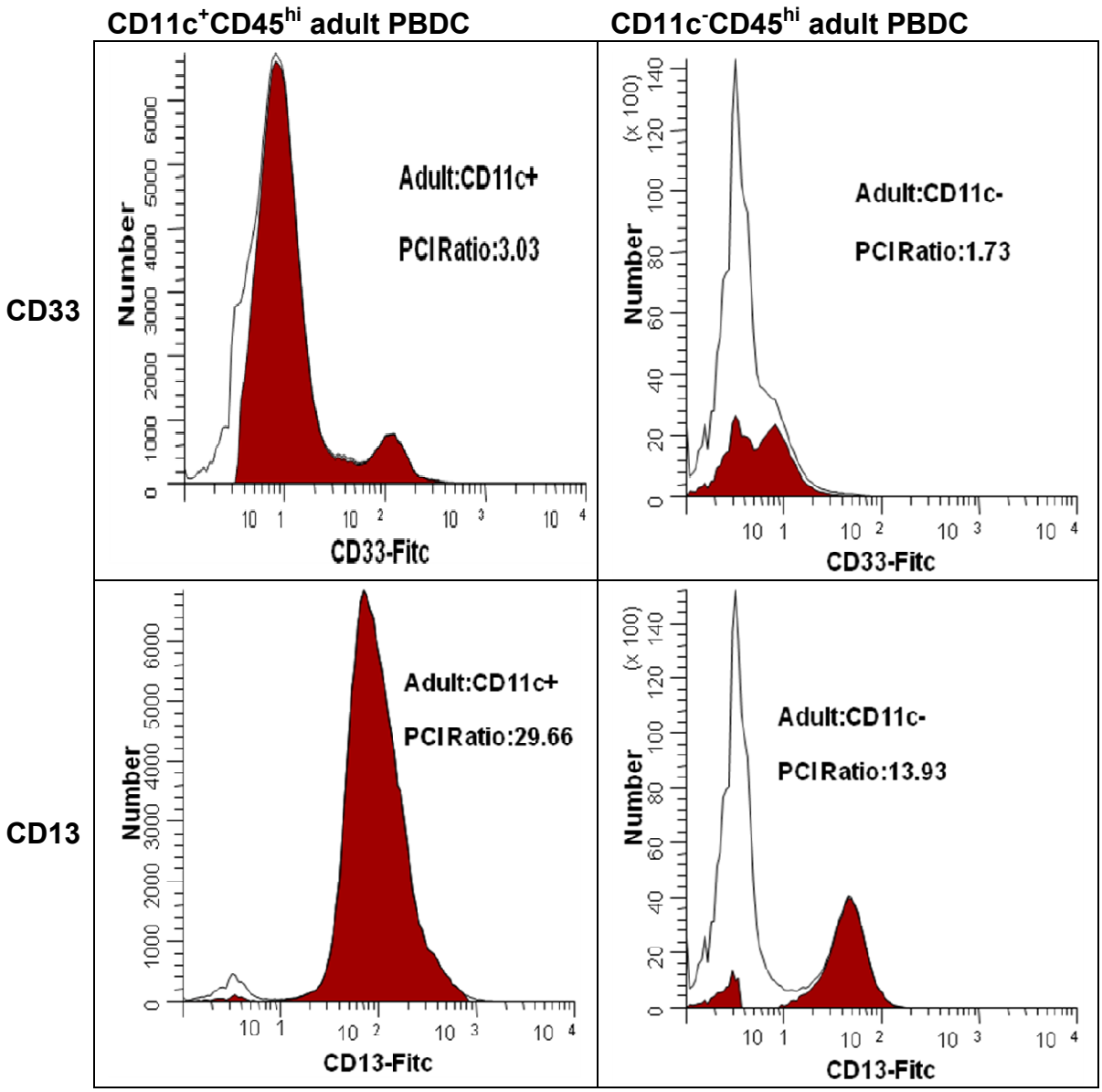


Figure 3.8 Expression of myeloid markers on the adult PB DC subsets

Representative histograms of three experiments are shown above. The PCI ratios for CD33 in the $CD11c^+$ and the $CD11c^-$ subset were low/negative at 3.03 and 1.73 respectively. The PCI ratios for CD13 in the $CD11c^+$ and the $CD11c^-$ subset were high/positive at 29.66 and 13.93 respectively

The $CD11c^+$ and the $CD11c^-$ subsets were negative for the expression of CD33 and positive for the expression of CD13.

3.4.2.3 Phenotypic characterisation of the novel $CD11c^-CD45^{inm}$ putative CBDCs

The $CD11c^-CD45^{inm}$ putative CBDC population was analysed for the expression of plasmacytoid and myeloid lineage markers. All comparisons were made with the known myeloid and plasmacytoid CBDC subsets and within the same individual sample.

3.4.2.3.1 $CD11c^-CD45^{inm}$ putative CBDCs do not express markers of plasmacytoid DC

To explore the potential relationship between $CD11c^-$ DC populations expressing high or intermediate levels of CD45, expression of CD123, BDCA2 and CD45RA, markers characteristic of the plasmacytoid DC, was examined.

The shaded histograms in Figure 3.9 represent the proportion of DCs in the subpopulation that was positive, i.e. the DCs exhibiting the marker. The $CD11c^-CD45^{inm}$ putative CBDCs were negative for BDCA2, CD45RA and CD123 (Figure 3.9). Taken together these data suggested that the $CD11c^-CD45^{inm}$ population represented a distinct subset, rather than the classical plasmacytoid DC expressing reduced levels of CD45.

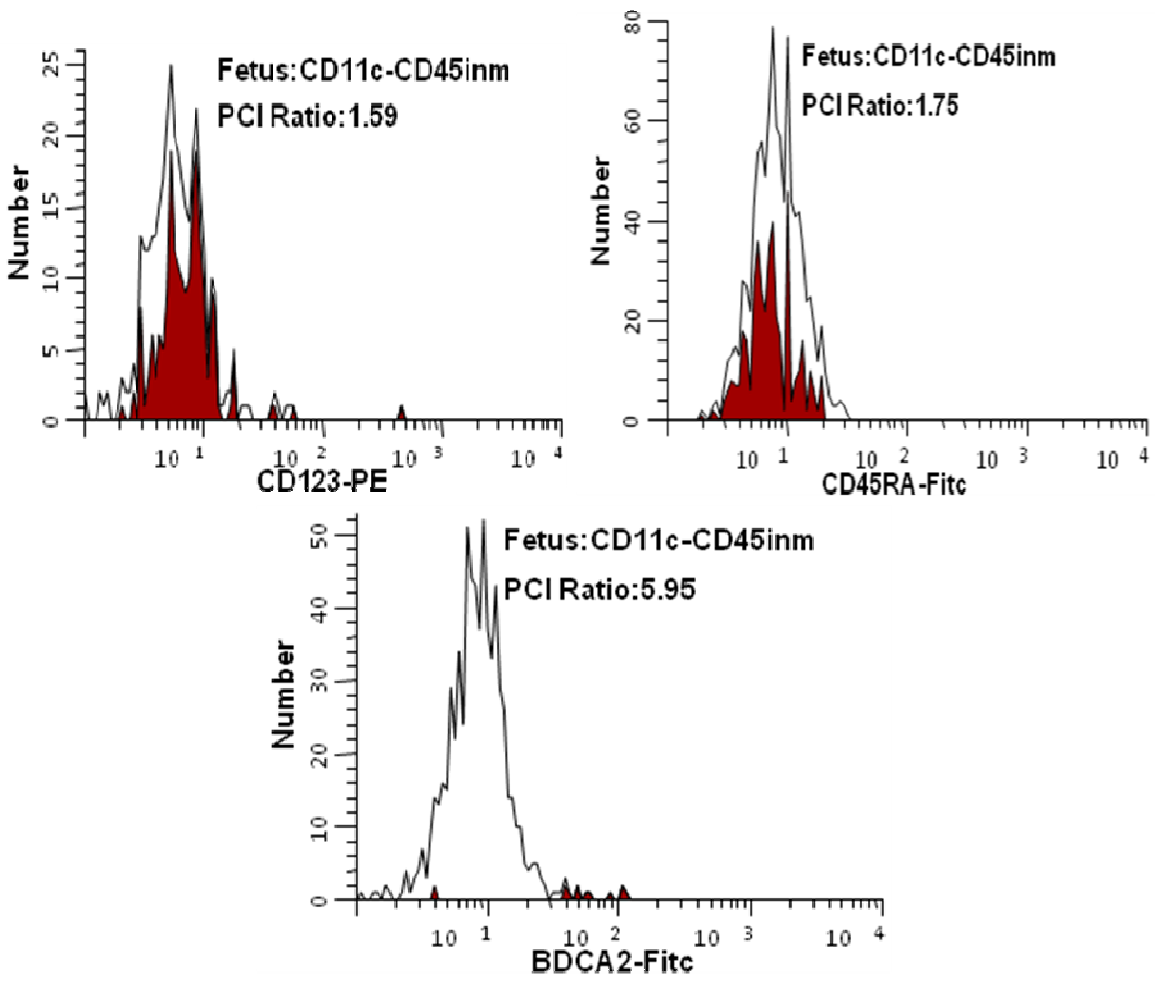


Figure 3.9 Expression of plasmacytoid markers on the CD11c$^-$CD45^{inm} putative CBDCs

Representative histograms of three experiments are shown above. The subset of CD11c$^-$ CD45^{inm} putative CBDC was negative for CD123 (average PCI ratio of 2.12), negative for CD45RA (average PCI ratio of 1.29) and negative for BDCA2 (average PCI ratio of 3.79)

3.4.2.3.2 CD11c^{-}CD45^{inm} putative CBDCs do not express markers of myeloid lineage

To explore the potential lineage of this putative DC subset, the expression of the myeloid markers CD33 and CD13 was examined.

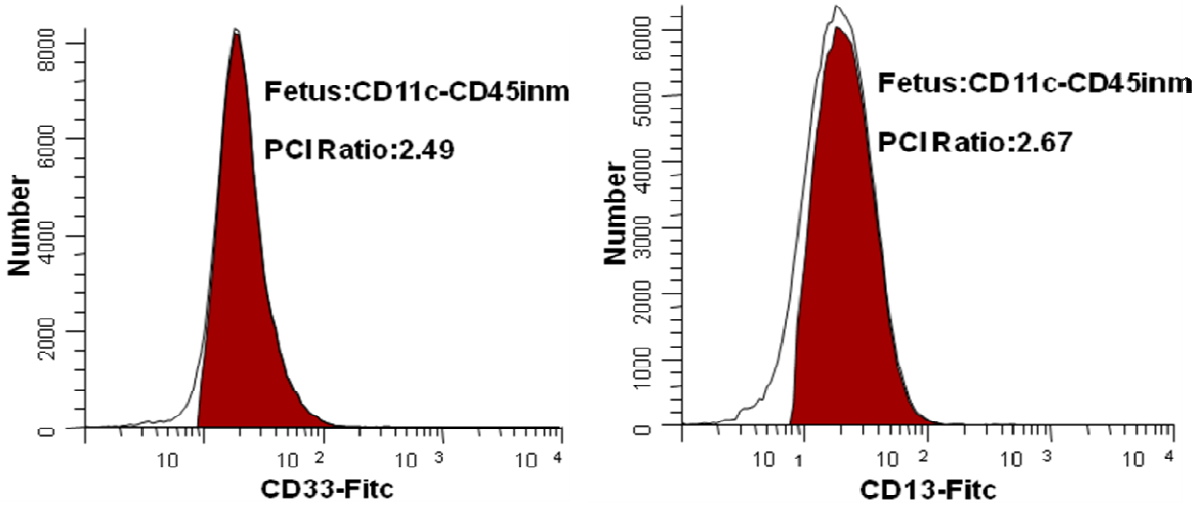


Figure 3.10 Expression of myeloid markers on the CD11c^{-}CD45^{inm} putative CBDCs

Representative histograms of three experiments are shown above. The CD11c^{-}CD45^{inm} putative DC subset was negative for CD33 (PCI ratio of 2.49) and negative for CD13 (PCI ratio of 2.67)

The shaded histograms in Figure 3.10 represent the proportion of DCs in the subpopulation that was positive, i.e. the DCs exhibiting the marker. The CD11c^{-}CD45^{inm} putative CBDCs were negative for CD33 and CD13 (Figure 3.10). Taken together these data suggest that the CD11c^{-}CD45^{inm} population represents a distinct subset, rather than the myeloid subset of CBDCs expressing reduced levels of CD45.

3.4.3 Functional characterisation of DC populations in CBDC and adult PB

3.4.3.1 Endocytic capacity

The endocytic activity of CB and adult PB DC subsets was assessed by measuring the temperature dependent uptake of FITC labelled Dextran.

3.4.3.1.1 Endocytic capacity of the $CD11c^{+}CD45^{hi}$ and the $CD11c^{-}CD45^{hi}$ DC populations

The endocytic activity of the $CD11c^{+}CD45^{hi}$ and the $CD11c^{-}CD45^{hi}$ DC populations in CB and adult PB was assessed by measuring the temperature dependent uptake of FITC labelled Dextran (Figure 3.11A and Figure 3.11B). Both these subsets exhibited endocytosis which exponentially increased at 30 minute. The endocytosis exhibited by the CBDC subsets was comparable to the adult PB DC activity. However the net MFI exhibited by the $CD11c^{-}$ DC populations (adult PB DC mean:5.91+/-0.63, *n*=4; CBDC mean:9.24+/-15.69, *n*=3) both in adult PB and in CB appear to be less than the endocytic activity exhibited by the $CD11c^{+}$ DC populations (adult PB DC mean:15.52+/-10.12, *n*=4; CBDC mean:34.69+/-45.29, *n*=3) (see section **3.4.3.1.3**).

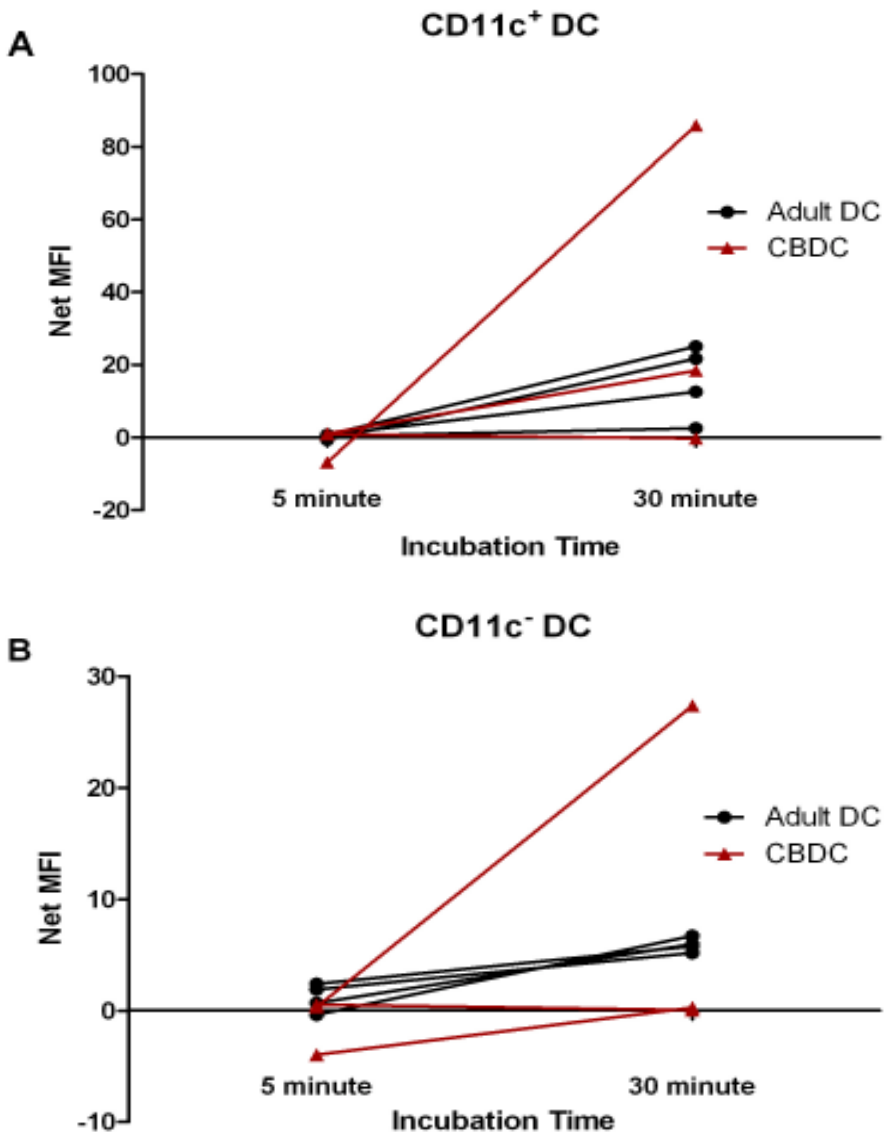


Figure 3.11 Endocytosis shown by the CD11c^{+}CD45^{hi} and the CD11c^{-}CD45^{hi} DC populations in adult PB and CB

Net MFI at 5 minute and 30 minute are shown. Data is shown from four adult samples and three CB samples from term fetuses. Both subsets exhibited antigen uptake mainly at 30 minute with the CD11c^{+} DC subset (Figure 3.11A) exhibiting higher MFI than the CD11c^{-} DC subset (Figure 3.11B). CBDCs show similar values of MFI compared to adult PB DCs

3.4.3.1.2 Endocytic capacity of the novel CD11c^{-}CD45^{inm} putative CBDC population

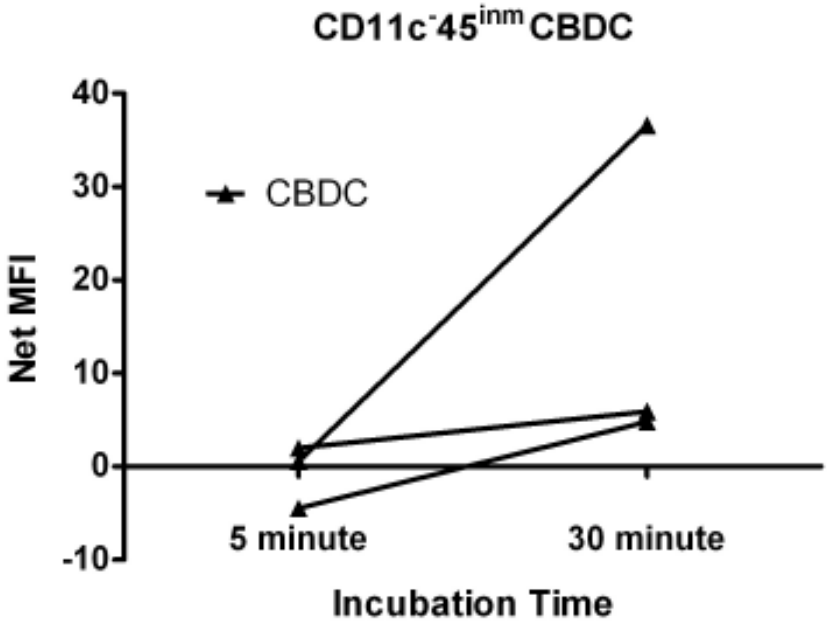


Figure 3.12 Enhanced antigen uptake by the CD11c^{-}CD45^{inm} putative CBDCs

Ficoll separated CBDCs were incubated with and without FITC-Dextran at 37^0 and 4^0 over the course of 30 minute to assay endocytic capacity. The uptake of FITC-Dextran was determined at 5 and 30 minute time intervals and the net MFI at each time point was calculated by substracting the net MFI at 4^0 from the net MFI at 37^0. The subtraction was performed to correct for passive absorption and temperature independent uptake. Data is displayed from three CBDC samples from term neonates

The cells in the CD11c^{-}CD45^{inm} subset exhibited the characteristic activity of the APC by endocytosing Dextran with the level of activity increasing at higher temperatures (mean: 15.78+/-18.05, *n*=3) (Figure 3.12).

3.4.3.1.3 The CD11c^{-}CD45^{inm} putative DC subset exhibited endocytic capacity comparable to adult PB DCs

The antigen uptake capacity of the novel CD11c^{-}CD45^{inm} subset was analysed by comparison to the endocytic capacity of the other known CD11c^{+} and CD11c^{-} DC subsets. Net uptake at 30 minutes was compared. In adult samples the CD11c^{+} DCs showed increased levels of activity (mean:15.52+/-10.12, *n*=4) when compared to the CD11c^{-} DC subset (mean:5.91+/-0.63, *n*=4) (p=0.007). The CB samples showed no such difference (CD11c^{+} CBDCs; mean:34.69+/-45.29, *n*=3; CD11c^{-} CBDC subset; mean:9.24+/-15.69, *n*=3) (p=0.410). The CD11c^{-}45^{inm} putative DCs (mean:15.78+/-18.05, *n*=3) exhibited activity comparable with the known endocytically active subset of adult CD11c^{+} DCs. That is both groups were not significantly different (p=0.982) (Figure 3.13).

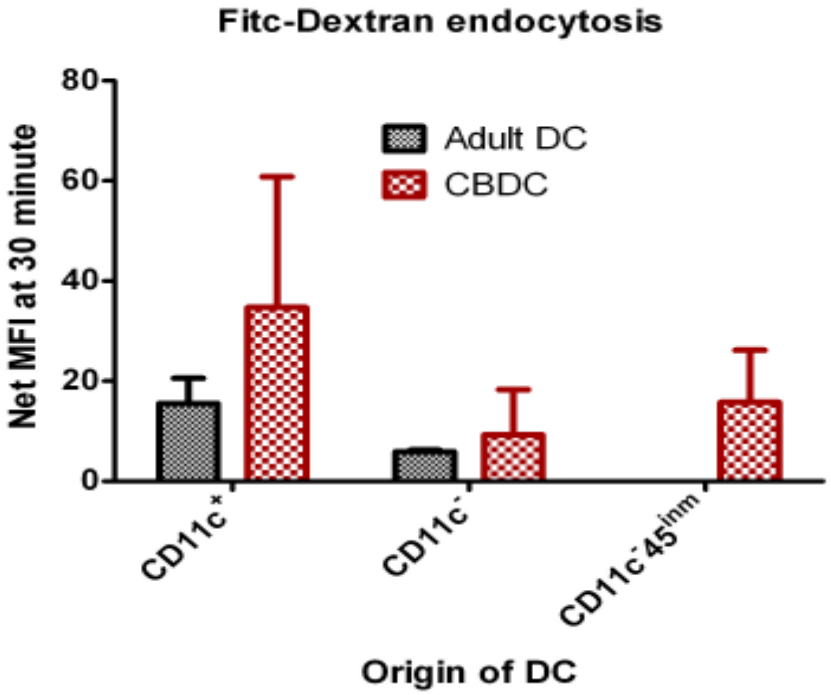


Figure 3.13 Comparison of the endocytic capacity of the DC populations

Net MFI at 30 minute is shown for all the DC subsets as the activity was negligible at 5 minute. Mean values with standard errors shown

3.4.3.2 Mixed Leukocyte Reaction

MLR is a lymphocyte proliferation assay which tests the ability of DCs to activate lymphocytes to undergo a clonal proliferation. Lymphocytes proliferate in response to antigenic peptides in association with class II MHC molecules on DCs. Lymphocytes were cocultured with DCs and pulsed with H^3-thymidine which is a radioactive nucleoside and a precursor of thymine found in DNA. Lymphocytes activated by DCs proliferate and replicate their chromosomal DNA, thus incorporating H^3-thymidine into the new strands. Using a scintillation beta-counter the extent of cell proliferation was measured as counts per minute (cpm) and is proportional to the number of proliferating cells, which in turn is a function of the number of lymphocytes that were stimulated to enter the proliferative response.

DCs were obtained using two methods:

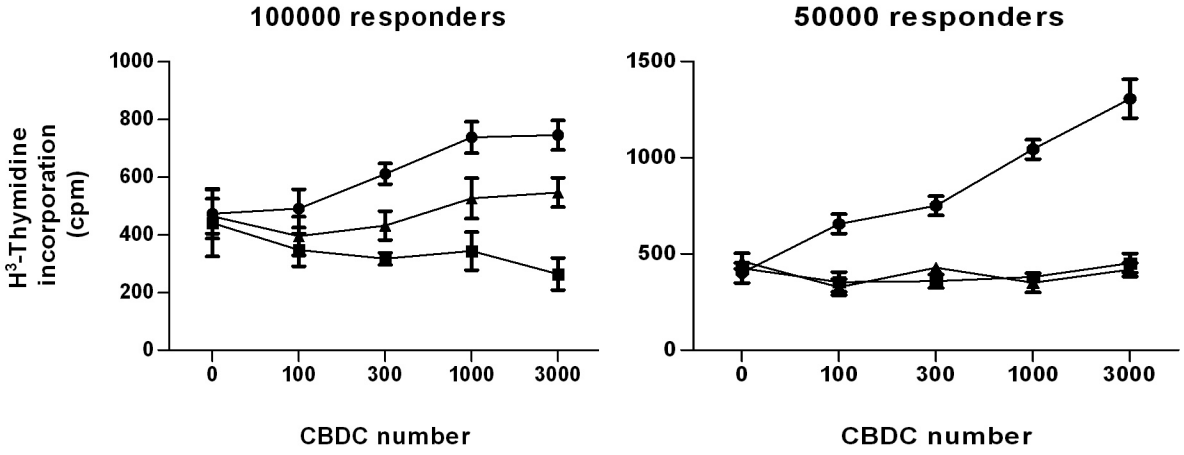
1] Density gradient separation of DCs: PBMCs in fetal venous CB were Ficoll separated (see **chapter 2, section 2.2.1.1** for protocol). The cell suspension was cultured overnight and the LDCs were separated out over a metrizamide gradient (see **chapter 2, section 2.2.1.2** for protocol) and cultured with responder PBMCs.

2] Flow cytometer sorted DCs: LDCs in suspension was serially diluted and labelled with monoclonal antibodies: HLA-DR and antibodies in the lineage cocktail (**chapter 2, Table 2.VII**). The rest of the DC cell suspension was sorted for fetal DCs through the flow

cytometer. Terasaki plates were plated with the unlabelled DCs, labelled DCs and sorted DCs. Detailed protocol for DC sorting is given in **chapter 2, section 2.2.4.2** and **Figure 2.7.** The antibody labelled DC plate functioned as a control to check the effects of antibody labelling on the stimulatory capacity of the DCs.

3.4.3.2.1 CBDCs are potent stimulatory cells

DCs were cultured in four decreasing concentrations (3000 cells/10µl, 1000 cells/10µl, 300 cells/10µl and 100 cells/10µl). Four concentrations of PBMCs per 10µl of cell suspension were used; 100,000, 50,000, 25,000 and 12,500.



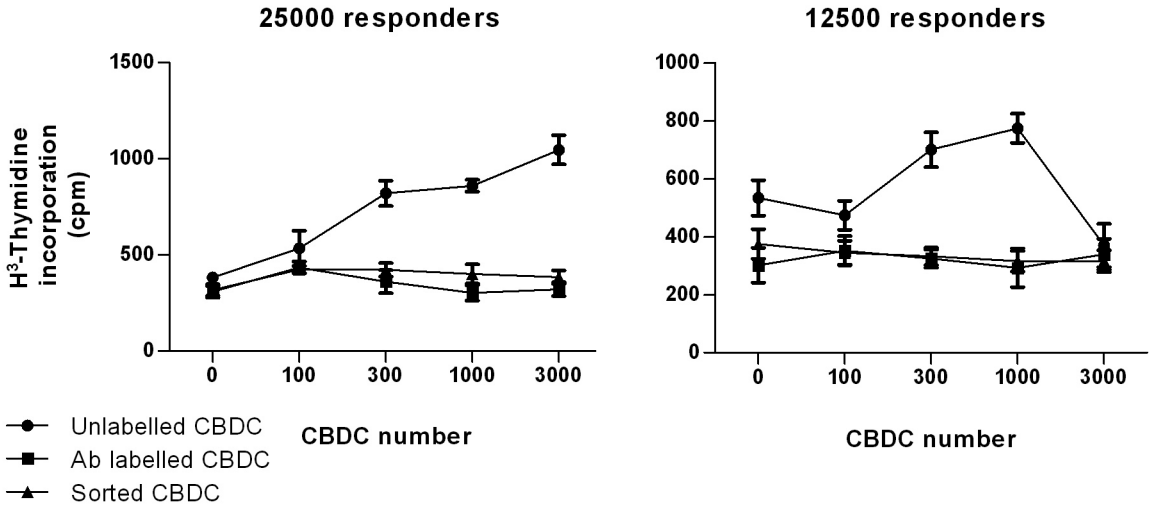


Figure 3.14 Lymphocytic stimulatory capacity of CBDCs

The multigraph details the stimulatory capacity of CBDCs related to the number of responder cells. Representative data from 2 experiments shown

CBDCs showed stimulatory capacity with unlabelled DCs inducing the highest level of proliferation and the highest level of stimulation was seen with 100,000 responders (Figure 3.14). As seen from the graphs above the stimulatory capacity is diminished with antibody labelling and sorting of the DCs through the flow cytometer.

3.4.3.2.2 CBDCs exhibit similar stimulatory capacity to adult PB DCs

The stimulatory capacity of unlabelled adult DCs and CDBCs were compared. CBDCs showed similar levels of stimulation when compared with adult PB DCs.

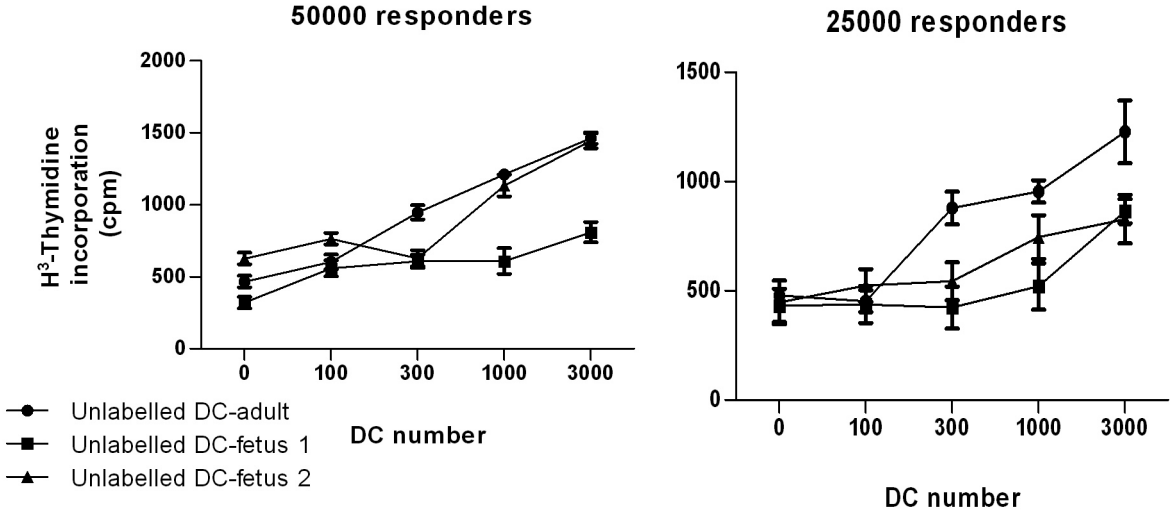


Figure 3.15 Comparison of the stimulatory capacity of CBDCs with adult DCs

The sample labelled fetus 2 showed a similar profile to stimulation by adult PB DCs in the coculture with higher numbers of responder cells. Data shown from two CB samples

Unlabelled DCs were used for coculture as antibody labelling and sorting of DCs were found to decrease their stimulatory capacity. Fetal DCs showed stimulatory capacity comparable to adult DCs.

3.5 DISCUSSION

This study has confirmed that comparable DC subsets can be identified in CB similar to adult PB. DC subsets in umbilical CB were analysed for their phenotype and functional characterisations. CB contained distinct populations of DCs, and we have identified the myeloid ($CD11c^{+}CD45^{hi}$) and the plasmacytoid ($CD11c^{-}CD45^{hi}$) DC subsets in fetal CB and in adult PB. In addition to the above two populations this study has identified an immature putative DC subset with a $CD11c^{-}CD45^{inm}$ phenotype in CB and seen only as a trace population in adult PB.

The tested method of monoclonal antibody labelling and multi colour flow cytometry was used to establish the fact that the phenotype of the CB subsets concurred with the conventional DC phenotype. To date studies in the fetus have been limited by the lack of availability of tissues with samples from the intrauterine period very difficult to access. CB samples have been traditionally used as tissue samples obtained immediately after delivery are deemed to reflect the intrauterine/fetal milieu. This usage has been limited by the small volumes usually available as well as techniques of analyses leading to modifications in the cellular properties. To overcome these issues analyses was performed on whole blood in order to reflect the *in vivo* environment as closely as possible as lack of a centrifugation step minimised *in vitro* induced changes of the surface phenotype. Studies using cell separation techniques have the attendant risk of loss of cell populations which can be avoided by analysing whole blood. Whole blood analysis has also enabled investigation of blood samples from very preterm neonates where the volume of blood available is usually minimal. This method has been shown to be reliable and reproducible with consistency throughout our study.

The myeloid and plasmacytoid CB subsets were identified as HLA-DR positive and negative for antibodies in the lineage cocktail and similar to the DC subsets in adult PB. Human DCs are

identified phenotypically based on their surface expression of HLA-DR and lack of expression of lineage associated markers (CD3, CD14, CD16, CD19, CD34 and CD56) (Knight, 1984; Reid, 1997; Banchereau, *et al*., 2000). The term 'lineage' includes all cells that express markers for lymphocytes, granulocytes, monocytes, NK cells and stem cells. In practice DCs do not appear as truly lineage negative as they show some labelling above background levels due to low levels expression of CD34 on both subsets and of CD14 on the $CD11c^{+}$ DC subset. Nevertheless, this minor degree of labelling did not prevent discrimination between the DCs and other lineage positive cells. Additional to the myeloid and plasmacytoid DC populations in CB, we have identified a third population of DCs consistently in all the CB samples. The cells in the novel CB subset of interest stained positive for HLA-DR and was negative for lineage associated markers and were readily identified as a discrete population of cells in the lower right quadrant of the lineage cocktail versus HLA-DR histogram.

All the major studies of fetal/CBDCs concur that fetal DCs are immature in their function and phenotype. Hence we analysed the expression of CD45 on our CBDCs. CD45 is a leukocyte specific protein tyrosine phosphatase present on the surface of all nucleated, haematopoietic cells. It is a regulator of TLR-mediated cytokine secretion in DCs and directs the outcome of the adaptive immune response (Cross, *et al*., 2008). CD45 has been shown to increase in intensity as maturation takes place, thus these putative DCs appear to be immature compared to the myeloid and plasmacytoid CBDC subsets (Borowitz, *et al*., 1993; Jennings and Foon, 1997). The $CD11c^{-}$ $CD45^{inm}$ putative DC subset exhibited lower levels of CD45 (enumerated further in **chapter 5**) and has thus been classified as CD45 intermediate (inm). The $CD11c^{-}CD45^{inm}$ putative DC subset was present only as a trace population in the adult.

Analysis was also performed for expression of plasmacytoid and myeloid lineage markers. The two main populations in CBDC and adult PB, the $CD11c^{+}CD45^{hi}$ myeloid and $CD11c^{-}CD45^{hi}$ plasmacytoid DC subsets were analysed for the plasmacytoid markers of CD123, CD45RA and BDCA-2. pDCs in blood and bone marrow are $CD11c^{-}$, $CD123^{high}$, $CD4^{+}$, Lin^{-}, $CD45RA^{+}$, CD304 (BDCA-4/Neuropilin-1)$^{+}$, CD141 (BDCA-3)^{low}, CD1c (BDCA-1)$^{-}$, $CD14^{-}$ and $CD2^{-}$. They express neither myeloid lineage markers (CD13, CD33) nor Fc receptors (CD32, CD64, FcRI). Human CD45RA is a transmembrane tyrosine phosphate and is expressed on all cells of haematopoietic origin, except erythrocytes. CD45RA is expressed on approximately 75% of CD8 lymphocytes and 50% of CD4 lymphocytes, all B cells and NK Cells, as well as on pDCs. BDCA-2 is a novel type II C-type lectin, expressed on pDCs in blood, lymphoid and non-lymphoid tissue. In blood, BDCA-2 is expressed on $CD11c^{-}$ $CD123^{bright}$ pDCs but not detectable on a third blood DC population, which is $CD1c^{+}CD11c^{bright}$ $CD123^{dim}$, or on any other cells in blood. Both the CBDC and adult PB DC subsets showed similar expressions of the plasmacytoid markers. The $CD11c^{+}$ myeloid subset was negative for and the $CD11c^{-}$ plasmacytoid subset was positive for the expression of CD123 and BDCA2. Both the subsets were negative for the expression of CD45RA.

The $CD11c^{-}CD45^{inm}$ novel CB subset was negative for the expression of the surface markers CD123, CD45RA and BDCA2, indicating that it is not plasmacytoid in origin (Dzionek, *et al*., 2000; Liu, et al., 2001; Comeau, *et al*., 2002). In literature to date, previous reports of increased $CD11c^{-}$ DC in CB have not distinguished pDCs from the distinct $CD45^{inm}$ population described here.

The two main populations in CBDC and adult PB, the $CD11c^{+}CD45^{hi}$ myeloid and $CD11c^{-}$ $CD45^{hi}$ plasmacytoid DC subsets were analysed for the myeloid lineage markers of CD33 and

CD13. CD33 is a transmembrane receptor; a 67 kDa glycoprotein expressed on cells of myeloid lineage, usually considered myeloid-specific and represents an important marker of myeloid cell differentiation. It is expressed on myeloid progenitors, monocytes, granulocytes, pDCs and mast cells and is absent on normal platelets, lymphocytes, erythrocytes and haematopoietic stem cells. CD13 is a 150-170 kD type II transmembrane glycoprotein expressed on the majority of peripheral blood monocytes, granulocytes, myeloid progenitors, endothelial cells, epithelial cells and subsets of granular lymphoid cells. It is not expressed on B cells, T cells, platelets or erythrocytes. In the fetus both the $CD11c^{+}$ myeloid and the $CD11c^{-}$ plasmacytoid subset were negative for the expression of CD33 and CD13. In the adult PB both subsets were negative for the expression of CD33 and positive for the expression of CD13.

Analysis of the novel $CD11c^{-}CD45^{inm}$ subset found their expression to be similar to the other CBDC subsets with the DCs negative for the myeloid lineage markers of CD33 and CD13.

The cardinal feature of the DC as an APC is antigen presentation, and endocytosis of antigen is integral to the two main pathways of MHC I and MHC II as well as the mechanism of cross presentation. Both the adult and fetal $CD11c^{+}$ DC subsets exhibited endocytotic activity as was expected of myeloid DCs. The $CD11c^{-}$ plasmacytoid DC subset in adult and CB exhibited endocytic activity, but the capacity was less on comparison with the myeloid DC subset.

The $CD11c^{-}CD45^{inm}$ putative DC subset was analysed for their capacity to endocytose FITC labelled dextran particles. In concurrence with the functioning of a conventional DC, cells in this subset showed endocytic capacity. The level of endocytosis exhibited by the novel subset was similar to that of the adult $CD11c^{+}$ DC subset. This finding is in line with the established observations of an immature phenotype of CBDCs, but with ability as potent stimulators of allogenic CB T cells (Pacora, *et al*., 2002b).

Analysis of the capacity of the CBDC to stimulate lymphocyte proliferation was assessed in a MLR. CBDCs were analysed in three conditions; as unlabelled DCs, DCs labelled with monoclonal antibodies and DCs sorted by the flow cytometer after labelling. The CBDCs stimulated lymphocytic proliferation and comparison with adult PB DCs showed similar levels of proliferation. Unlabelled DCs induced the highest levels of proliferations, with labelled DCs showing reduced levels and sorted DCs exhibiting the lowest level of proliferation.

These results indicated that labelling and manipulation of DCs decreased their lymphocytic stimulatory capacity. This is probable as labelling with HLA-DR antibody blocks the HLA-DR receptors and this decreases the receptor sites available for lymphocyte activation. This could also be the case for other receptor sites used in lymphocyte activation. Additionally further cell handling and washing with chemicals as occurs during cell sorting could affect the DC's ability to activate lymphocytes. The results could also be affected by the degree of maturity of the CBDCs. Many DC functions such as antigen uptake and lymphocyte activation are regulated during DC maturation. As the maturation stage of DC may vary from sample to sample, especially in a rapidly changing milieu such as the fetal immune system, this could affect the functioning of the DC. To minimise the above, all the CB samples used in the functional studies were acquired from full term neonates with no other pathology.

Further functional analysis of the $CD11c^{-}CD45^{inm}$ CBDC subset would have been pertinent to firmly establish the identity of these cells as DCs. Lymphocytic proliferation assays on a DC subset necessitate labelling and sorting of the DCs. For the reasons explained above the lymphocytic capacity diminishes and is almost negative with antibody labelling and sorting. Thus the stimulatory capacity of CBDCs as a whole could be assessed as unlabelled DCs were

used, but the stimulatory capacity of the individual CBDC subsets could not be assessed as labelling was required to separate the subsets.

Taken together these data indicate that CB contained discrete subsets of DCs which were comparable to adult CB subsets and can be identified in whole blood. The myeloid and plasmacytoid DC subsets exhibited similar phenotypic and functional characteristics in adult PB and fetal CB. Additionally the third population of $CD11c^{-}CD45^{inm}$ CBDCs exhibited phenotypic and functional characteristics identifying it as a population of putative DCs in CB. As this population was present mainly in the fetus, these putative DCs appear to be immunologically active in the fetus as opposed to the adult. This also raises the question of the role of this immature cell population in the stimulation and organisation of the fetal immune system. As DCs have been found to be central in orchestrating the immune response, and the subset described above is seen in the fetal circulation, these putative DCs could play an important role in the early development of the fetal immune system especially the establishment of the adaptive arm. It is possible that these immature putative DCs are evolving into a mature form as the immune system of the fetus ages with increasing gestational age. This could mean that these cells are central to the fetal defence against infection and the evolving change from immature to mature is necessary to mask fetal antigens in the initial temporal sequence of gestation. Fetal immune system has a general haemopoietic immaturity which inhibits immune responses and maintains a tolerogenic environment essential for the survival of the fetus with its 'foreign' antigens within a potential hostile maternal environment. The $CD11c^{-}CD45^{inm}$ immature DC population could facilitate mechanisms through which tolerogenic signals are promoted and presented to the maternal immune surveillance. Further evaluation of this subset and their response to increasing gestational age and exposure to antigenic stimuli *in vivo* could contribute far towards our

understanding of the fetal immune system and its development from an immature innate form to a mature adaptive form.

CHAPTER 4

RESULTS II

NEUTROPHILIC ACTIVATION MARKERS FOR INFECTION AND THE VALIDATION OF THE CLASSIFICATION OF EXPOSURE TO 'STRESSORS'

4.1 SUMMARY

The classification of CB samples into those exposed and not exposed to stressor stimuli was based on clinical criteria. The response of the CBDC subsets to stressor stimuli was studied based on that classification (see **chapters 5** and **6**). It would add to the validity of the classification if in the same samples, indicators of infection were positive using another cellular marker other than the DC profiling. Downregulation of neutrophilic activation antigens have been used as markers of infections with high sensitivity and specificity (Cui, *et al*., 2003; Huang, *et al*., 2009). Hence the neutrophilic activation markers; CD11b and CD16 were analysed in CB. Labelling of whole blood with monoclonal antibodies and flow cytometry analyses was used to identify the neutrophils and analyse their expression of CD11b and CD16 surface markers. Analysis was performed on 29 of the 37 CB samples classified into stressed and non stressed and used for the DC studies.

Correlation analyses of the change in numbers of CD11b and CD16 with the change in numbers of $CD11c^{-}CD45^{inm}$ CBDCs in the same samples was performed to assess the validity of the clinical classification. The preterm stressed samples showed a statistically significant negative correlation; with the CD11b and CD16 numbers decreasing with increasing $CD11c^{-}CD45^{inm}$ numbers on exposure to stressors (*p* values 0.0218 and 0.0262 respectively). The preterm non stressed samples showed no such correlation.

For the purposes of our study, analyses of the neutrophilic markers and their significant correlation with the CBDC numbers on exposure to stressors, validates our clinical classification of CBs into stressed and non stressed.

4.2 INTRODUCTION

Polymorphonuclear neutrophils constitute the majority of blood leukocytes, 95% of circulating granulocytes and have a characteristic multilobed nucleus containing azurophilic and secondary cytoplasmic granules used in phagocytosis (Malech and Gallin, 1987). This first line of defence depends on the two mechanisms of toxic oxygen metabolites produced by the NADPH oxidase system and the release of proteolytic enzyme granules (Henson and Johnston, 1987). Resting neutrophils are activated by chemotactic stimuli resulting in margination, diapedesis and congregation at sites of injury/infection where they show adherence to matrix components such as fibronectin and laminin (Yamashiro, *et al*., 2001). The chemotactic factors include; C5a released on complement activation, products of platelets, bacteria and other leucocytes (Colotta, *et al*., 1992). Cytokines, mainly IL-8 also known as the neutrophil activating factor as well as IL-1, TNF-α and IL-6; lead to activation of neutrophil β_2 integrins (Weissman, *et al*., 1980). Neutrophils respond to and are modulated by different bacterial antigens through TLRs which stimulate neutrophils to recruit immune cells to the sites of infection (Hayashi, *et al*., 2003). Bacterial DNA induces neutrophil activation evidenced by L-selectin shedding, CD11b upregulation and IL-8 secretion (Trevani, *et al*., 2003).

The innate neonatal immune system is functionally immature and the leukocytes of stressed newborn infants are deficient in their bactericidal activity and this intrinsic immaturity contributes to the neonatal increased susceptibility to infections (Hill, 1987; Levy, *et al*., 1999). Neutrophil function and phagocytosis are well established to be deficient in the neonate, especially chemotaxis (Falconer, *et al*., 1995). Neonates express less CD11b/CD18 on their neutrophils which contributes to their deficient diapedesis, bactericidal activity and chemotaxis

(Sacchi, *et al*., 1982; Reddy, *et al*., 1998). In particular, the immune function of the preterm infant is functionally immature compared to the term fetus (Abughali, *et al*., 1994; Kotiranta-ainamo, *et al*., 1999). This is seen in the decreased amount of total cell content of CD11b/CD18 in preterm infants compared to term infants (McEvoy, *et al*., 1996). Preterm infants exhibit a lower neutrophilic cell mass and a decreased capacity to increase progenitor proliferation resulting in the premature neonates' response to infection with neutropenia (Carr, 2000). Preterm neutrophils also exhibit a diminished upregulation of CD11b expression after stimulation which reflects in the functional defects and the increased susceptibility to infections (Smith, *et al*., 1991; Kaufman, *et al*., 1999).

Binding of immunoglobulin to cell surface receptors improves clearing of bacteria by neutrophils. These surface antigens play a key role in the recruitment and endothelial transmigration of neutrophils into tissues and their upregulation indicate neutrophil activation (Simms and D'Amico, 1995). Important cell surface receptors responsible for binding immunoglobulin include CD11b and FC g-receptor III (CD16). CD11b is a β_2 integrin which are type 1 transmembrane glycoproteins mediating intercellular and cell-substrate interactions and expressed mainly on granulocytes as well as on monocytes and NK cells (Smyth, *et al*., 1993). It is part of the CD11b/CD18 heterodimer (Mac-1), also known as the C3 complement receptor (Petty and Todd, 1996). Integrins are heterodimeric cell surface receptors that are composed of noncovalently associated α and β subunits (Hynes, 1987). The leukocyte integrins CD11a,b,c,d are associated with CD18 β_2 chains and promote neutrophil attachment to the endothelium with subsequent transmigration (Bevilacqua, 1993). Neutrophil degranulation is channelled through CD11b/CD18 (Arnaout, 1990). The functional importance of the subset of integrins, CD11b/CD18 (Mac-1) is emphasised by the recurrent bacterial infections and impaired wound

healing seen in patients with Mac-1 deficiency (Crowley, *et al*., 1980). The expression of Mac-1 is upregulated by infectious stimuli, and chemoattractants mobilise the Mac-1 from the intracellular granules to the cell surface (Corbi, *et al*., 1988). As the molecule serving as an iC3b-receptor, the CD11b/CD18 integrin is responsible for recognising microbial pathogens opsonised with iC3b (Wright, *et al*., 1983). PB polymorphonuclear cells activated with activating agents such as zymosan activated plasma show a marked increase in the expression of the CD11b/CD18 integrins (Klut, *et al*., 1997). Hence monitoring of the CD11b levels also indicate the functional state and priming of the neutrophil (Wittman, *et al*., 2004). CD11b is stored in the secondary and tertiary intracellular granules and the cell surface expression changes within minutes of activation by soluble stimuli (Wright and Meyer, 1986) enabling levels of CD11b expression to be used as a diagnostic marker for early onset neonatal sepsis (Weirich, *et al*., 1998; Nupponen, *et al*., 2001).

The CD16 is a neutrophilic surface antigen (the Fc gammareceptor III) which induces phagocytosis of bacteria and binds to the Fc region of immunoglobulin G. It is expressed largely on neutrophils and hence used as a surface marker for their identification and activation status. It is a heavily glycosylated protein of 50–70 kd that is linked to the plasma membrane of the neutrophil. (Huizinga, *et al*., 1990; De Haas M, *et al*., 1995). Preformed intracellular stores of CD16 are mobilised to the cell surface to balance the rates of shedding and thus maintain surface membrane levels (Middelhoven, *et al*., 1999; Moldovan, *et al*., 1999; Middelhoven, *et al*., 2001).

For a holistic view of the neutrophilic interactions leading to immune activation, we have analysed the changes in CD11b and CD16 expression with advancing age. CD11b has been shown to be downregulated in prolonged complicated labour (Banasik, *et al*., 2000) and hence the effect of labour on the expression of these markers was also analysed.

Neutrophil activation results in physical changes of the cell characterised by the rounding and condensation of the characteristic multi-lobed nucleus, decrease in granularity and cell shrinkage. These physical changes were assessed by analysing the size and granularity of the cells in accordance with their light scatter properties.

The research focuses on analysing the expression of CD11b and CD16 on CB neutrophils and their change when exposed to infectious/stressor stimuli. Analysis of the response to stressors of another cellular marker other than the DC would serve to validate the clinical classification of exposure to stressors.

4.3 SUBJECTS

Umbilical CB was obtained and collected as described previously. Umbilical CB from 18 preterm babies and 11 term babies were analysed. Of the 18 preterm CB samples, 9 were exposed and 9 were not exposed to stressors. All the 11 term samples were not exposed to stressors.

Adult blood obtained from 18 healthy non-pregnant volunteers was analysed. All the blood samples taken have been analysed within 1-2 hours of delivery of the fetus, to eliminate changes in immunological activity occurring with time.

4.4 RESULTS

4.4.1 Flow cytometry analysis

Whole blood was labelled with monoclonal antibodies to CD11b and CD16 and analysed using the flow cytometer (See **chapter 2, section 2.2.2.1.4** for a detailed description).

4.4.1.1 Gating strategy for neutrophils

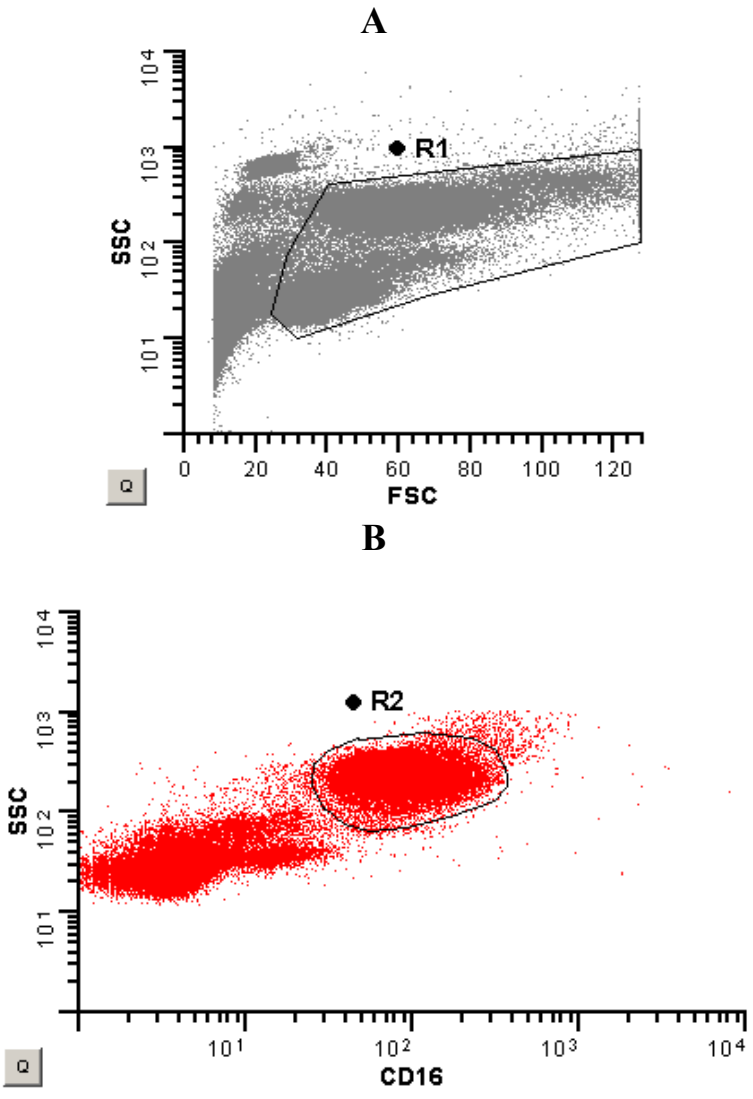


Figure 4.1 Identification of neutrophils

Region R1 shows the viable cell gate in Figure 4.1A. The histogram in Figure 4.1B shows the neutrophils identified as Region R2, on their basis of granularity (SSC) and expression of CD16 and gated on the viable cell region. Representative histograms from 29 experiments shown

Neutrophils were identified based on their light scattering properties as discussed above in Figure 4.1. The mean SSC positively identified the cells based on their granularity.

4.4.1.2 Analysis of CD11b expression

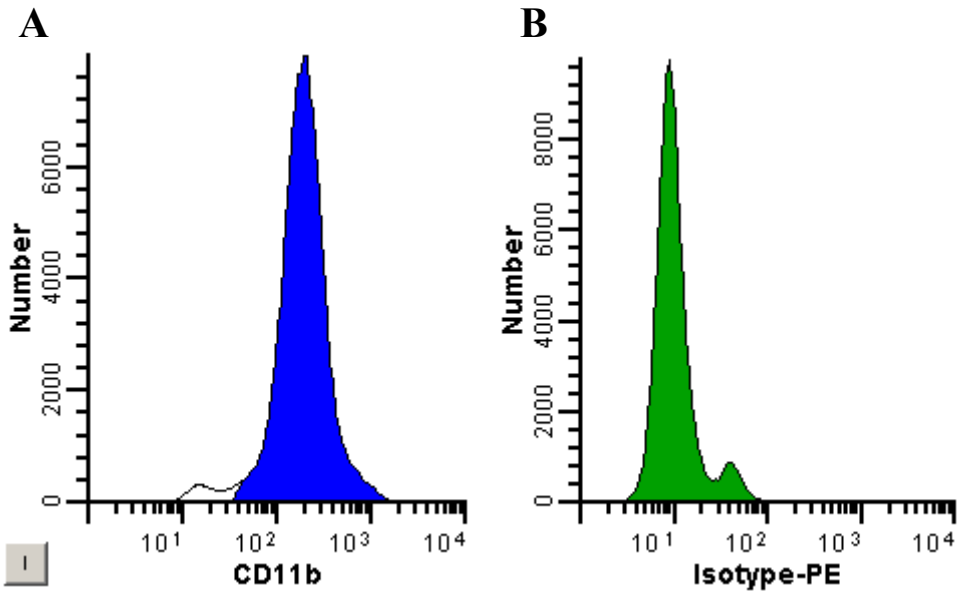


Figure 4.2 Analysis of CD11b expression

The subtraction histogram for the expression of the CD11b surface marker on neutrophils is shown above. The shaded region in Figure 4.2A represents the subtracted histogram of the isotype control from the histogram of the number of neutrophils positively expressing

the CD11b surface marker. Single parameter histogram of the isotype staining of CD11b is shown in Figure 4.2B. Representative histograms of CB neutrophils from 29 experiments shown

Isotype controls were used to delineate the negative expression of the marker to be identified (CD11b). To measure the expression of the marker on the required cell population, the isotype control (negative) histogram is subtracted from the histogram expressing the marker (test) histogram (Figure 4.2A). Subtraction estimates the number of positive cells for the measured marker, in the test histogram. The frequency histogram is a graphical representation of the number of events occurring for each channel. The median values from both frequency histogram (test and control) distributions were linearised and the positive linearised median was divided by the control median. The intensity of the positive staining, termed the PCI ratio measures the ratio of linearised positive median to the linearised control median. Thus subtraction estimates the number of positive cells for the measured marker, in the test histogram (see **chapter 2, section 2.2.3.2.2** for a detailed explanation).

4.4.1.3 Analyses of CD16 expression

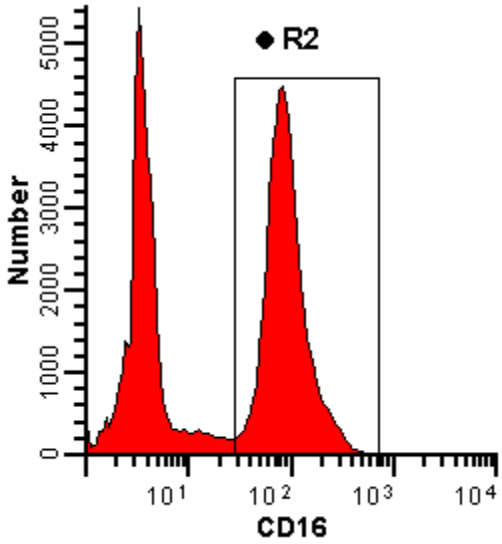


Figure 4.3 Analysis of CD16 expression

The above single parameter histogram shows the expression of the CD16 surface marker on neutrophils gated on the total viable cells. The region marked R2 outlines the cells positively expressing CD16. Representative histogram of CB sample from 29 experiments shown

Neutrophils identified on their light scattering properties and the positive expression of CD16 antigen as discussed above are gated on all the viable cells (Figure 4.3). The region R2 measures the number of cells positively expressing CD16.

4.4.2 Validation of the clinical CB classification

4.4.2.1 CB classification into 'stressors' and 'non stressors'

As infections and its sequelae are retrospective diagnoses at the time of birth when the CB for analysis is taken, various factors were used to classify the neonates into the two groups of infected/stressed or non infected/non stressed. The following criteria were employed to differentiate those neonates considered to have been 'stressed' or exposed to intrauterine infectious/stressor stimuli and those neonates who were 'non-stressed'. Essentially the first criteria used to classify neonates as 'stressed' were clinical signs and symptoms of infection without or without confirmation by laboratory tests. Neonates with PROM, albeit with a full course of antibiotics, were considered in the group labelled as 'stressed'. Neonates with an abnormal or suboptimal antenatal and intrapartum period or with any positive lab tests for infection necessitating neonatal or maternal antibiotic treatment were classified in the stressed group. In order to broaden our understanding of the immunogenic stimuli and its response in the fetal microenvironment, we have also analysed various pathological factors which could result in stress to the fetus, in addition to the known factor of infection. Thus neonates with prolonged complicated labours or other clinical findings indicating a higher infection risk and intrauterine growth restriction with compromised pattern on fetal monitoring necessitating urgent delivery were also classified as infected/stressed. Other obstetric complications such as severe pre eclampsia necessitating delivery were also classified in the stressed group. Preterm neonates delivered for maternal indications or acute events not affecting the fetus such as APH and vaginal deliveries with no signs of infection were classified in the non stressed group. Neonates

with an uncomplicated intrapartum period and a normal antenatal period were classified in the non stressed group.

Detailed table of patient demographics and CB classification is given in **Chapter 2, Table 2.8.**

4.4.2.2 Validation of the CB classification into 'stressors' and 'non stressors'

It was felt that validation of this clinical classification was needed and assessment of the response to stressors in the same group of CBs using another cellular marker would serve to validate the classification. Neutrophilic activation markers have been used as markers of infection and there is ample evidence in the literature of their change with neonatal exposure to infection (Carr and Davies, 1990; Weirich, *et al*., 1998; Nupponen, *et al*., 2001). Hence we have analysed the expression of CD11b and CD16 on CB neutrophils and their change on exposure to infectious/stressor stimuli. Neutrophils from adult PB samples were labelled and analysed concurrently to act as controls.

Of the 43 CB samples used in this body of work, 37 were classified into stressed and non stressed groups and changes on CBDC populations on exposure to stressors were analysed (See **chapters 5** and **6**). The CD11b and CD16 neutrophilic activation markers were assessed in 29 of the 37 CB samples which were classified into stressed and non stressed.

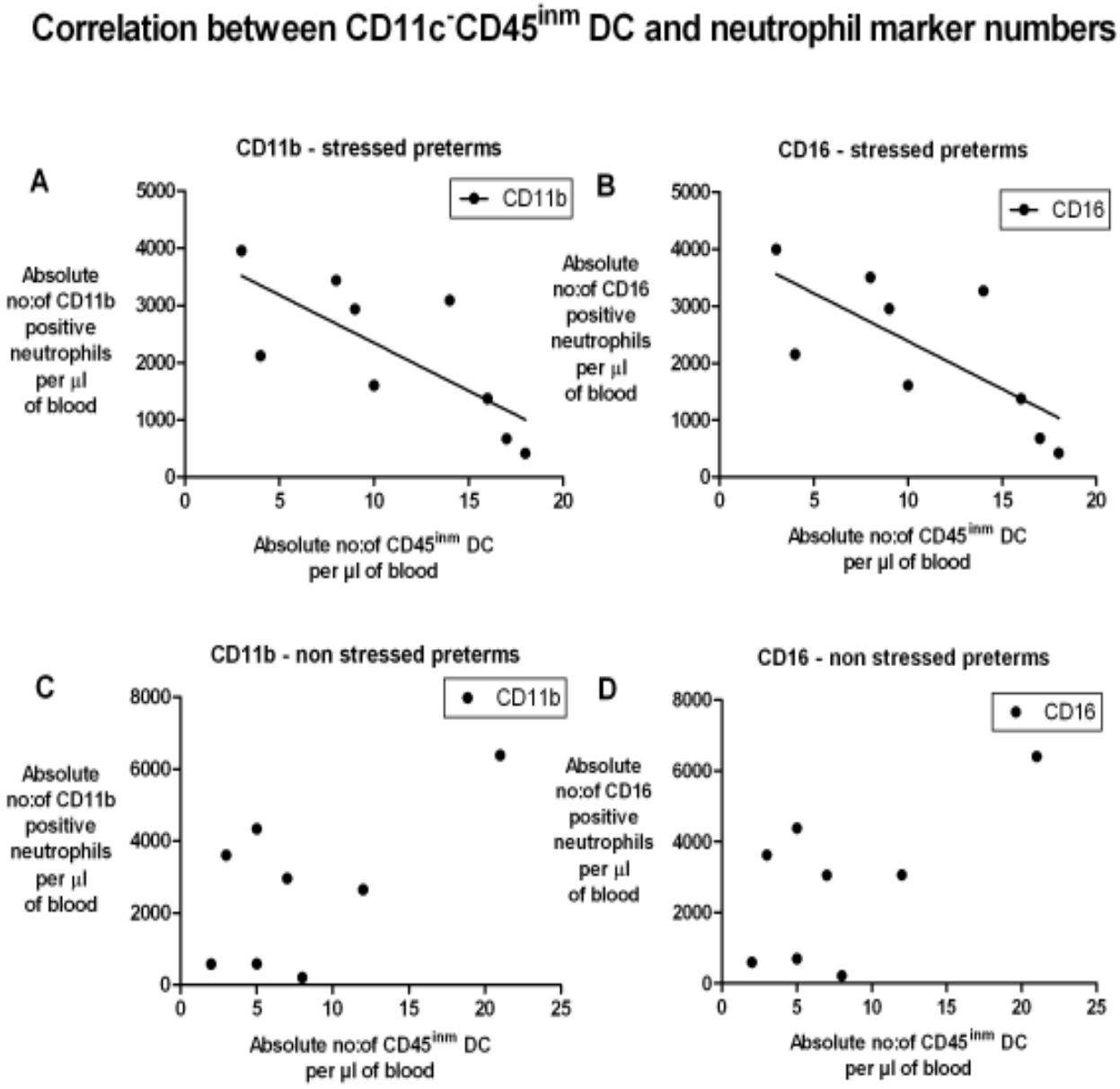


Figure 4.4 Multigraph showing correlation between neutrophilic markers and CBDC numbers

The above multigraph with correlation point plots show the change in absolute numbers of CD11b and CD16 together with the change in numbers of the $CD11c^{-}CD45^{inm}$ CBDC population on exposure to stressors. Pearson Product Moment Correlation was used as the data passed the test of normality

Table 4.1 Data on analyses of correlation

Parameters of analyses	Stressed preterm		Non stressed preterm	
	CD11b	**CD16**	**CD11b**	**CD16**
Correlation coefficient - *r*	-0.7430	-0.7278	0.6163	0.6306
p value	0.0218	0.0262	0.1037	0.0937

Correlation coefficients of the change in absolute numbers of CD11b and CD16 with the change in numbers of DC in the $CD11c^{-}CD45^{inm}$ CB population are shown above

Of the three identified CBDC populations, the $CD11c^{-}CD45^{inm}$ CBDC population was found to be the most responsive to stressor stimuli (see **chapter 6**). Thus the numbers of this CBDC population was used to analyse the correlation with numbers of CD11b and CD16 on exposure to stressors. Comparison was made within the preterm samples (stressed and non stressed). The term stressed and non stressed samples could not be compared as term stressed samples were not available for the current study.

Figures 4.4A and 4.4B show that in the preterm stressed CBs the numbers of CD11b and CD16 showed a significant correlation with DC numbers. The numbers of both neutrophilic activation markers decreased on exposure to infection and correlated with the increase in DC numbers on exposure to stressors (see **chapter 5** for the change in DC numbers). The preterm non stressed samples showed no such correlation (Figures 4.4C and 4.4D).

It can be seen that the neutrophilic markers indicate exposure to stressor stimuli in the same subset of CB samples classified as stressed and analysed for DC phenotypic and functional changes. Hence the justifiable conclusion can be drawn that the additional parallel changes seen on another cellular marker validates the clinical classification of CB samples into those exposed and not exposed to stressors.

4.4.3 Analysis of neutrophil activation markers

Alterations in the neutrophilic activation markers were assessed with age and on exposure to stressors.

4.4.3.1 Alterations of the CD11b marker with age

Table 4.2 Data on change in CD11b levels with age

Origin of sample	Mean+/-SD			No : of samples (*n*)
	Absolute numbers	% positive	PCI Ratio	
Adult	3149.94+/-1423.97	99.24+/-0.98	58.88+/-44.82	18
Term fetus	6012.82+/-1737.17	98.17+/-2.67	56.31+/-41.45	11
Preterm fetus	2301.83+/-1700.92	95.93+/-5.68	26.48+/-34.24	18

Absolute numbers of neutrophils positive for CD11b expression, percentage positive and PCI ratios are given above

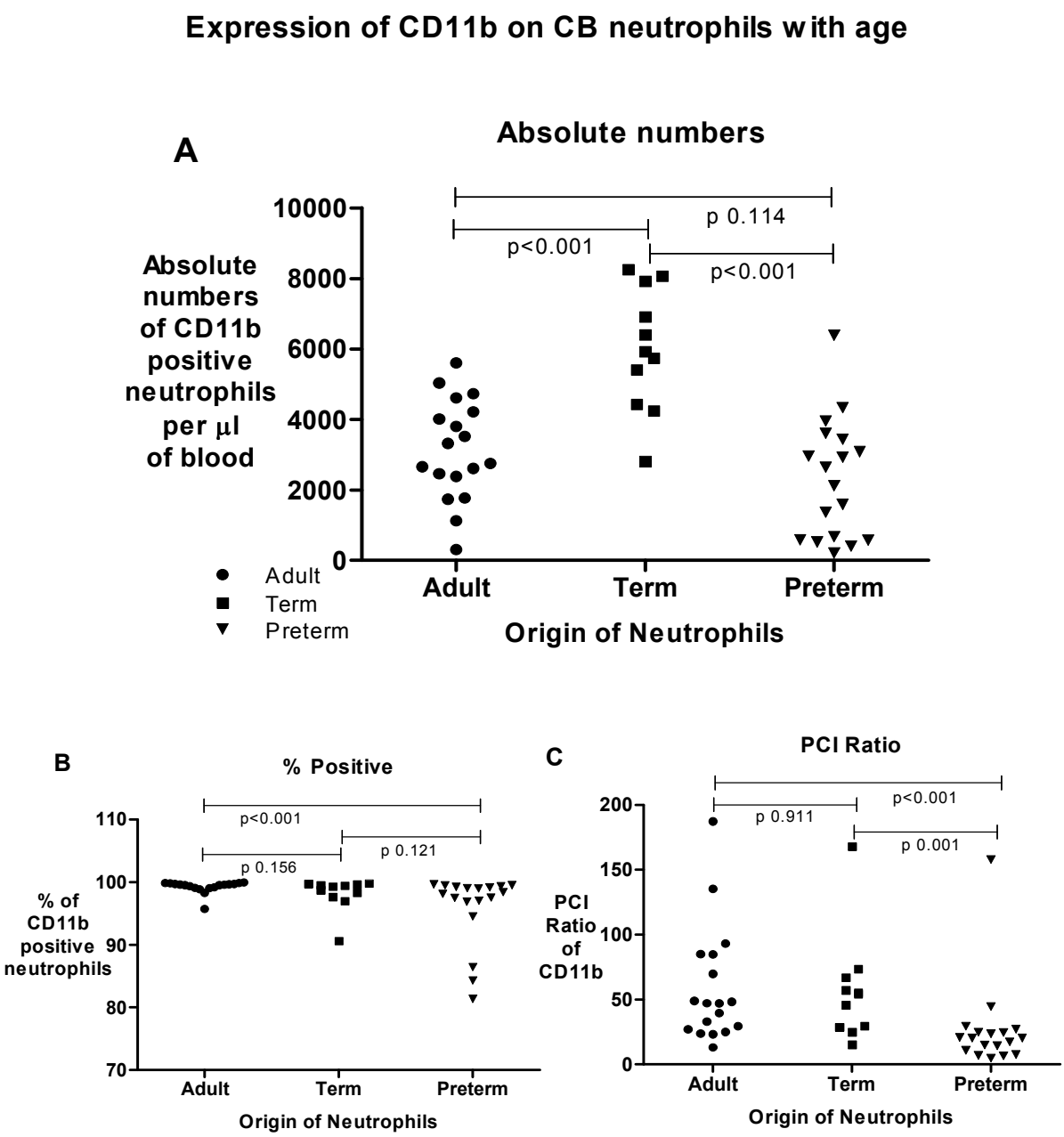


Figure 4.5 Change in CD11b expression with age

Multigraph with dot plots and *p* values delineating the expression of CD11b in adult, term and preterm fetuses. Absolute numbers of CD11b positive neutrophils (Figure 4.5A),

percentage positive (Figure 4.5B) and PCI ratios (Figure 4.5C) are given. Both stressed and non stressed samples were included in the fetal samples

Figure 4.5 exhibit that the preterm samples significantly expressed the lowest levels of CD11b in all three parameters of absolute numbers, percentage positive and PCI ratios. The expression increases with advancing age. The absolute numbers were highest in term samples and decreases again significantly in the adult (Figure 4.5A and Table 4.2).

The percentage of positive cells and the PCI ratios were highest in adult samples. Although adults showed the highest expression in the above two parameters the increase from term samples to adult samples was not statistically significant (Figures 4.5B, 4.5C and Table 4.2).

4.4.3.2 Alterations of the CD11b marker on exposure to stressors

As discussed above a subset (*n*-29) of the 37 stressed/non stressed samples used for DC analyses was used to analyse the changes in CD11b expression on exposure to stressors (term non stressed: *n*-11; preterm non stressed: *n*-9; preterm stressed: *n*-9). All the term samples used were non stressed.

Table 4.3 Data on change in CD11b levels with stressors

Origin of sample	Mean+/-SD			No : of samples (*n*)
	Absolute numbers	% positive	PCI Ratio	
Term non stressed	6012.82+/-1737.17	98.17+/-2.67	56.31+/-41.45	11
Preterm non stressed	2428.78+/-2132.78	93.32+/-7.13	37.04+/-46.4	9
Preterm stressed	2174.89+/-1250.16	98.53+/-1.59	15.92+/-9.35	9

Absolute numbers of neutrophils positive for CD11b expression, percentage positive and PCI ratios are given above

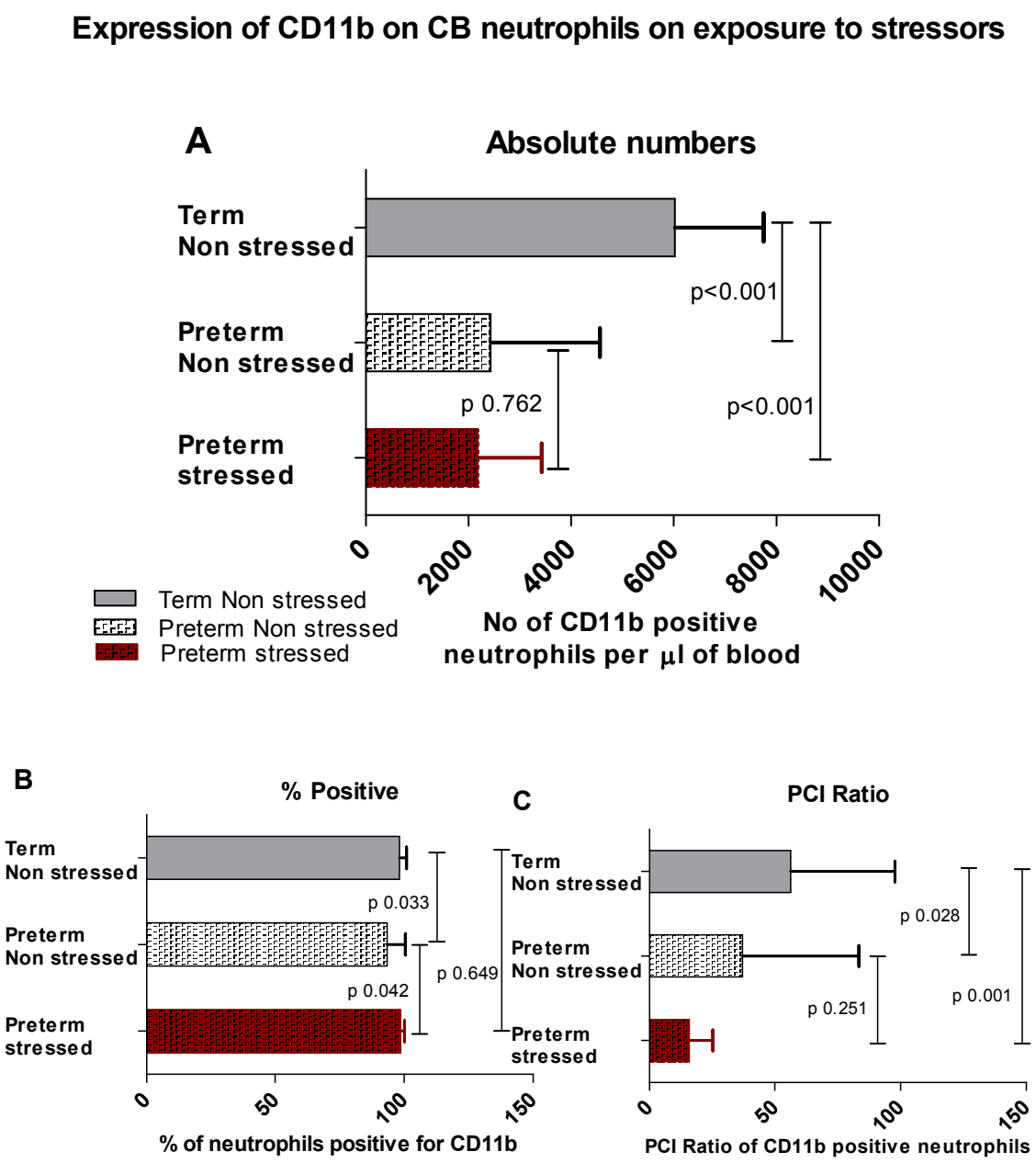


Figure 4.6 Change in CD11b expression with stressors

Multigraph with column bars and *p* values showing CD11b expression on preterm stressed, preterm non stressed and term non stressed CB

The absolute numbers and PCI ratios showed similar results with the preterm stressed samples having the lowest expression of CD11b (Figures 4.6A, 4.6C and Table 4.3). The values did not reach statistical significance on comparison with the preterm non stressed samples, and this could be an effect of the very small numbers of samples.

There was an age related increase with the term samples expressing more CD11b than the preterm non stressed samples in absolute numbers and PCI ratios.

The percentages of positive cells for CD11b were highest in the preterm stressed samples, this could be a result of a change in another cell population and not thought to be significant (Figure 4.6B and Table 4.3).

4.4.3.3 Alterations of the CD16 marker with age

Table 4.4 Data on change in CD16 levels with age

Origin of sample	Mean+/-SD		No : of samples (*n*)
	Absolute numbers	% of viable cells	
Adult	3322.39+/-1238.2	66.93+/-10.68	18
Term fetus	6138.55+/-1863.5	57.33+/-12.53	11
Preterm fetus	2367.89+/-1709.9	32.24+/-19.52	18

Absolute numbers of neutrophils positive for CD16 expression and percentage positive neutrophils of total number of viable cells are given above

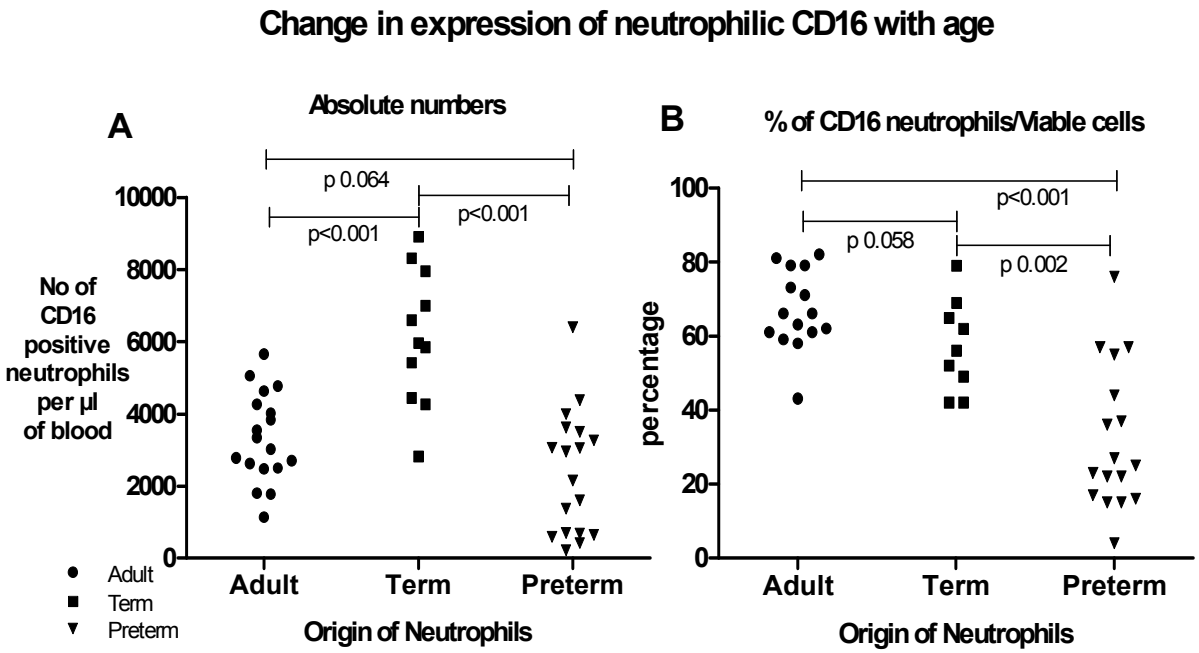


Figure 4.7 Change in CD16 expression with age

Multigraph with column bars and *p* values delineating CD16 expression in adult, term and preterm fetuses. Absolute numbers of CD16 positive neutrophils (Figure 4.7A) and percentage positive in total number of viable cells (Figure 4.7B) are given. Both stressed and non stressed samples were included in the fetal samples

The change in absolute numbers of neutrophils expressing CD16 was analysed with advancing age. Additionally the percentage of CD16 positive neutrophils in total number of viable cells have been assessed as fetal CB was found to have increased number of viable cells when compared to adult PB (see **chapter 5, section 5.4.2.4**). The total number of viable cells in the CB was significantly increased when compared to the adult. There was also a significant increase with advancing gestational age with term samples having more number of viable cells than preterm samples (fetus: mean-7630.89+/-3960.52, n-37 [preterm: mean- 6530.32+/-3786.48,

n-25; term: mean-9923.75+/-3400.81, n-12]; adult: mean-4409.36+/-1960.57, n-25) (see **chapter 5, Figure 5.7A**).

The absolute numbers showed a significant upregulation from preterm to term (Table 4.4). The numbers decreased significantly in the adult compared to term samples (Figure 4.7A).

The preterm samples showed the least percentage and represented only 32% of the total viable cell number compared to term CB which represented 57% of the total viable cells (Figure 4.7B). Adult samples showed the highest percentage representation of 67% which could be due to the decreased total number of viable cells in the adult.

4.4.3.4 Alterations of the CD16 marker on exposure to stressors

As discussed above a subset (*n*-29) of the 37 stressed/non stressed samples used for DC analyses was used to analyse the changes in CD16 expression on exposure to stressors (term non stressed: *n*-11; preterm non stressed: *n*-9; preterm stressed: *n*-9). All the term samples used were non stressed.

Table 4.5 Data on change in CD16 levels with stressors

Origin of sample	Mean+/-SD		No : of samples (*n*)
	Absolute numbers	% of viable cells	
Term non stressed	6138.55+/-1863.5	57.33+/-12.53	11
Preterm non stressed	2520.78+/-2125.49	37.38+/-24.84	9
Preterm stressed	2215+/-1281.68	27.67+/-13.12	9

Absolute numbers of CD16 positive neutrophils and percentage positive neutrophils of total number of viable cells are given above

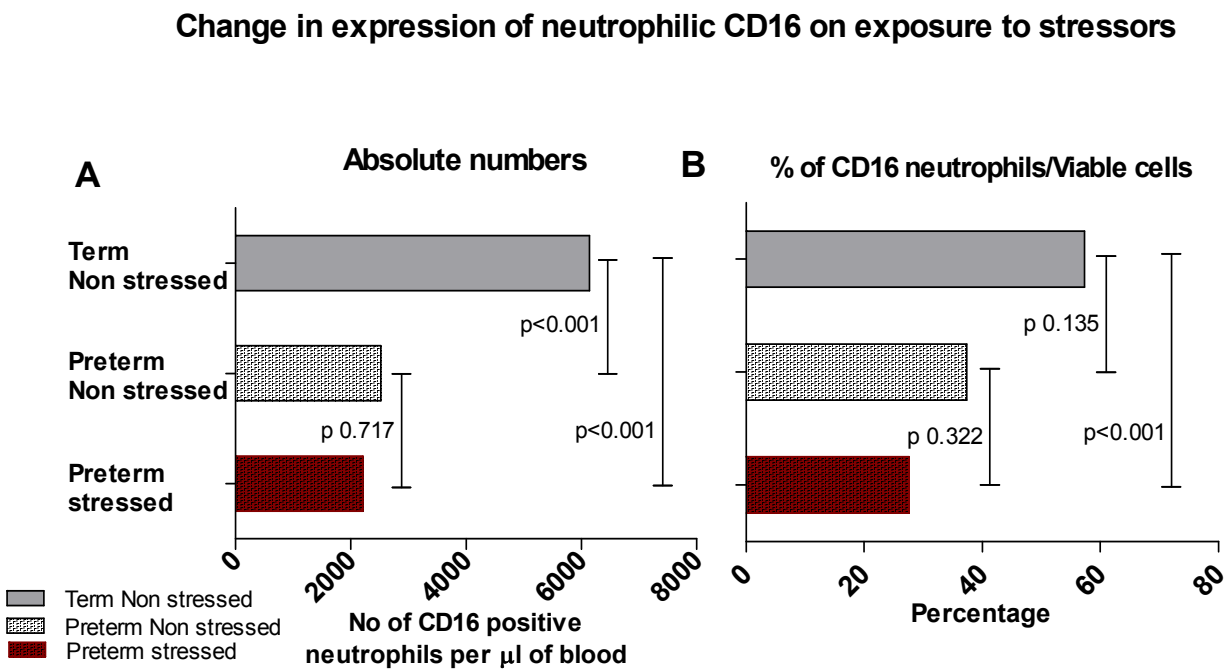


Figure 4.8 Change in CD16 expression with stressors

Multigraph with column bars and *p* values showing CD16 expression on preterm stressed, preterm non stressed and term non stressed CB samples

The preterm stressed samples showed the lowest expression of CD16 in absolute numbers when compared with that of term but the results did not achieve statistical significance when comparing stressed vs non stressed. There was also an age related increase with the term samples expressing more CD16 than the preterm non stressed samples in absolute numbers (Figure 4.8A and Table 4.5).

The percentages showed the preterm stressed samples to be the lowest proportion at 27.67% compared with that of term, but did not achieve statistical significance on comparison with the preterm non stressed samples (Figure 4.8B and Table 4.5).

4.4.4 Alteration in neutrophilic size and granularity with age and stressors

As infection and stress are high turnover states and result in neutrophil activation, which is reflected in the decrease in size and granularity of neutrophils, we have analysed the size and granularity by flow cytometry. FSC assessed the size of the neutrophil and SSC assessed the granularity. Geometric means of the single parameter histograms of the above were analysed for the changes in size and granularity.

4.4.4.1 Alterations in neutrophilic size

Table 4.6 Data on alterations in neutrophilic size

Change with age	**Absolute numbers (mean+/-SD)**	**No : of samples (*n*)**
Adult	57.54+/-7.5	18
Term	61.68+/-10.57	11
Preterm	60.61+/-11.82	18
Exposure to stressors	**Absolute numbers (mean+/-SD)**	**No : of samples (*n*)**
Term non stressed	61.68+/-10.57	11
Preterm non stressed	63.19+/-10.66	9
Preterm stressed	57.39+/-13.12	9

Neutrophilic size at different age groups and with stressors are given above

There was no significant difference in the size of the neutrophils with advancing age on comparison between the three groups of preterm, term and adult (p 0.516).

There was also no significant difference in the size of the neutrophils with exposure to stressors on comparison between the three groups of preterm stressed, preterm non stressed and term non stressed CB samples (*p* 0.551).

4.4.4.2 Alterations in neutrophilic granularity

Table 4.7 Data on alterations in neutrophilic granularity

Change with age	**Absolute numbers (mean+/-SD)**	**No : of samples (*n*)**
Adult	156.79+/-42.56	18
Term	163.26+/-44.84	11
Preterm	138.5+/-39.5	18
Exposure to stressors	**Absolute numbers (mean+/-SD)**	**No : of samples (*n*)**
Term non stressed	163.26+/-44.84	11
Preterm non stressed	151.3+/-28.15	9
Preterm stressed	122.51+/-47.44	9

Neutrophilic granularities at different age groups and with stressors are given above

There was no significant difference in neutrophilic granularity with advancing age on comparison between the three groups of preterm, term and adult (*p* 0.251).

There was also no significant difference in the neutrophilic granularity with exposure to stressors on comparison between the three groups of preterm stressed, preterm non stressed and term non stressed CB samples (*p* 0.112).

4.4.5 Differences in markers of neutrophil activation with labour

There is evidence in the literature to support the hypothesis that labour itself could activate neutrophils (Weinschenk, *et al.,* 1998; Gronlund, *et al*., 1999). Hence the change in expressions of both CD11b and CD16 were analysed and compared between CB of neonates who underwent labour with those who were delivered prior to the start of labour.

Table 4.8 Data on CD11b and CD16 alterations with labour

Samples	Absolute numbers (mean+/-SD)		No : of samples (*n*)
	CD11b expression	CD16 expression	
Labourers	3762.53+/-2469.38	3841.37+/-2465.98	19
Non labourers	3608.6+/-2655.6	3716+/-2827.3	10

Absolute numbers of neutrophils expressing CD11b and CD16 in labourers and non labourers are given above

There was no change in CD11b expression (p 0.878) and CD16 expression (p 0.902) with the difference in labour.

4.5 DISCUSSION

This study confirms a statistically significant negative correlation between the neutrophilic activation markers numbers and $CD11c^{-}CD45^{inm}$ DC numbers in the same CB samples classified as stressed and used for the DC studies. Evidence in literature supports a downregulation of the neutrophilic activation markers; CD11b and CD16 in infectious states. There was a decrease of the neutrophilic activation markers; CD11b and CD16 in the same CB samples that exhibited the rise in CBDC numbers and these findings confirm the exposure to infectious/stressor stimuli in these CB samples and validate the above clinical classification.

Analysis was done on whole blood without cell separation in order to mimic the *in vivo* environment as closely as possible. Studies using cell separation techniques have the attendant risk of loss of cell populations which has been avoided by analysing whole blood. Centrifugation on density gradients have been shown to upregulate surface antigens, especially CD16, CD45 and the CD18 integrins (Kuijpers, *et al*., 1991). Flow cytometric techniques have been used throughout to measure the phenotypic expressions. Unlike the use of morphological criteria to identify cells, the use of monoclonal antibodies enables the accurate and objective analysis and eliminates observer variation. Heparinised tubes were used for blood collection as EDTA has been shown to activate CD11b expression (Repo, *et al*., 1995). All the blood samples have been analysed within two hours of collection and at stable room temperature as studies have shown that CD11b is spontaneously upregulated after two hours in room temperature (Weirich, *et al*., 1998). It is submitted that the results of the investigations using these methods reflect the *in vivo* state, without any modulations.

Our analysis did not show a statistically significant change in the expression of CD11b on neutrophils when fetuses had been exposed to intrauterine stress or infections. This could be due to the small numbers of samples in the study. Previous studies show decreased bactericidal and phagocytic activity of stressed new born infants (Wright, *et al*., 1975; Cui, *et al*., 2003). The functional deficiency of the neonatal immune system, in particular the activation of the neutrophils, is reflected in the decreased upregulation of the CD11b integrin and is proven by the immune response of neutropenia to sepsis seen in the neonate as opposed to the neutrophilia mounted by the more mature adult immune system (Carr, 2000). Evidence in literature points to a modulation of neutrophilic surface receptors expression across the continuum of sepsis, and enhanced or decreased expression is dependant on the stage of the disease. In the early stages of sepsis, studies have identified an upregulation of CD11b indicating an active immune system (Weirich, *et al*., 1998; Nupponen, *et al*., 2001). In profound sepsis especially with the added conundrum of an immature immune system with prematurity, a significant downregulation has been identified (Wright, *et al*., 1975; Cui, *et al*., 2003; Huang, *et al*., 2009).

Analysis of the CD16 marker paralleled the CD11b analysis. Studies have shown that in patients with severe bacterial infections CD16 is partially lost from the surface (Wagner, *et al*., 2003). Carr and Davies showed that stress (respiratory distress syndrome or sepsis) in extremely premature babies was associated with a reduction of neutrophilic CD16 surface expression that persisted over at least the first 3 weeks of post-natal life (Carr and Davies, 1990). This finding correlates with the shedding of the antigen from the neutrophil surface on neutrophil activation due to an immunogenic stimulus.

To further understand the mechanics of the fetal immune system we have analysed the differences in the neutrophilic expression of CD11b and CD16 seen with advancing age. The

analysis of CD11b expression on term and preterm neutrophils revealed an upregulation in the term CB. The numbers of CD11b positive neutrophils decrease in the adult but the percentage of positive cells and their PCI ratio is higher when compared to preterm CB. This again indicates the comparative greater functional maturity of the adult neutrophils. Thus the alterations of neutrophilic CD11b with age indicated the functional immaturity and decreased priming of the fetal neutrophils compared to the adult blood. It also indicated an ongoing process of maturation along with fetal development and increasing gestation, as the term neutrophils express more CD11b than the preterm blood.

Analysis of CD16 expression with age was similar and revealed an upregulation in term CB compared to preterm CB. This finding is in line with the reasons expanded on above and indicates that premature neutrophils are functionally immature and mature with increasing gestation. Henneke and Berner in their study showed that CD16 expression can be significantly lower in polymorphonuclear cells from very premature neonates as compared with term neonates, and the expression rises towards adult levels over the first post-natal weeks (Henneke and Berner, 2006).

Previous studies have shown the mode of delivery and length of labour to affect neutrophil activation (Weinschenk, *et al.,* 1998; Gronlund, *et al*., 1999; Banasik, *et al*., 2000). In our study there were no significant differences seen with the mode of delivery in the expression of CD11b and CD16 in any of our group of neonates, but this could be due to the small numbers of samples.

As infectious states result in a high neutrophil turnover and increased apoptosis we have analysed the neutrophilic size and granularity according to their light scatter properties. The enzymes in the intracellular neutrophilic granules play the central role in phagocytosis and

degranulation indicates an activated functional neutrophil (Stephen, *et al*., 1999). Also apoptotic cells have reduced forward scatter properties due to their loss of water and they become less granular resulting in reduced side scatter (Gorman, *et al*., 1997). There is a 10-100 fold increase in neutrophilic turnover during acute infections (McAfee and Thakur, 1976) and ageing neutrophils develop morphological features typical of apoptosis (Whyte, *et al*., 1993). Hence we investigated the SSC and FSC properties of the adult and CB neutrophils. In our study there were no significant differences with age or exposure to stressors, but again this could be due to the small numbers of samples.

The phenotypic and functional immaturity of the fetal and neonatal immune system has been evidenced in numerous studies and contributes to the higher incidence of infections seen in neonates compared to adults. Preterm neonates of 28 to 34 weeks gestational age exhibit decreased polymorphonuclear opsonic capacity, phagocytosis and respiratory burst compared with term neonates and adults, and this may be worse in very premature neonates (Drossou, *et al*., 1997; Bialek and Bartmann, 1998; Kallman, *et al*., 1998; Henneke and Berner, 2006). It has been shown that the regulation of phenotypical parameters such as the adhesion molecules, CD11b and L-selectin indicates upregulation of the functional status or increasing maturity on activation by infectious or immunogenic stimuli. Thus analysing the changes of CD11b expression would serve to identify the group of babies exposed to infectious stimuli, prior to the onset of clinical infection. Cui *et al* showed that the detection of CD11b (</= 600 MFI) for suspected sepsis showed a sensitivity of 86.3%, a specificity of 100%, a positive predictive value of 100%, and a negative predictive value of 68.2%. The positive rate of CD11b detection was 86.3%, which was higher than the blood culture test ($p< 0.05$) (Cui, *et al*., 2003).

There was a statistically significant negative correlation between the neutrophilic activation markers numbers and $CD11c^{-}CD45^{inm}$ DC numbers in the same CB samples classified as stressed and used for the DC studies, indicating a real pathology in the chosen samples. Thus for the purposes of this study, the significant correlation between the changes in neutrophilic markers and CBDC numbers in the CB samples classified as stressed validates the clinical classification and adds credence to the CBDC studies discussed in the later chapters.

CHAPTER 5

RESULTS III

ALTERATIONS OF CORD BLOOD DENDRITIC CELL POPULATIONS WITH AGE AND EXPOSURE TO 'STRESSORS'

5.1 SUMMARY

The myeloid and plasmacytoid subpopulations of DCs in adult PB and CB and the additional $CD11c^{-}CD45^{inm}$ CBDC subpopulation were identified and their phenotypic and functional characteristics established previously. The aim of this study was to assess the changes on the CBDC subpopulations with advancing age and exposure to stressors. Analysis was performed to assess the change in the expression of the maturation markers, CD45 and HLA-DR and the change in numbers.

All analysis was performed using labelling with monoclonal antibodies and flow cytometry acquisition.

Analysis of the expression of maturation markers showed the myeloid and plasmacytoid CBDC and adult PB DC populations to express similar amounts of CD45 and HLA-DR. The $CD11c^{-}CD45^{inm}$ DCs were immature compared with the myeloid and plasmacytoid subset, expressing significantly lower levels of CD45 and HLA-DR. There was no significant change in the expression of CD45 and HLA-DR with age and on exposure to stressors in the fetal CBDCs and adult PB DCs.

Analysis of the numbers showed that the myeloid $CD11c^{+}CD45^{hi}$ DC subset predominated in adult PB when compared to CBDC. There was no significant change seen in the $CD11c^{-}CD45^{hi}$ plasmacytoid DC subset between adult and CBDC. The myeloid and plasmacytoid CBDC subsets did not change with increasing gestation from preterm to term. The novel $CD11c^{-}CD45^{inm}$ putative CBDC subset decreased significantly with increasing gestational age as they were less prominent in the term fetus compared to the preterm fetus and almost absent in the adult. This subset represented 31.33% of the total number of DC in the preterm CB, but

represented 21.26% of the total number of DC in the term CB samples and only 1.54% of the total number of DC in adult PB.

Taken together, these data indicated that the myeloid and plasmacytoid CBDC populations were comparable in maturity to the adult PB DC subsets. They showed no significant difference in numbers between adult and fetus and no change in numbers with advancing gestational age. Both the subsets increased in numbers on exposure to stressor stimuli. The novel $CD11c^{-}CD45^{inm}$ CBDCs were immature expressing lower levels of CD45 and HLA-DR and their numbers decreased with increasing gestational age. They also significantly increased in numbers on exposure to stressor stimuli. Thus, of the three subsets of CBDCs, the novel $CD11c^{-}CD45^{inm}$ CBDC subset seemed to be the most kinetic, changing with advancing gestation as well as exposure to stressor stimuli.

5.2 INTRODUCTION

CBDCs have been identified by the presence of the cell surface marker HLA-DR and lack of the CD3, CD14, CD16, CD19, CD34, and CD56 markers, as has been followed in the identification of adult DCs. This population was a minority and represents only about 0.3% of CB mononuclear cells (Sorg, *et al*., 1999). The composition and maturity of CBDC subpopulations vary when compared to the adult DC populations. The majority of CBDCs show a plasmacytoid phenotype resulting in an inverted $CD11c^{+}/CD11c^{-}$ ratio with predominant $CD11c^{-}$ DCs, when compared with adult PB DCs; in adults $CD11c^{+}$ DCs dominate with a predominant myeloid phenotype (Borras, *et al*., 2001; Drohan, *et al*., 2004). CBDCs are immature when compared with adult DCs; showing high levels of CD34, low CD4, low CD83, and lower levels of HLA-DR, CD40 and CD86 (Holloway, *et al*., 2000). A lower functional capacity has been attributed to CBDCs when compared to adult DCs. They have a reduced ability to attain a fully mature adult phenotype and to activate $CD4^{+}$ T cells to produce IFN-α (Langrish, *et al*., 2002).

This study investigated the above background literature of differing composition and maturity of CBDC subsets when compared to adults. The aim of this study was to identify and further analyse the changes occurring on CBDC subsets with increasing gestational age and on exposure to stressors. As discussed previously three subsets of CBDCs were identified and of those the $CD11c^{-}CD45^{inm}$ subset was of interest as to date it had not been identified in CB. The immature $CD11c^{-}$ CBDC subsets described in the literature has been identified as either $CD123^{+}$ (plasmacytoid) or $CD123^{dim}$, but this putative subset was negative for the expression of CD123. Thus it was of interest to investigate the evolution of this novel subset and the change in numbers and in maturation markers occurring with age and on exposure to stressors.

It was hypothesised that CBDC populations change with increasing gestational age, during the intrauterine period and into the neonatal period. It was also hypothesised that exposure to 'stressors' such as intrauterine infection and immunogenic stimuli can accelerate and modify these changes. If proven, these changes which occur due to infectious/immunogenic stimuli may serve to identify immature infants exposed to infections *in utero.* The ability of DCs to alter their phenotype and function in response to microbial exposure suggests that changes in CBDC populations may be useful markers of exposure to intrauterine infection or stress and hence the risk of adverse neonatal sequelae (Huang, *et al*., 2001). Also understanding the composition and change in CBDC populations would further our understanding of the genesis and organisation of the immune system.

5.3 SUBJECTS

Blood from the umbilical cord of 25 preterm babies and 12 term babies was analysed for numbers of the DC subsets and their change with age and exposure to stressors. Blood from the umbilical cord of 26 preterm babies and 14 term babies was analysed for expression of CD45 and HLA-DR. Adult peripheral venous blood for analysis was obtained from 32 healthy non-pregnant volunteers. Changes in numbers of the fetal CBDC subsets on exposure to stressor stimuli were evaluated. All the term fetuses evaluated (*n*-12) were not exposed to stressor stimuli *in utero*, while of the preterm fetuses, 17 were exposed to stressor stimuli and 8 were not exposed to stressor stimuli. Detailed discussion about the criteria for classification and validation by neutrophilic activation markers is given in **Chapter 4**.

5.4 RESULTS

5.4.1 Expression of CD45 and HLA-DR on CBDC and adult PB DC population

In the preceding chapter, the CBDC subsets were identified based on their expression of HLA-DR and Lineage cocktail and further identification of subsets were done based on their expression of CD45 and CD11c. The levels of expression of the CD45 and HLA-DR markers was analysed on the CBDC subsets as differential expression by these subsets were noted on the histograms. It was felt that analyses of the levels of expression of CD45 and HLA-DR will give information on the comparative maturation states of the CBDC subsets. The usually studied DC markers of maturation will be analysed in the next chapter.

5.4.1.1 Expressions of CD45 and HLA-DR on the myeloid and plasmacytoid DC populations

Expression of CD45 and HLA-DR was assessed by comparison of the geometric x mean of the positive histograms (*n*-40).

Table 5.1 Data on expression of CD45 and HLA-DR on myeloid and plasmacytoid subsets

	Mean+/-SD		No : of samples (*n*)
	CD45	**HLA-DR**	
Myeloid DC subset			
Adult	2409.06+/-1641.63	640.83+/-459.81	32
Fetus	1904.64+/-1474.94	781.11+/-626.08	40
p values adult vs fetus	0.231	0.360	
Plasmacytoid DC subset			
Adult	2295.06+/-1588.19	612.18+/-496.94	32
Fetus	2037.95+/-1502.52	736.72+/-722.78	40
p values adult vs fetus	0.603	0.449	

Expressions of CD45 and HLA-DR by CBDCs in the various age groups

The myeloid and the plasmacytoid DC subsets in adult and CB expressed similar amounts of CD45 and HLA-DR.

5.4.1.2 Comparison of CD45 and HLA-DR expression on CBDC populations

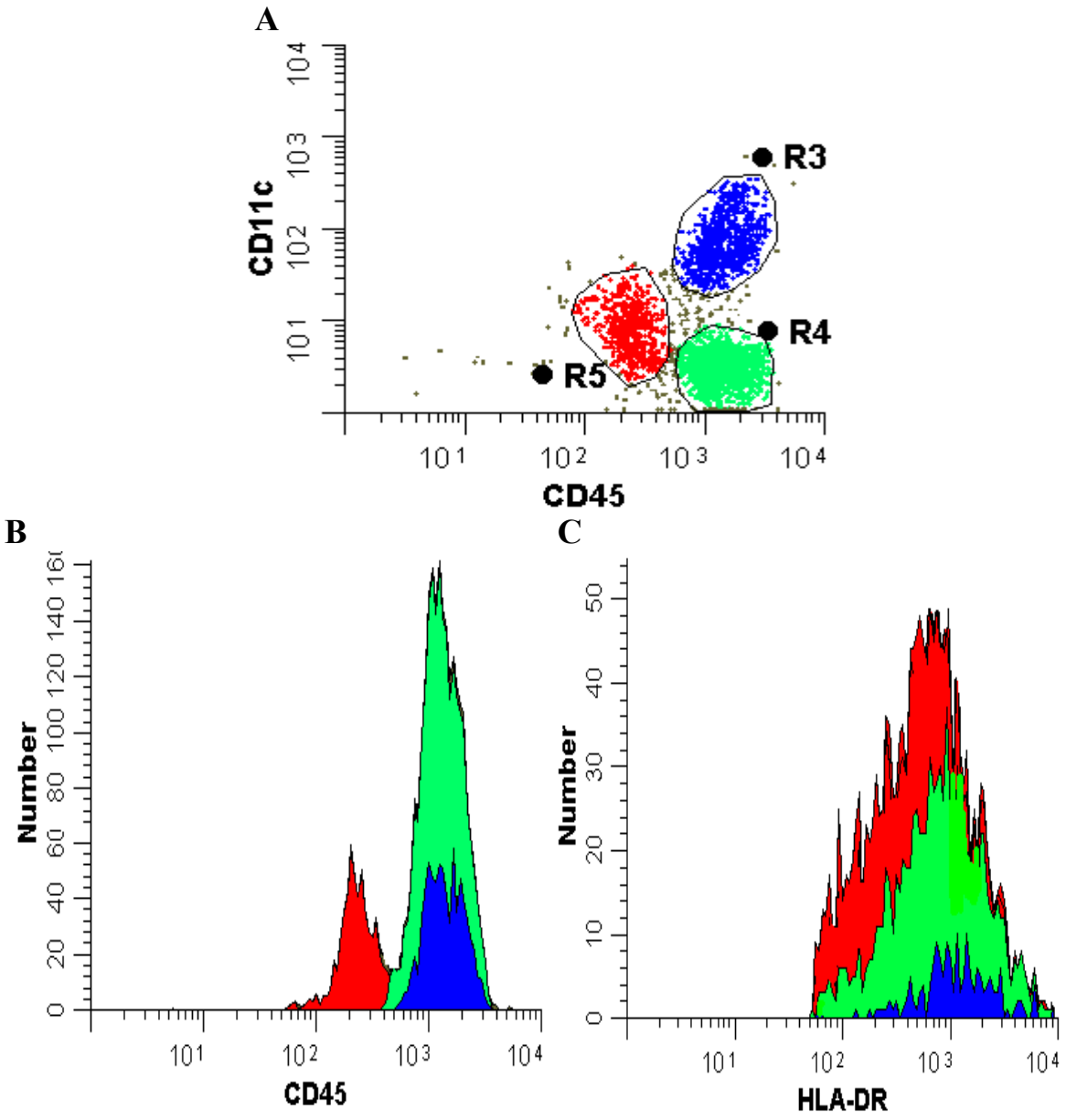


Figure 5.1 Backgated histograms of CD45 and HLA-DR expressions on CBDCs

The two parameter histogram above delineate the three CBDC subsets (R3: $CD11c^{+}CD45^{hi}$, R4: $CD11c^{-}CD45^{hi}$, R5: $CD11c^{-}CD45^{inm}$) (Figure 5.1A) and the backgated single parameter histogram show the expressions of CD45 (Figure 5.1B) and HLA-DR (Figure 5.1C). Representative histograms shown from 40 experiments

The back gated histograms gave a clear visual impression of lower level of expression of these maturation markers on the CD11c^{-}CD45^{inm} subset. Comparison of intensity values were made to investigate the above observation further.

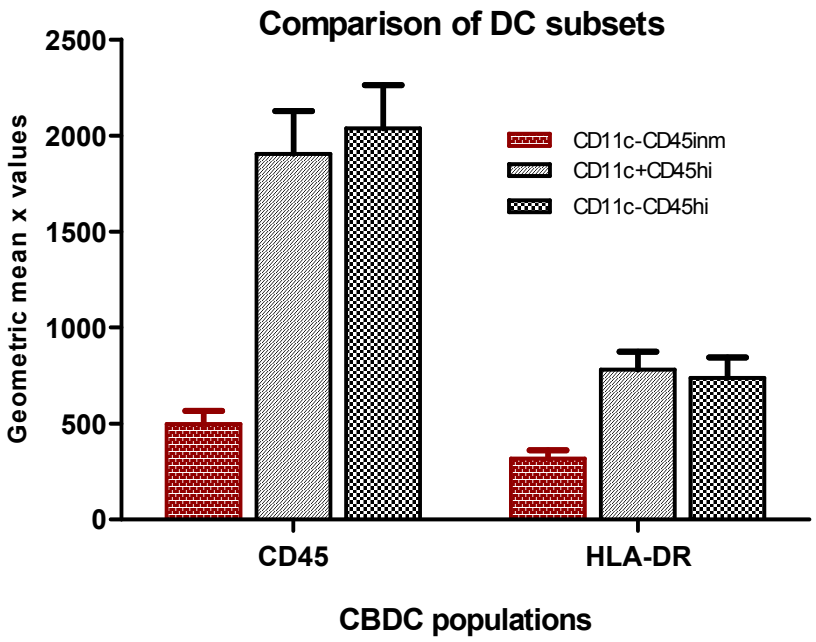


Figure 5.2 Levels of CD45 and HLA-DR on the CBDC subsets

Bar graphs of CD45 and HLA-DR levels on the three CBDC subsets are shown. Expression was assessed by comparison of the geometric x mean of the positive histograms (*n*-40). Mean values with standard error shown (CD11c^{-}CD45^{inm}: CD45 p<0.001; HLA-DR p<0.001; myeloid vs plasmacytoid: CD45 p 0.602; HLA-DR p 0.512)

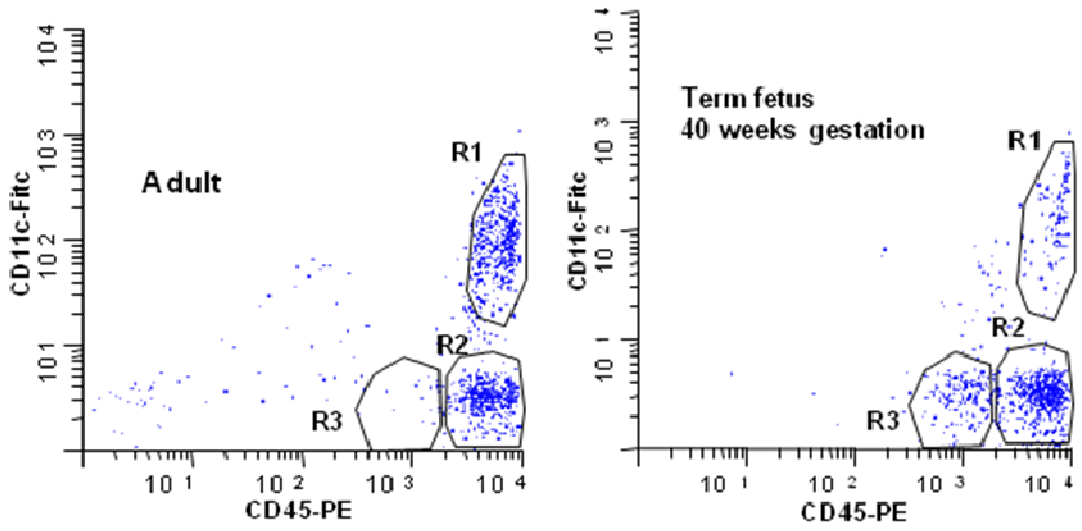
The results show that CD11c^{-}CD45^{inm} DCs expressed significantly lesser amounts of both CD45 and HLA-DR when compared with the myeloid and plasmacytoid DC populations (CD45: mean-497.34+/-460.53, p<0.001; HLA-DR: mean-318.95+/-274.52, p<0.001). Both the myeloid and plasmacytoid subsets expressed higher amounts and were similar (CD45: p 0.602; HLA-DR: p

0.512). Taken together the data expressed in Figures 5.1 and 5.2 suggest that the $CD11c^{-}CD45^{inm}$ DCs were immature compared to the myeloid and plasmacytoid CBDC populations.

5.4.2 Alterations in numbers of CBDC populations with age

The changes seen in numbers of cells were analysed in the CBDC subsets and also on adult PB DCs for comparison.

5.4.2.1 $CD11c^{+}CD45^{hi}$ DC population predominated in adult PB compared to fetal CB



A **B**

Figure 5.3 CD11c versus CD45 histogram of CBDC populations

Two parameter histograms shown of the adult PB (Figure 5.3A) and CBDC subsets (Figure 5.3B) based on their expression of CD45 and CD11c phenotypic markers. Representative histograms shown from 43 experiments

Figure 5.3 showed an apparent decrease in the myeloid subset in the term fetus when compared to adult PB. This was analysed further. Both absolute numbers as well as the percentage of total viable cells and of the total number of DCs that the subset represented were compared. Comparison was made between adult, term and preterm samples to assess the change with advancing age.

Table 5.2 Data on change in myeloid CBDCs with age

Origin of sample	**Mean+/-SD**			**No : of samples (*n*)**
	Absolute numbers	**% of viable cells**	**% of total DC**	
Adult	20.88+/-12.22	0.58+/-0.52	52.88+/-17.94	25
Term fetus	17.83+/-8.99	0.24+/-0.30	32.97+/-8.04	12
Preterm fetus	28.32+/-37.41	0.61+/-0.91	29.44+/-14.34	25
Fetus (term & preterm)	24.92+/-31.34	0·48+/-0·78	31.20+/-12.62	37

The difference in numbers of $CD11c^{+}CD45^{hi}$ DCs with age is shown in absolute numbers, percentage of total viable cells and percentage of total DCs

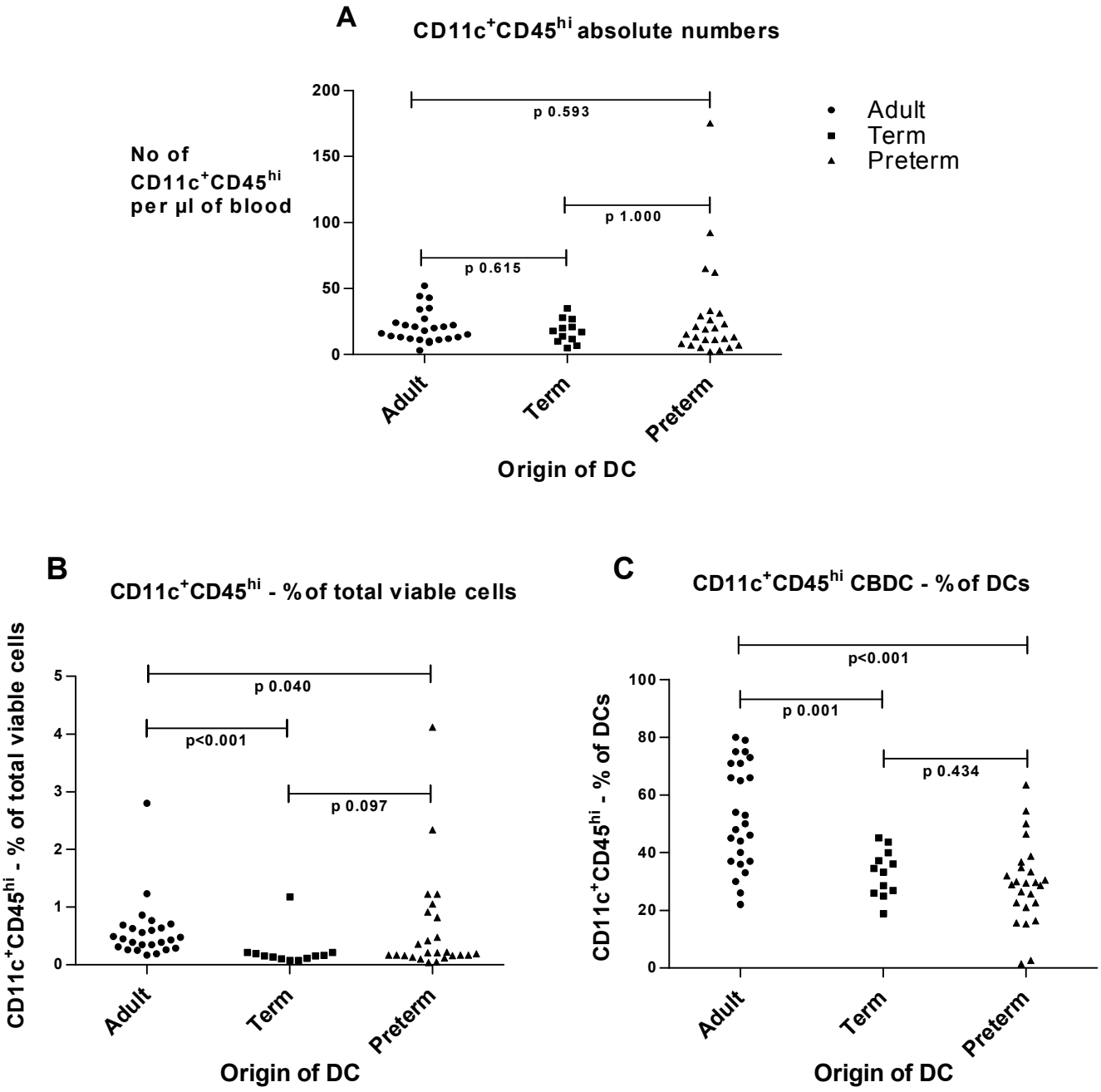


Figure 5.4 Differences in CD11c^{+}CD45^{hi} subset with age

Multigraph with point plots and *p* values showing the difference in the numbers of myeloid DCs as absolute numbers, percentage of total viable cells and percentage of total DCs

The absolute numbers of $CD11c^{+}CD45^{hi}$ DC did not show a significant difference between adult, preterm and term CB (Figure 5.4A).

The percentage of $CD11c^{+}CD45^{hi}$ DC in the total viable cells showed a significant difference between adult and preterm and between adult and term. Percentage was highest in preterm CB (Figure 5.4B).

The percentage of $CD11c^{+}CD45^{hi}$ DC in the total number of DC showed a significant difference between adult and preterm and between adult and term. Percentage was highest in the adult and this subset formed 52.88% of the total adult DCs (Figure 5.4C).

Comparison was made between adult and fetal samples to assess the change with age. The absolute numbers showed no significant difference (p 0.532). On comparison as proportion of the total number of viable cells (p 0.001) and as proportion of the total number of DCs ($p<0.001$) the myeloid DCs showed predominance in adult blood when compared to CB.

This data indicates that although the differences in absolute numbers of cells did not reach statistical significance, the myeloid population is the predominant DC population in the adult when compared to the fetus.

5.4.2.2 $CD11c^{-}CD45^{hi}$ DC population does not change with age

Table 5.3 Data on change in plasmacytoid CBDCs with age

Origin of sample	Mean+/-SD			No : of samples (*n*)
	Absolute numbers	% of viable cells	% of total DC	
Adult	18.56+/-14.26	0.59+/-0.58	45.58+/-18.38	25
Term fetus	25+/-16.69	0.33+/-0.44	45.77+/-13.22	12
Preterm fetus	43.4+/-59.4	1.17+/-1.84	39.23+/-16.82	25
Fetus (term & preterm)	37.43+/-50.13	0.9+/-1.57	40.74+/-15.86	37

The difference in numbers of $CD11c^{-}CD45^{hi}$ DC subset with age is shown in absolute numbers, percentage of total viable cells and percentage of total DCs

There was no significant change seen in the $CD11c^{-}CD45^{hi}$ DC subset with respect to increasing age and gestational age. There were no significant differences between adult, term and preterm samples as well as between adult and fetus in the above parameters.

P values between term and preterm for change with increasing gestation (absolute numbers: *p* 0.147; % of viable cells: *p* 0.188; % of total DCs: *p* 0.246).

P values between adult and fetus for change with age (absolute numbers: *p* 0.053; % of viable cells: *p* 0.491; % of total DCs: *p* 0.344).

5.4.2.3 CD11c^{-}CD45^{inm} CBDC population decreases with advancing gestational age

Table 5.4 Data on change in CD11c^{-}CD45^{inm} CBDCs with age

Origin of sample	Mean+/-SD			No : of samples (*n*)
	Absolute numbers	% of viable cells	% of total DC	
Adult	0.64+/-1.02	0.01+/-0.02	1.54+/-2.26	25
Term fetus	10.25+/-5.96	0.13+/-0.11	21.26+/-14.71	12
Preterm fetus	40.56+/-53.58	0.59+/-0.77	31.33+/-19.72	25
Fetus (term & preterm)	25.22+/-42.26	0.44+/-0.67	28.06+/-18.66	37

The difference in numbers of CD11c^{-}CD45^{inm} DC subset with age is shown in absolute numbers, percentage of total viable cells and percentage of total DCs (*p* values: Absolute numbers: adult vs term- <0.001, adult vs preterm - <0.001, term vs preterm – 0.039; % of total viable cells: adult vs term - <0.001, adult vs preterm - <0.001, term vs preterm – 0.026; % of total DC – adult vs term - <0.001, adult vs preterm - <0.001, term vs preterm – 0.035)

The two parameter histograms in Figure 5.5 below showed the CD11c^{-}CD45^{inm} CBDC subset to change with advancing gestational age. The subset appeared more prominent in the preterm fetus and less so in the term fetus. This change in numbers seemed to be evolving into the neonatal period as the histogram of the adult DCs revealed the subset to be almost absent.

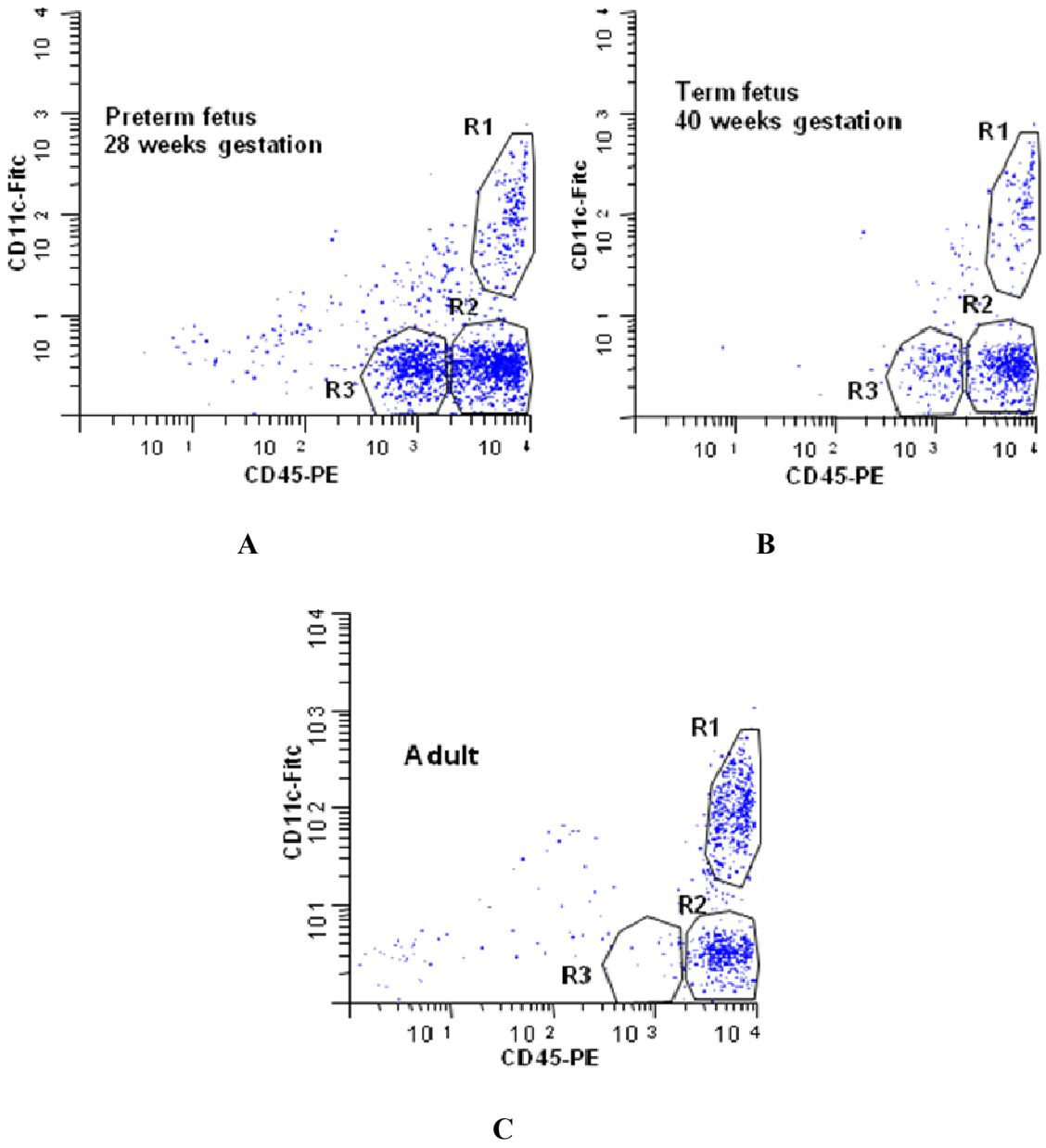


Figure 5.5 CD11c^{-}CD45^{inm} DC numbers decrease with increasing gestational age

In Figure 5.5, this subset (R3) was more pronounced in the preterm fetus and becomes less pronounced as gestation increases (compare subset in R3 in Figure 5.5A with subset in R3 in Figure 5.5B). Additionally the subset was absent in the adult (compare R3 in Figure 5.5A with R3 in Figure 5.5C). Representative histograms shown of 43 experiments

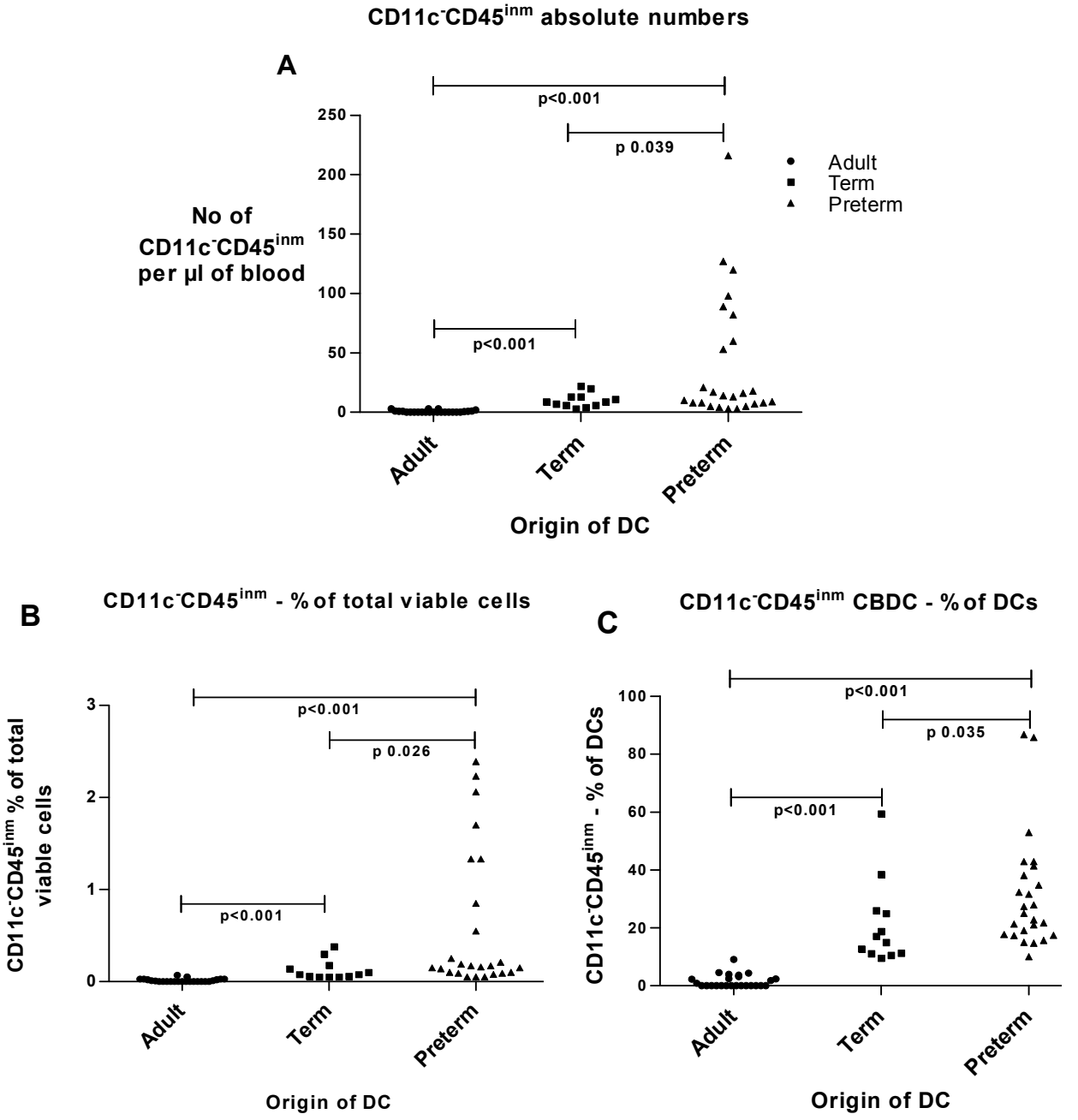


Figure 5.6 Changes in CD11c^{-}CD45^{inm} CBDCs with increasing age

Multigraph with point plots and *p* values showing the difference in the numbers of CD11c^{-} CD45^{inm} DC subset as absolute numbers, percentage of total viable cells and percentage of total DCs

To further ascertain the changes seen in the two parameter histograms, comparison of the absolute numbers of the $CD11c^{-}CD45^{inm}$ subset per µl of blood between the preterm, term CB and adult PB was performed (Figure 5.6A). To rule out the possibility that the increase in numbers observed in both preterm and term CBs was a simple reflection of the increased cellularity of fetal samples, the $CD11c^{-}CD45^{inm}$ numbers was examined as a proportion of total viable cells and as a proportion of total number of DCs (Figure 5.6B and Figure 5.6C).

Comparison of absolute numbers and percentage of viable cells showed a statistically significant decrease with advancing gestational age on comparison between preterm and term samples. The decrease continued after birth as adult blood showed a statistically significant decrease when compared with both preterm as well as term CB. (Figures 5.6A and 5.6B).

There was a significant decrease in the proportions of total DC with gestational age on comparison between preterm and term samples. The proportions showed a significant increase in the preterm as well as the term CB samples, compared with adult PB (Figure 5.6C). This subset represented 31.33% of the total number of DC in the preterm CB, but represented 21.26% of the total number of DC in the term CB samples and only 1.54% of the total number of DC in adult PB (Table 5.4).

Taken together these results highlighted in Figure 5.6 indicate that there is a real decrease in the numbers of the $CD11c^{-}CD45^{inm}$ population with increasing age. The numbers in the fetus decreases towards term and the changes continue into the neonatal and adult period as reflected by their almost absence in the adult.

5.4.2.4 Changes in the total cell numbers with increasing age

To assess the validity of the changes in the different CBDC subsets in relation to global changes in cell numbers with age, changes in the total numbers of viable cells and total number of DCs were analysed between the adult PB and CB samples.

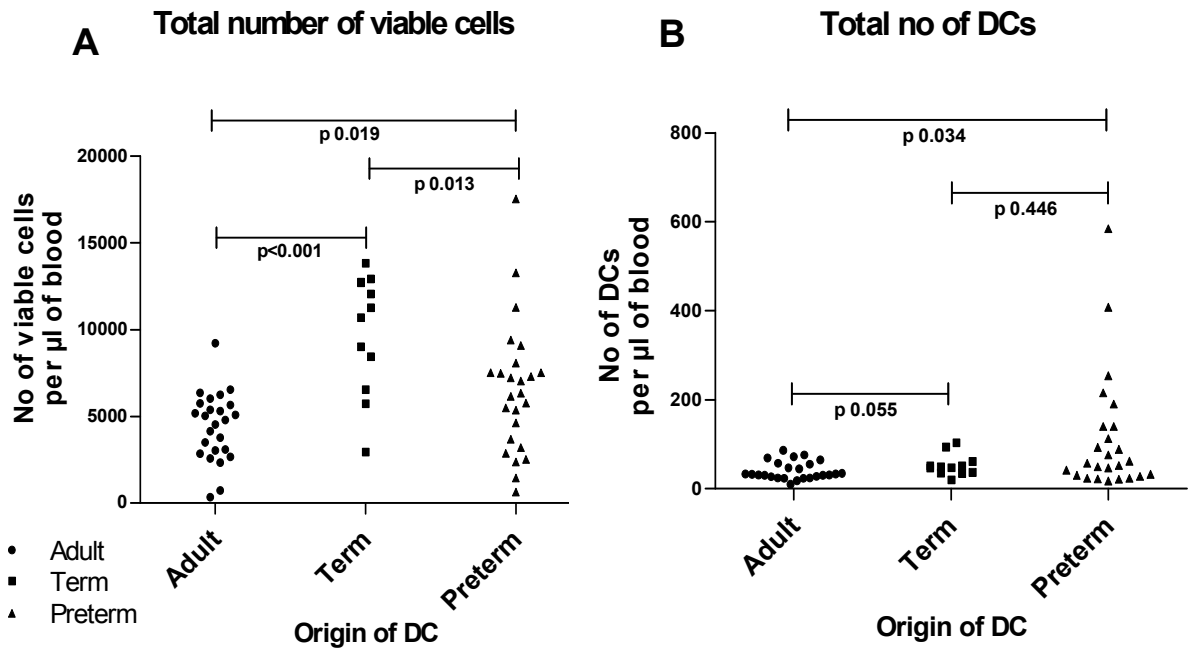


Figure 5.7 Differences in total cell numbers with increasing age

The above point plots with *p* values show the difference in the total number of viable cells and in the total number of DCs in adult PB and CB samples

Table 5.5 Data on change in total no:of viable cells and total no:of DCs with age

Origin of sample	Mean+/-SD		No : of samples (*n*)
	Total no:of viable cells	Total no:of DC	
Adult	4409.36+/-1960.57	40.12+/-20.25	25
Term fetus	9923.75+/-3400.81	53.08+/-24.11	12
Preterm fetus	6530.32+/-3786.48	112.28+/-134.73	25
Fetus (term & preterm)	7630·89+/-3960.52	93.08+/-114.32	37

The total numbers of viable cells and the total numbers of DCs in the various age groups are shown above

Fetal CB was found to have significantly increased cellularity when compared to adult PB. Within the gestational period the cellularity significantly increased with term samples having more number of viable cells than preterm samples (Figure 5.7A).

The total number of DCs was statistically increased in the preterm compared to the adult. But there was no significant difference between adult and term samples as well as between term and preterm samples (Figure 5.7B).

Taken together with the data discussed above, this finding showed that the significant increase in the DC numbers in the preterm is probably a reflection of the increased $CD11c^{-}CD45^{inm}$ subset, as the myeloid and plasmacytoid subsets showed no significant change. The increase in cellularity in the fetus was not a reflection of a change in DC populations, but a probable change in numbers of other cellular groups which was not investigated further in our study.

5.4.2.5 Comparison of the numbers in the DC populations in CB and adult PB

Comparison of the percentages and numbers were made to further understand the composition of CBDCs and adult PB DCs.

5.4.2.5.1 Comparison of percentage composition

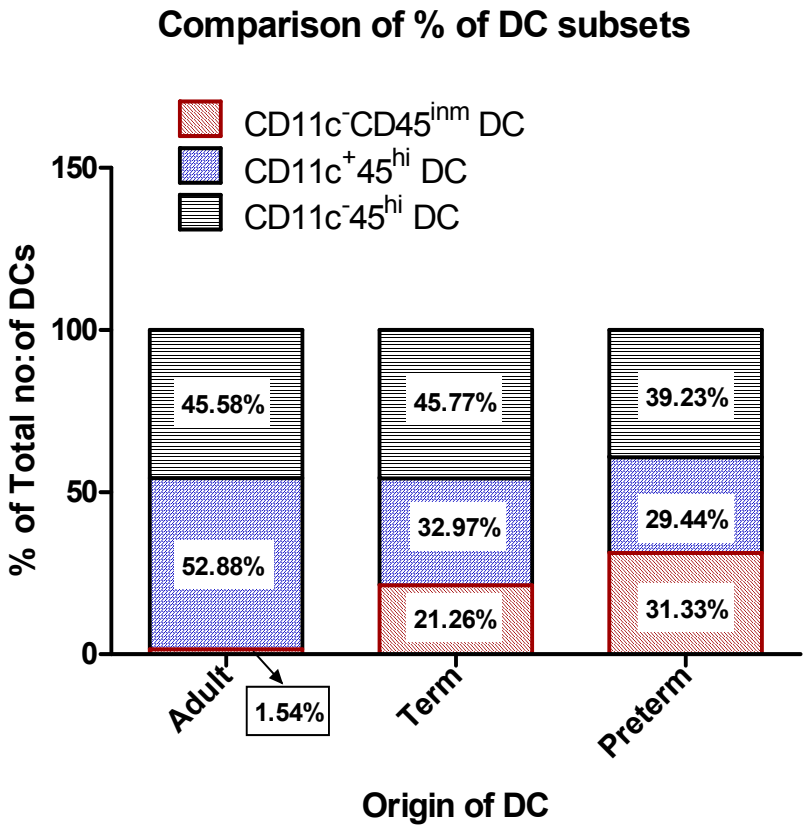


Figure 5.8 Percentage composition of DC subsets in adult, term and preterm

In adults the predominant DC population is myeloid followed by plasmacytoid. Conversely the plasmacytoid DC population predominated in both preterm and term CB. The $CD11c^{-}CD45^{inm}$ population formed a larger percentage in the preterm compared to the term fetus

Table 5.6 Data on proportions of CBDC subsets in total no:of DC

Origin of sample	Mean+/-SD			No : of samples (*n*)
	Myeloid subset	**Plasmacytoid subset**	**CD11c$^-$CD45^{inm} subset**	
Adult	52.88+/-17.94 (52.88%)	45.58+/-18.38 (45.58%)	1.54+/-2.26 (1.54%)	25
Term fetus	32.97+/-8.04 (32.97%)	45.77+/-13.22 (45.77%)	21.26+/-14.71 (21.26%)	12
Preterm fetus	29.44+/-14.34 (29.44%)	39.23+/-16.82 (39.23%)	31.33+/-19.72 (31.33%)	25
Fetus (term & preterm)	31.20+/-12.62 (31.20%)	40.74+/-15.86 (40.74%)	28.06+/-18.66 (28.06%)	37

The percentage of the CBDC subsets in the total number of DCs for the various age groups are given above

As is shown by Figure 5.8 above, the plasmacytoid subset forms similar percentages and predominates in the fetal blood. The myeloid subset forms similar proportion in CB, but predominates in the adult. The CD11c$^-$CD45^{inm} population decreases with increasing age and is seen most predominantly in the preterm fetus and almost absent in the adult.

5.4.2.5.2 Comparison of $CD11c^+/CD11c^-$ ratio

The ratio of $CD11c^+$ to $CD11c^-$ numbers was analysed as literature to date supports a reversal of ratio in fetal circulation with predominance of $CD11c^-$ numbers.

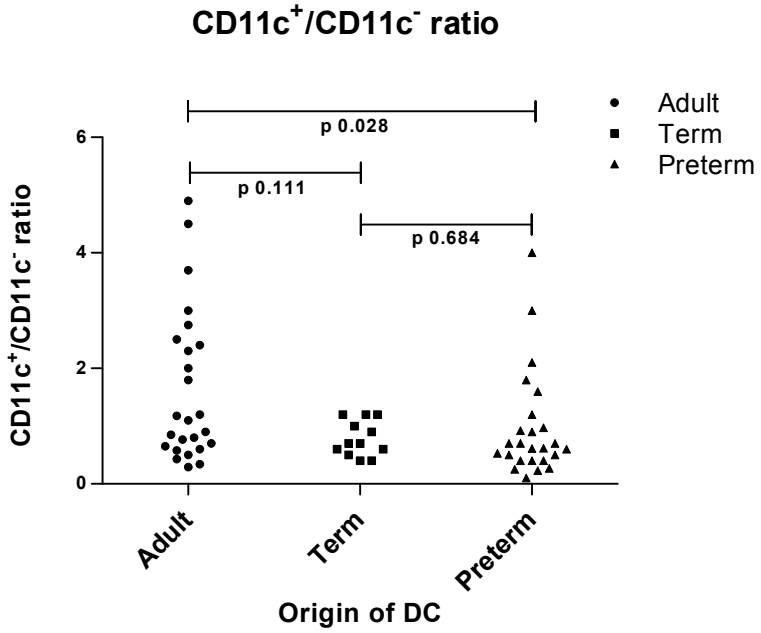


Figure 5.9 Change in the ratio of $CD11c^+/CD11c^-$ DC numbers

The above point plot with *p* values shows the difference in the $CD11c^+/CD11c^-$ ratio between preterm (*n*-25), term (*n*-12) and adult blood (*n*-25)

The ratio of $CD11c^+/CD11c^-$ was increased significantly in adults compared to the fetus (fetus: ratio-0·90; adult ratio-1·63, $p<0·020$), and appeared to be due to the increased numbers of $CD11c^+$ DCs in comparison to the $CD11c^-$ DCs. Thus the ratio in adults is positive as the $CD11c^+$ DCs predominate. The ratio is less than one in all the CBDC samples as the $CD11c^-$ DC subset predominates (preterm ratio - 0.96; term ratio - 0.78).

5.4.3 Change in the CD45 and HLA-DR expression of CBDC populations with age

As the various CBDC populations were found to vary in their expressions of the above markers, their potential alteration with advancing gestational age was analysed.

The myeloid and plasmacytoid subset showed no alterations in CD45 and HLA-DR with increasing age and advancing gestational age. The $CD11c^{-}CD45^{inm}$ CBDC population showed no significant change in CD45 and HLA-DR with advancing gestational age.

Table 5.7 Data on change of CD45 and HLA-DR expression with age

Origin of sample	**CD45 expression (Mean+/-SD)**			**No : of samples (*n*)**
	Myeloid subset	**Plasmacytoid subset**	**$CD11c^{-}CD45^{inm}$ subset**	
Adult	2409.06+/-1641.63	2295.06+/-1588.19	NA	32
Term fetus	1937.01+/-1543.79	2184.71+/-1630.72	552.85+/-617.71	14
Preterm fetus	1884.25+/-1459.49	1945.54+/-1440.22	462.39+/-335.66	26
Fetus (term & preterm)	1904.64+/-1474.94	2037.95+/-1502.52	497.30+/-460.50	40
Origin of sample	**HLA-DR expression (Mean+/-SD)**			**No : of samples (*n*)**
	Myeloid subset	**Plasmacytoid subset**	**$CD11c^{-}CD45^{inm}$ subset**	
Adult	640.82+/-459.81	612.18+/-496.94	NA	32
Term fetus	645.62+/-413.64	616.68+/-422.76	285.87+/-270.29	14
Preterm fetus	866.42+/-723.48	812.3+/-859.45	339.78+/-280.20	26
Fetus (term & preterm)	781.11+/-626.08	736.72+/-722.78	318.90+/-274.50	40

The absolute numbers of CD45 and HLA-DR expression by CBDCs in the various age groups are given above

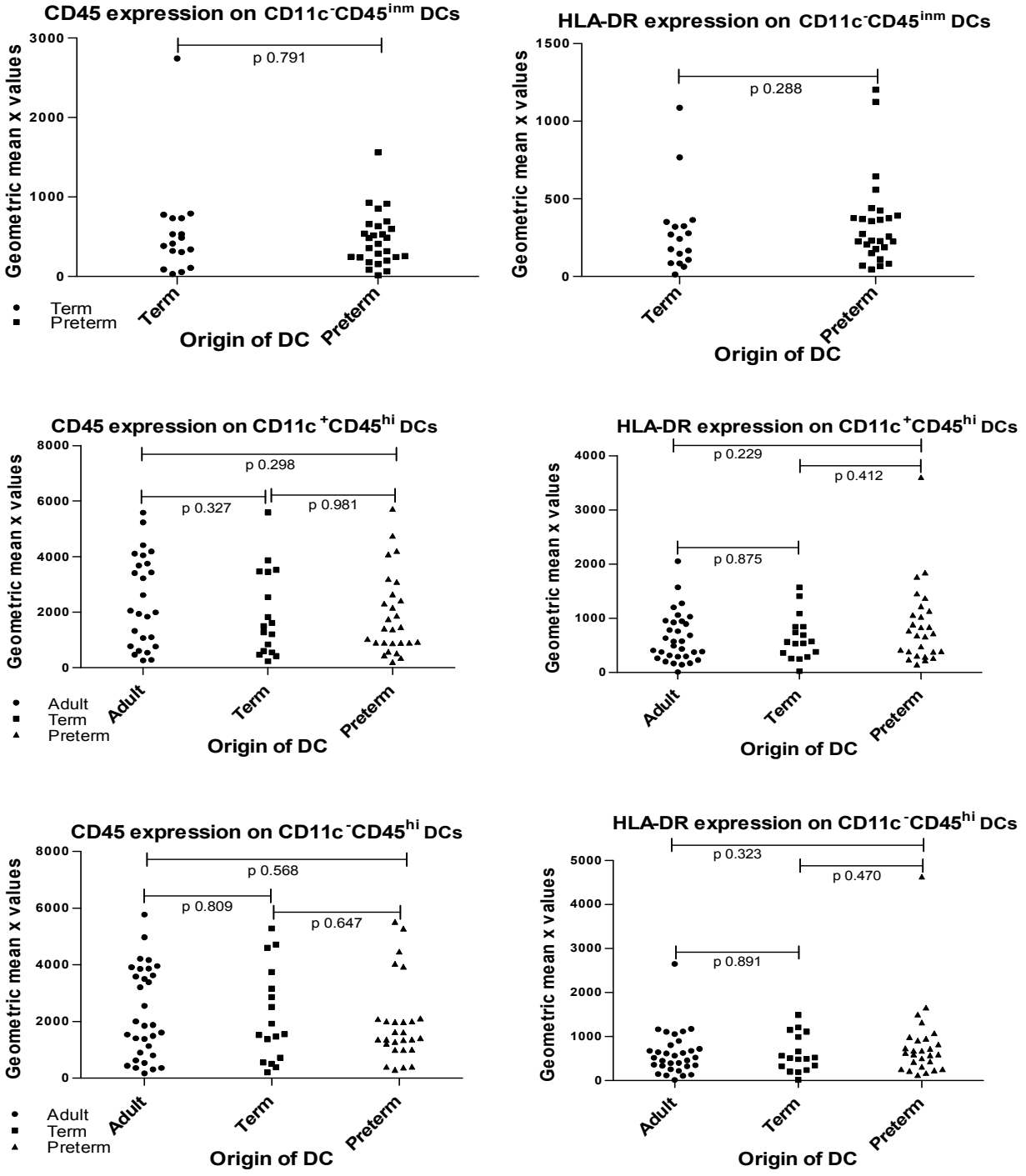


Figure 5.10 Changes of the expression of CD45 and HLA-DR with age

Multigraph of point plots with *p* values showing CD45 and HLA-DR expression on DC subsets in CB and adult PB

5.4.4 Alterations in numbers of CBDC populations on exposure to stressor stimuli

It was postulated that increasing gestational age as well as fetal/intrauterine stressors could lead to identifiable changes in CBDC subsets. To distinguish between the effects of exposure to stressors and of increasing gestational age all the preterm CB samples (*n*-25) were analysed in two groups; samples from fetuses with (*n*-17) and without (*n*-8) stressor stimuli. All the term CB analysed did not have any exposure to stressor stimuli (*n*-12).

To rule out the possibility that the increase in CBDC numbers was a simple reflection of the increased cellularity of fetal samples, we also examined the CBDC numbers as a proportion of total number of viable cells and of total number of DCs.

5.4.4.1 $CD11c^{+}CD45^{hi}$ CBDCs increased in numbers with stressors

Table 5.8 Data on change of myeloid CBDC subset with stressors

Origin of sample	Mean+/-SD			No : of samples (*n*)
	Absolute numbers	% of viable cells	% of total DC	
Term non stressed	17.83+/-8.99	0.24+/-0.30	34.5+/-10.08	12
Preterm non stressed	8.88+/-5.72	0.13+/-0.06	30.63+/-16.31	8
Preterm stressed	37.47+/-42.5	0.84+/-1.03	37.24+/-12.17	17

The change in myeloid CBDC subset with stressors is given in absolute numbers, percentage of viable cells and percentage of total number of DCs

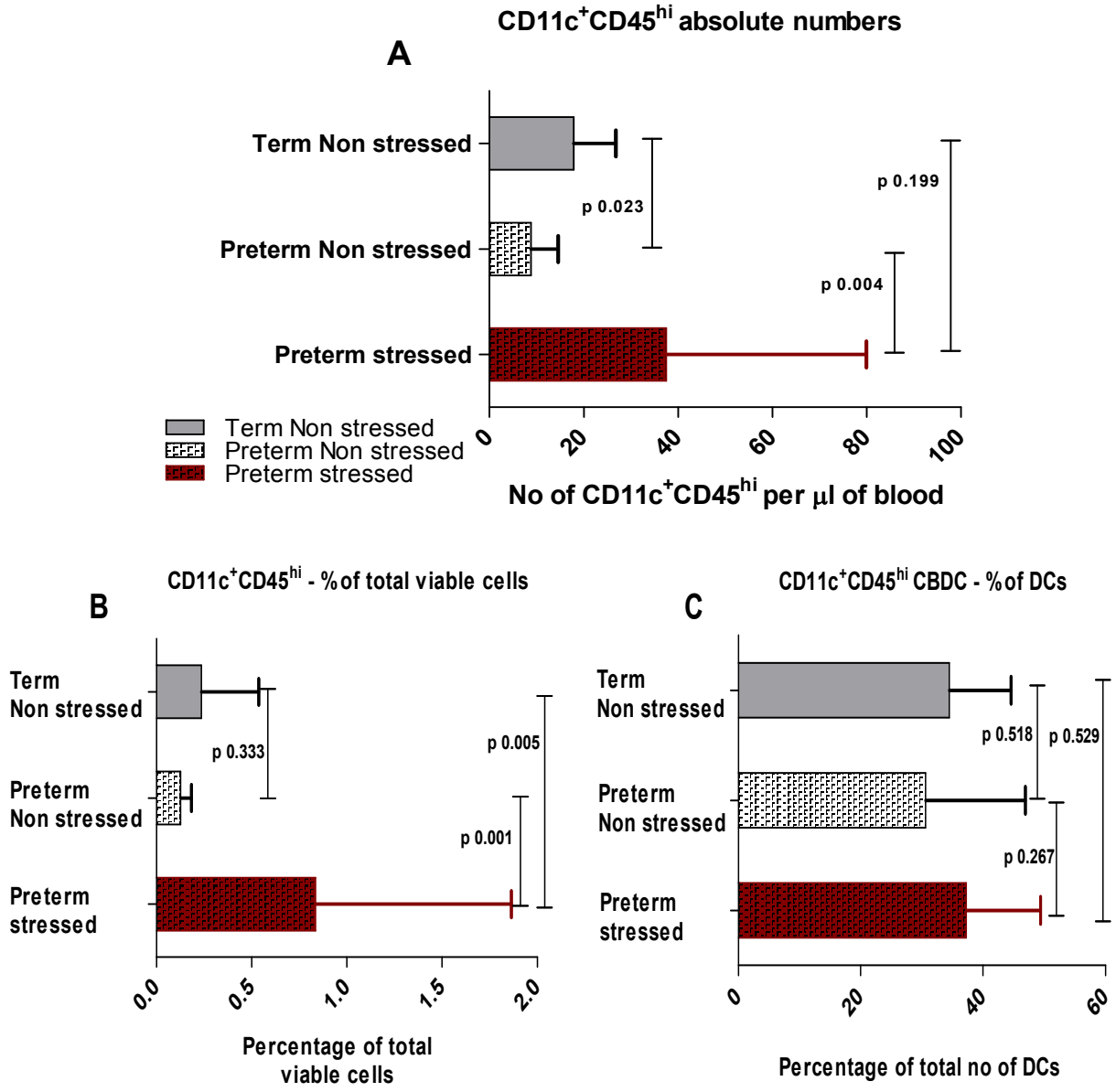


Figure 5.11 CD11c^{+}CD45^{hi} DC subset on exposure to stressor stimuli

The above multigraph with column bars and *p* values shows the difference in numbers of myeloid subset. Figure 5.11A shows the absolute numbers of DCs per µl of blood and Figures 5.11B and 5.11C show the proportion of total viable cells and of total DCs respectively

On comparison of absolute numbers the two groups that significantly differed were the preterm stressed vs preterm non stressed and the term vs preterm non stressed (Figure 5.11A).

The numbers as a proportion of total viable cells showed an increase in the preterm stressed samples (Figure 5.11B), while the proportions of total number of DCs were not significantly different (Figure 5.11C).

This data evidences that exposure to stressor stimuli increases the numbers of the $CD11c^{+}CD45^{hi}$ DCs as the preterm stressed CB showed a significant increase compared to the preterm non stressed CB.

5.4.4.2 $CD11c^{-}CD45^{hi}$ CBDCs increased in numbers with stressors

Table 5.9 Data on change of plasmacytoid CBDC subset with stressors

Origin of sample	Mean+/-SD			No : of samples (*n*)
	Absolute numbers	% of viable cells	% of total DC	
Term non stressed	25+/-16.69	0.33+/-0.44	43.25+/-10.15	12
Preterm non stressed	12.13+/-5.96	0.25+/-0.23	43.24+/-22.43	8
Preterm stressed	58.12+/-67.51	1.61+/-2.10	24.99+/-6.39	17

The change in plasmacytoid CBDC subset with stressors is given in absolute numbers, percentage of viable cells and percentage of total number of DCs

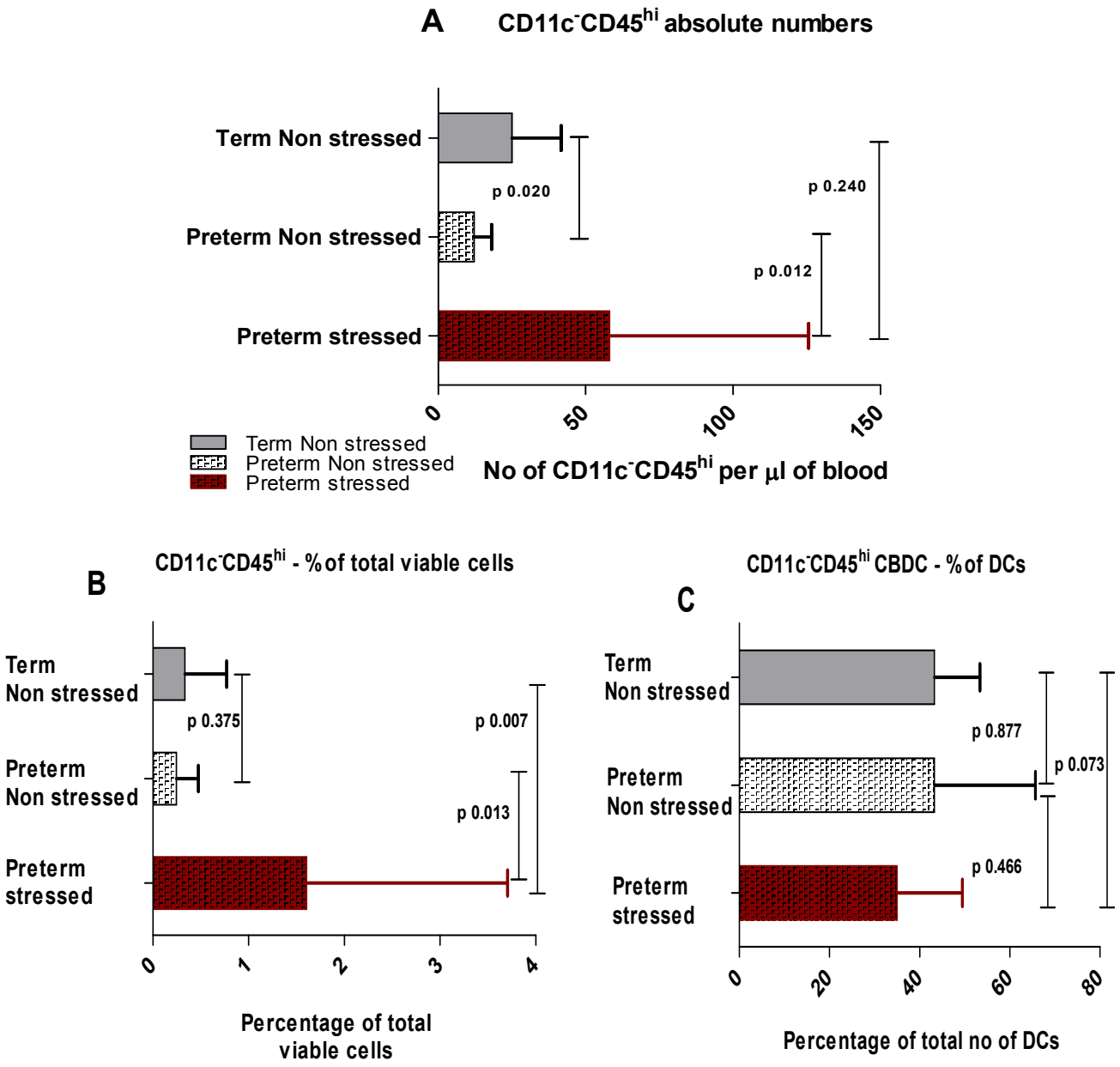


Figure 5.12 CD11c^{-}CD45^{hi} DC subset on exposure to stressor stimuli

The above multigraph with column bars and *p* values shows the difference in numbers of plasmacytoid subset. Figure 5.12A shows the absolute numbers of DCs per µl of blood and

Figures 5.12B and 5.12C show the proportion of total viable cells and of total DCs respectively

The changes in the plasmacytoid CBDC population paralleled the changes in the myeloid CBDC population. On comparison of absolute numbers the two groups that significantly differed were the preterm stressed vs preterm non stressed and the term vs preterm non stressed (Figure 5.12A).

The numbers as a proportion of total viable cells showed an increase in the preterm stressed samples (Figure 5.12B), while the proportions of total number of DCs were not significant (Figure 5.12C).

This data evidences that exposure to stressor stimuli increases the numbers of the $CD11c^{-}CD45^{hi}$ DC subset as the preterm stressed CB showed a significant increase compared to the preterm non stressed CB.

5.4.4.3 $CD11c^{-}CD45^{inm}$ CBDCs increased in numbers with stressors

Table 5.10 Data on change of $CD11c^{-}CD45^{inm}$ CBDC subset with stressors

Origin of sample	Mean+/-SD			No : of samples (*n*)
	Absolute numbers	% of viable cells	% of total DC	
Term non stressed	10.25+/-5.96	0.13+/-0.11	22.25+/-15.25	12
Preterm non stressed	7.88+/-6.15	0.11+/-0.06	26.13+/-14.05	8
Preterm stressed	43.94+/-57.33	0.81+/-0.85	37.77+/-26.84	17

The change in $CD11c^{-}CD45^{inm}$ CBDC subset with stressors is given in absolute numbers, percentage of viable cells and percentage of total number of DCs

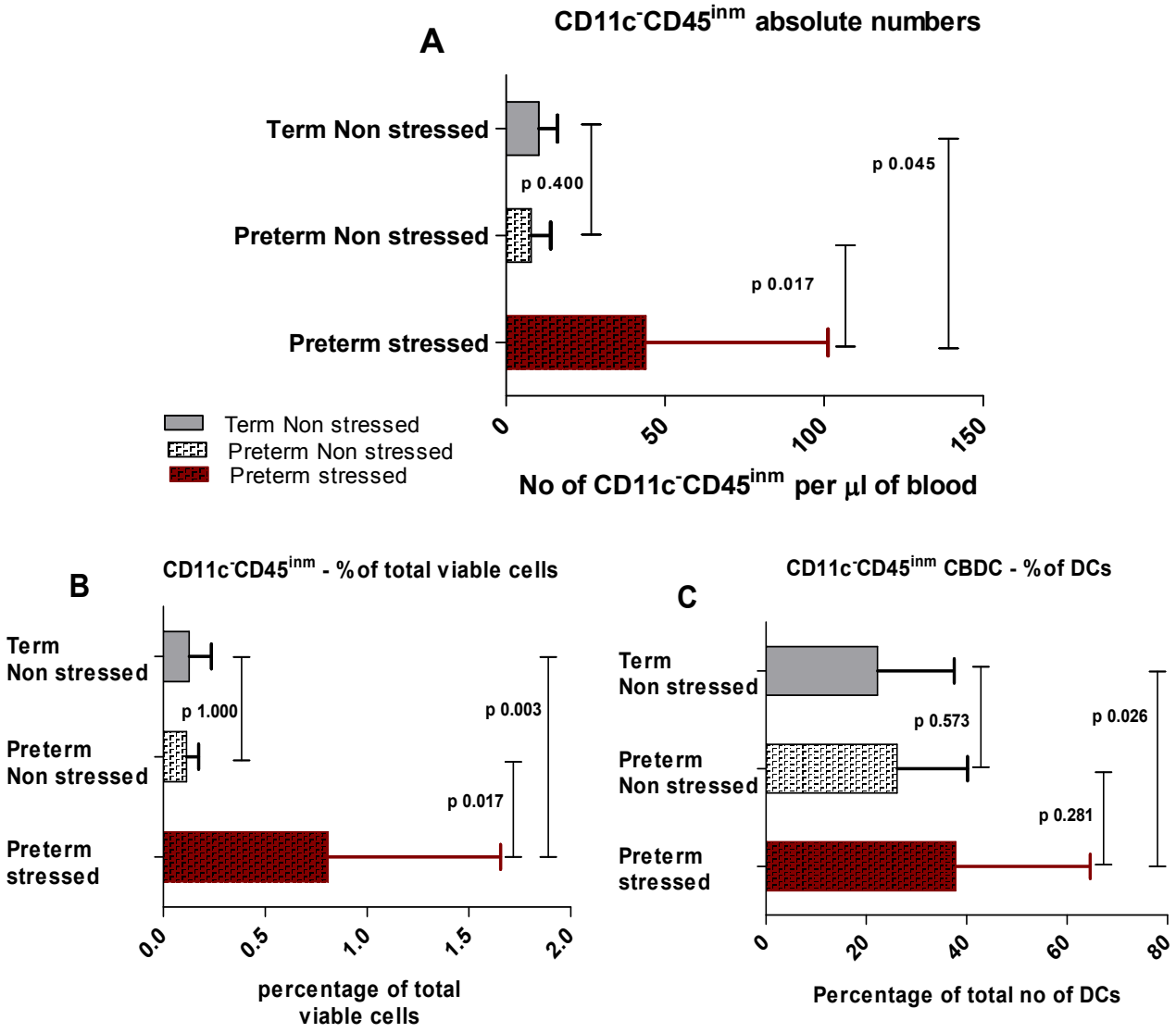


Figure 5.13 CD11c^{-}CD45^{inm} DC subset on exposure to stressor stimuli

The above multigraph with column bars and *p* values shows the difference in numbers of CD11c^{-}CD45^{inm} DCs. Figure 5.13A shows the absolute numbers of DCs per µl of blood and Figures 5.13B and 5.13C show the proportion of total viable cells and of total DCs respectively

Differences with adult PB $CD11c^{-}CD45^{inm}$ DCs could not be ascertained due to the significant lack of the $CD11c^{-}CD45^{inm}$ subset in the adult.

On analyses of absolute numbers, preterm stressed CB showed a statistically significant increase in absolute numbers when compared with preterm non stressed CB (Figure 5.13A).

The $CD11c^{-}CD45^{inm}$ subset as a percentage of the total number of viable cells was significantly increased in preterm stressed CB when compared with the preterm non stressed CB (Figure 5.13B). Analysis of the subset as a percentage of the total number of DCs showed no significant change in preterm stressed CB (Figure 5.13C).

Taken together the data above indicate that exposure to stressors increased the numbers of $CD11c^{-}CD45^{inm}$ CBDCs significantly.

5.4.4.4 Changes in total cell numbers with stressors

To assess the validity of the changes in the different CBDC subsets in relation to global changes in cell numbers, the change in the total numbers of viable cells and total DCs were analysed between the three groups of term non stressed, preterm non stressed and preterm stressed.

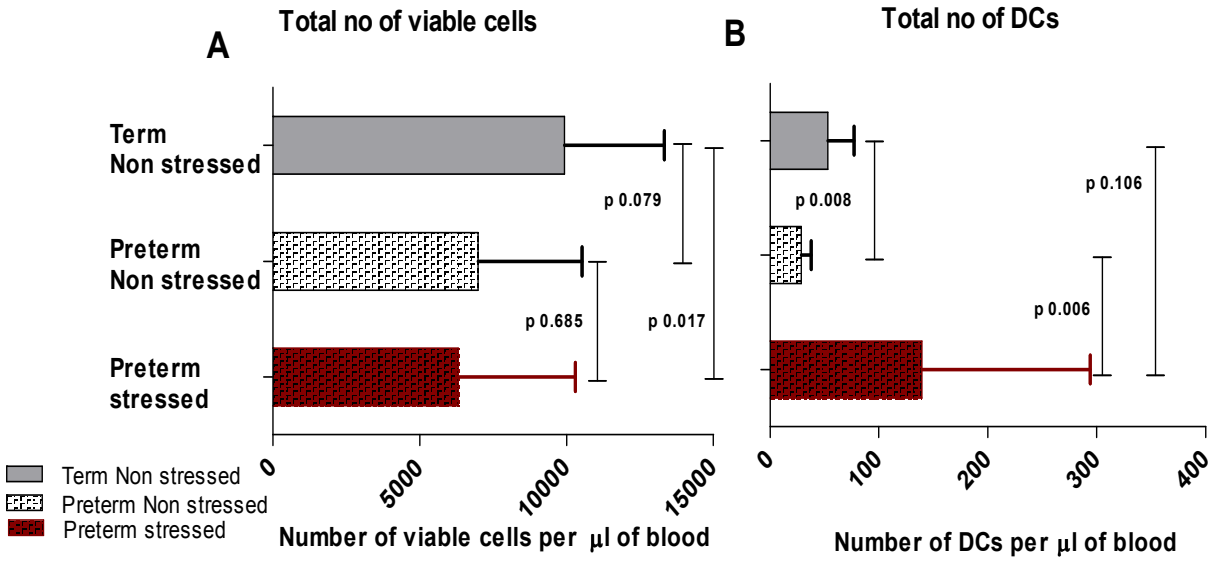


Figure 5.14 Differences in total cell numbers with stressors

The above multigraph with column bars and *p* values shows the difference in the total number of viable cells and the total number of DCs per µl of blood

Table 5.11 Data on change in total no:of viable cells and total no:of DC with stressors

Origin of sample	Mean+/-SD		No : of samples (*n*)
	Total no:of viable cells	Total no:of DC	
Term non stressed	9923.75+/-3400.81	53.08+/-24.10	12
Preterm non stressed	6991.63+/-3539.15	28.88+/-9.08	8
Preterm stressed	6313.24+/-3983.68	139.53+/-154.41	17

The total numbers of viable cells and the total numbers of DCs in the various age groups are shown above

On comparison of the total number of viable cells there was no significant effect of exposure to stressors. The effect of advancing gestational age was seen with the term CB showing more cellularity (Figure 5.14A).

On comparison of the total number of DCs, the two groups that significantly differed were the preterm stressed vs preterm non stressed and the term vs preterm non stressed (Figure 5.14B). Thus exposure to stressor stimuli was shown to increase the total DC numbers.

5.4.5 Changes in CD45 and HLA-DR expression of CBDC populations with stressors

As the various CBDC populations were found to vary in their expressions of the above maturation markers, their potential alteration on exposure to stressors was analysed.

Table 5.12 Data on change of CD45 and HLA-DR expression with stressors

Origin of sample	CD45 expression (Mean+/-SD)			No : of samples (*n*)
	Myeloid subset	Plasmacytoid subset	$CD11c^{-}CD45^{inm}$ subset	
Term non stressed	1977+/-1544	2185+/-1631	552.8+/-617.7	14
Preterm non stressed	2809+/-1953	2548+/-1743	648.3+/-481	26
Preterm stressed	1497+/-1029	1692+/-1259	384.1+/-225.7	40
Origin of sample	**HLA-DR expression (Mean+/-SD)**			**No : of samples (*n*)**
	Myeloid subset	**Plasmacytoid subset**	**$CD11c^{-}CD45^{inm}$ subset**	
Term non stressed	645.6+/-413.6	616.7+/-422.8	285.9+/-270.3	14
Preterm non stressed	828.2+/-381.7	748.6+/-375.3	271.3+/-231.3	26
Preterm stressed	882.5+/-835.7	839.1+/-1005	368.6+/-299.4	40

The expressions of CD45 and HLA-DR by the CBDC subsets in the various age groups are given above

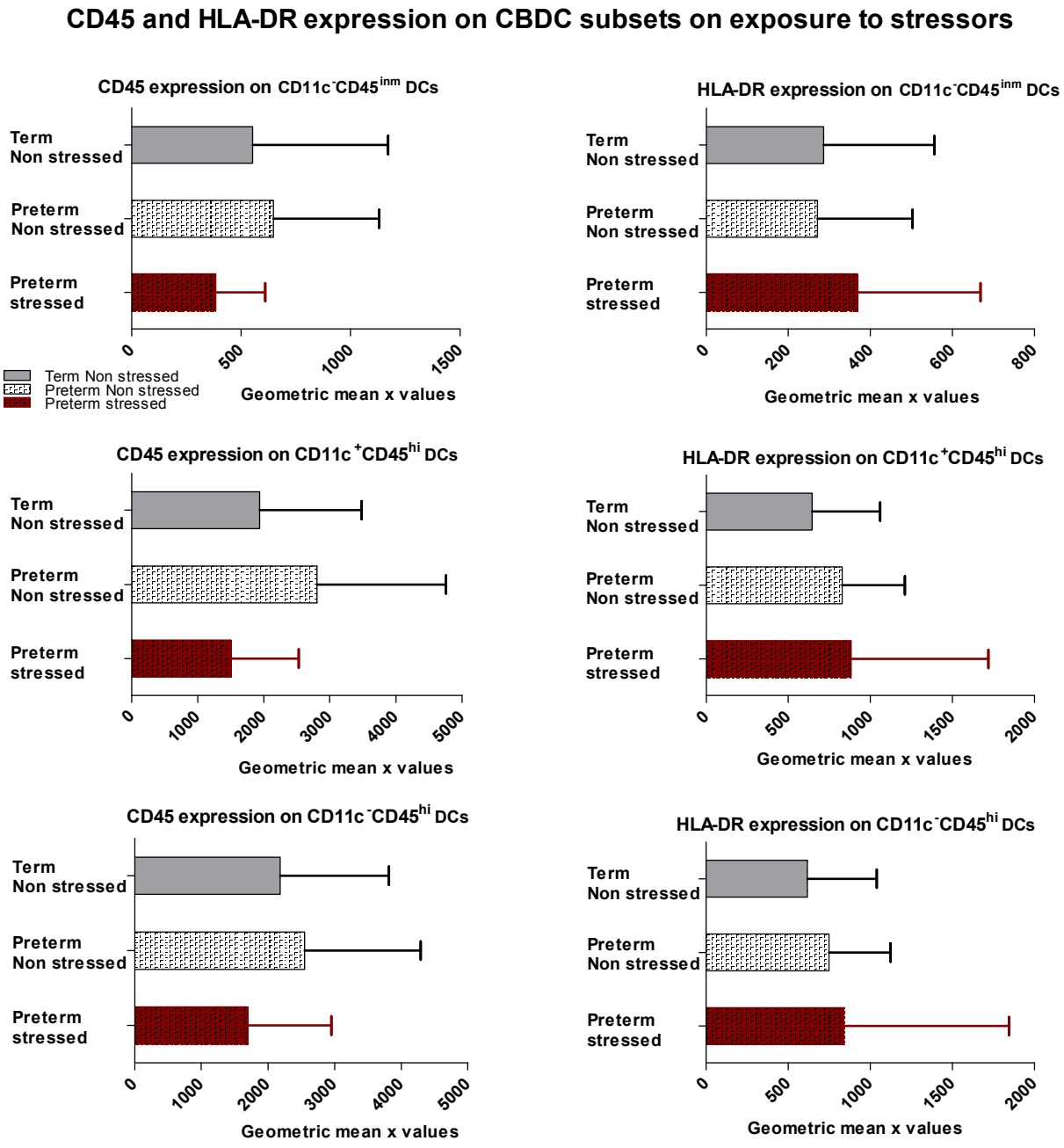


Figure 5.15 Expression of CD45 and HLA-DR on exposure to stressor stimuli

Multigraph with column bars shown. All comparisons showed non significant *p* values, i.e *p* values were over 0.05

In summary from the data shown above, the myeloid, plasmacytoid and the $CD11c^{-}CD45^{inm}$ CBDC subsets did not show any alterations in their expression of CD45 and HLA-DR on exposure to stressors.

5.5 DISCUSSION

This study analysed the changes that occurred in the umbilical CBDC subsets with increasing gestational age and exposure to stressor stimuli. The $CD11c^{-}CD45^{inm}$ subset decreased significantly with increasing gestational age and was seen only as a trace population in the adult. The myeloid and plasmacytoid CBDC subsets showed no change with increasing gestational age. All three CBDC subsets increased in numbers on exposure to stressor stimuli. The myeloid and plasmacytoid subsets expressed similar amounts of CD45 and HLA-DR when compared with the adult PB DCs, while the $CD11c^{-}CD45^{inm}$ subset expressed lower levels indicating an immature phenotype. These changes may be a reflection of the maturation and activation of the immune system with increasing gestational age or with intrauterine stressor stimuli.

Analysis was performed on whole blood in order to reflect the *in vivo* environment as closely as possible with its attendant advantages and with the appropriate controls as discussed previously.

CD45 (PTPRC, leukocyte common antigen, B220, T200) is a signalling molecule expressed on all nucleated haematopoietic cells and is therefore widely used as a pan-leukocyte maturation marker molecule. It has a central role in antigen stimulation of T lymphocytes and is a marker of functional maturation (Trowbridge and Thomas, 1994). CD45 mutations result in severe combined immunodeficiency phenotype, systemic lupus erythematosus, and many other diseases (Cale, *et al*., 1997; Kung, *et al*., 2000; Tchilian, *et al*., 2001). Cross *et al* have shown that CD45, though not required for the development of DCs, does influence DC maturation induced by TLR agonists and that CD45-null DCs had a reduced ability to activate NK and Th1 cells to produce IFN-γ indicating functional immaturity (Cross, *et al*., 2008). CD45 has been shown to increase in intensity as maturation takes place, thus the $CD11c^{-}CD45^{inm}$ CBDCs appear to be immature

compared to the myeloid and plasmacytoid CBDC subsets (Borowitz, *et al*., 1993; Jennings and Foon, 1997).

There were no differences in the expression of the markers of maturity i.e. CD45 and HLA-DR on myeloid and plasmacytoid CBDC subsets when compared with adult PB DCs. As the $CD11c^{-}$ $CD45^{inm}$ subset expressed both CD45 and HLA-DR at a lower intermediate level, they appear to be a group of immature DCs undergoing change. By its very definition HLA is necessary for the main DC function of antigen presentation and reduced expression of which could readily result in functional immaturity. Unfortunately adult PB did not have enough numbers of cells within the novel subset to compare the expression of the maturation markers. The conclusion could be drawn that as the myeloid and plasmacytoid CBDC subsets have the same level of maturity as adult PB DCs, the immature $CD11c^{-}CD45^{inm}$ subset must be a major contributing factor to the immature functioning of fetal DCs. The probable key role played by this subset in the fetal immune system was in line with the finding that this subset formed a large percentage in the fetus and was almost absent in the adult. The study did not identify any significant change in the expression of these maturation markers with age or with exposure to stressors. The lack of a significant change in these markers could be a real effect or a reflection of the inadequate power of the study and analyses of a larger number of samples could be more sensitive in elucidating these changes.

In order to investigate the changes in the CBDC subsets occurring in parallel with the development of the fetal immune system, we have analysed the changes in numbers occurring with increasing gestational age. Analyses of the myeloid and plasmacytoid CBDC subsets showed no change with increasing gestational age. Drohan *et al* found that adult blood had a significantly higher proportion of myeloid DC than did CB (Drohan, *et al*., 2004) which was

confirmed in this study. As had been observed in other studies there was a reversal of ratio in the fetus with the plasmacytoid subset predominating. In adults the predominant DC population is $CD11c^{+}$ myeloid (52.88%) followed by the $CD11c^{-}$ plasmacytoid (45.58%). Conversely the $CD11c^{-}$ DC population predominated in the fetus (40.74%).

We have studied the numbers and percentages of the $CD11c^{-}CD45^{inm}$ DC subset in preterm and term CB and also adult PB. In line with the observation of an evolving, maturing DC subset, we have found this subgroup predominantly in preterm CB when compared with term CB. Comparison of this subset in the CB with adult PB revealed an almost absence of these DCs in adult blood. This subset represented 21.26% of the total DC population in the term CB, but formed 31.33% of the total DC population in the preterm CB, and only 1.54% in adult blood. It could be argued that the decreasing number of cells indicates the population to be evolving into a more mature phenotype.

This study also analysed the change in numbers of the CBDC subsets on exposure to stressor stimuli. The myeloid and plasmacytoid subsets showed a significant increase in numbers on exposure to stressors. A significant increase in numbers of the novel $CD11c^{-}CD45^{inm}$ DC subset in response to stressor stimuli was also identified. This DC subset increased in absolute numbers as well as percentage of total viable cells in the preterm stressed CB, when compared with the preterm non stressed. Thus this subset appears to respond to stressor stimuli with an increase in numbers.

CB was more cellular as the total number of viable cells was significantly more than seen in the adult and there was a significant increase in the viable cells in the term CB as opposed to preterm CB. Whether this indicates preparation for parturition and delivery with an increase in leukocytes in anticipation of infectious stimuli is a question which cannot currently be answered. There was

no significant change in the total number of DCs with increasing gestational age and on comparison between fetal and adult samples, but exposure to stressors increased the numbers. This increase in numbers on exposure to stressors could be due to the identified increase of the CBDC subsets by peripheral recruitment in response to infection and again this hypothesis needs to be elucidated further.

The neonatal immune system can be deficient in its efficacy and is also functionally different from the adult system (Hunt, *et al*., 1994). The innate neonatal immune system is functionally and phenotypically immature (Levy, *et al*., 1999; Sorg, *et al*., 1999). CBDCs tend to express lower levels of the γ-chain IL-2 receptor (CD132) and of the CD86 costimulatory molecule, supporting a higher degree of immaturity as compared to adult DCs (Crespo, *et al*., 2004). As a result of the immaturity, it would appear that development and maturity of the fetal immune system involves specific cell populations being upregulated and exhibiting a more mature phenotype. This idea is supported by our findings of decreasing percentages of the $CD11c^{-}$ $CD45^{inm}$ subset with advancing gestational age. Additionally exposure to stressor stimuli increased the numbers of all three CBDC subsets. This increased response of CBDC subsets to stressor stimuli mean that fetal DCs are immunologically active, changing with the rapidly changing fetal environment which needs to adapt swiftly from immunotolerance to response against infections. Thus the positive response on exposure to stressors seen in these subsets confers a central role for these DCs in the response to stressor stimuli and fetal defences against infections.

The above observations from this study raise the question whether the level of maturity and numbers of the immature $CD11c^{-}CD45^{inm}$ DC subset could be used to indicate the immunocompetence of and exposure to infectious stimuli of immature, preterm infants. The

responsiveness of this DC subset to stressor stimuli and the additional decrease in numbers with a maturing immune system would suggest that this subset is most active immunologically and investigating this subset would be indicative of an *in utero* exposure to infectious/stressor stimuli. This in turn would contribute towards a more accurate diagnosis and management with improved prognosis for the premature neonate.

CHAPTER 6

RESULTS IV

PROINFLAMMATORY CHANGES ON CORD BLOOD DENDRITIC CELL POPULATIONS WITH AGE AND EXPOSURE TO 'STRESSORS'

6.1 SUMMARY

The three DC subsets in CB of myeloid, plasmacytoid and the novel $CD11c^{-}CD45^{inm}$ subset were analysed for potential proinflammatory changes on exposure to stressor stimuli. This involved analyses of the costimulatory markers, CD40 and CD86 expression and the production of the intracellular cytokine, IL-12 with advancing age as well as exposure to stressors.

Analyses of whole blood with monoclonal antibody labelling and flow cytometer acquisition were performed on all the CB samples.

As advancing gestational age itself alters the CBDC subsets, analysis of their change in expressions of CD40 and CD86 and IL-12 production with age was performed. The myeloid DC subset increased, and the plasmacytoid subset decreased expression of CD40 with advancing age to adult. Both the above subsets showed no change in the expression of CD86. Only the $CD11c^{-}CD45^{inm}$ showed a change with advancing gestational age from preterm to term by increasing in numbers and upregulating CD40 and CD86 expression. The myeloid and the plasmacytoid population increased IL-12 production with increasing age, while the $CD11c^{-}CD45^{inm}$ CBDC population showed no change in IL-12 production with advancing gestational age.

Changes of the CBDC subsets in response to exposure to stressors were identified. The numbers of $CD11c^{-}CD45^{inm}$ DCs expressing CD40 and CD86 increased significantly on exposure to stressors. Conversely the myeloid and the plasmacytoid CBDC subsets showed no upregulation of CD40 and CD86 on exposure to stressors. The numbers of CBDC in the $CD11c^{-}CD45^{inm}$ CBDC population producing IL-12 increased significantly on exposure to stressors independent of gestation. The myeloid DC subsets decreased while the plasmacytoid DC subset increased the numbers of IL-12 producing DCs on exposure to stressors.

Thus the $CD11c^{-}CD45^{inm}$ DCs exhibited immunogenic capacity both by their upregulation of the costimulatory markers and their production of IL-12 on exposure to stressors. These changes in the $CD11c^{-}CD45^{inm}$ CBDC subset in response to stressors and the changes in numbers with increasing gestational age confirmed the postulation that increasing gestational age as well as exposure to stressors induced proinflammatory and maturation changes in this subset. Significantly only the $CD11c^{-}CD45^{inm}$ DC subset showed a costimulatory response to the stressor stimuli suggesting that this evolving DC subset in the fetus could be key to the antigen presenting capacity of the fetal immune system.

6.2 INTRODUCTION

A Th2 bias or immunoprotective function is the hallmark of the fetal immune system and is essential for masking the fetal antigens from the maternal immune system, thus ensuring a successful pregnancy. This fetal innate immune system response may result in deficient immunocompetence against infections and underlie the neonatal susceptibility to infections. The relative inefficiency of CBDCs in the activation of T cells has been shown to be linked to their low cell surface expression of MHC and cell adhesion molecules (Petty and Hunt, 1998). Fetal biology changes with the onset of maternal/intrauterine infection, with a rise in IL-8 concentration in CB (Dembinski, *et al*., 2002). Infection is a major cause of neonatal morbidity and the immunodeficiency of the fetal immune system seems to play a major part in these adverse outcomes.

The ability of DC to alter their phenotype and function in response to microbial exposure suggests that changes of CBDC populations will be useful markers of intrauterine infection or stress and neonatal sequelae (Huang, *et al*., 2001). Thus we have compared the DC phenotype of CB from neonates who have undergone intrauterine stress or exposure to infections *in utero* with the DC phenotype of CB from neonates with a normal antenatal and intrapartum period. The previously described changes on the CBDC subsets with increasing gestational age raised the question whether an antigen stimulus could result in a consistently identifiable phenotypic change.

The expression of the costimulatory markers, CD40 and CD86 have been analysed on the DCs, in conjunction with the changes induced by an infectious or immunogenic stimulus. Products of infection and inflammation such as LPS induce DC maturation, release chemokines and

cytokines and exhibit costimulatory molecules on their surface such as CD40 and CD86. The upregulated costimulatory molecules are presented by MHC class II proteins in DCs and initiation of the adaptive immune system takes place with activation of naïve $CD4^{+}$T cells. Mature DCs exhibit class I and class II MHC and costimulatory molecules such as CD40, CD80 and CD86 (Almeida, *et al*., 1999; Almeida, *et al*., 2001). Immature DCs exhibit decreased expression of the costimulatory molecules (Banchereau, *et al*., 2000).

IL-12 is a proinflammatory cytokine that induces the differentiation of Th1 cells and forms a link between innate and adaptive immunity (Kobayashi, *et al*., 1989; Hsieh, *et al*., 1993). IL-12 is produced mainly from activated DCs and phagocytes (monocytes/macrophages and neutrophils). IL-12 has been shown to increase not only in preterm labour and infections, but also on exposure of the fetoplacental unit to stressor stimuli such as in severe pre-eclampsia (Dudley, *et al*., 1996).

Our overall aim was to test the hypothesis that changes in CBDC populations are consistently identifiable and can be used as biomarkers of intrauterine infection or exposure to stressor stimuli. The research focuses on analysing the phenotypic changes of the costimulatory markers CD40 and CD86 on CBDC subsets and the production of IL-12 by CBDCs, to identify that group of neonates who were potentially exposed to intrauterine stressor and may provide an indicator of the risk of developing short and long term infectious sequelae.

6.3 SUBJECTS

Blood from the umbilical cord of 14 preterm neonates and 7 term neonates were analysed for costimulatory markers. Blood from the umbilical cord of 8 preterm neonates and 10 term neonates were analysed for production of IL-12. Adult blood for analysis was obtained from 14 non-pregnant healthy volunteers between the age groups of 22 to 56 years.

Clinical criteria were employed to differentiate those neonates considered to have been 'stressed' and those neonates who were 'non-stressed' (Detailed discussion about the criteria for classification and validation by neutrophilic activation markers is given in **Chapter 4**).

6.4 RESULTS

6.4.1 Costimulatory marker expression on CBDC populations

6.4.1.1 Analysis of expression

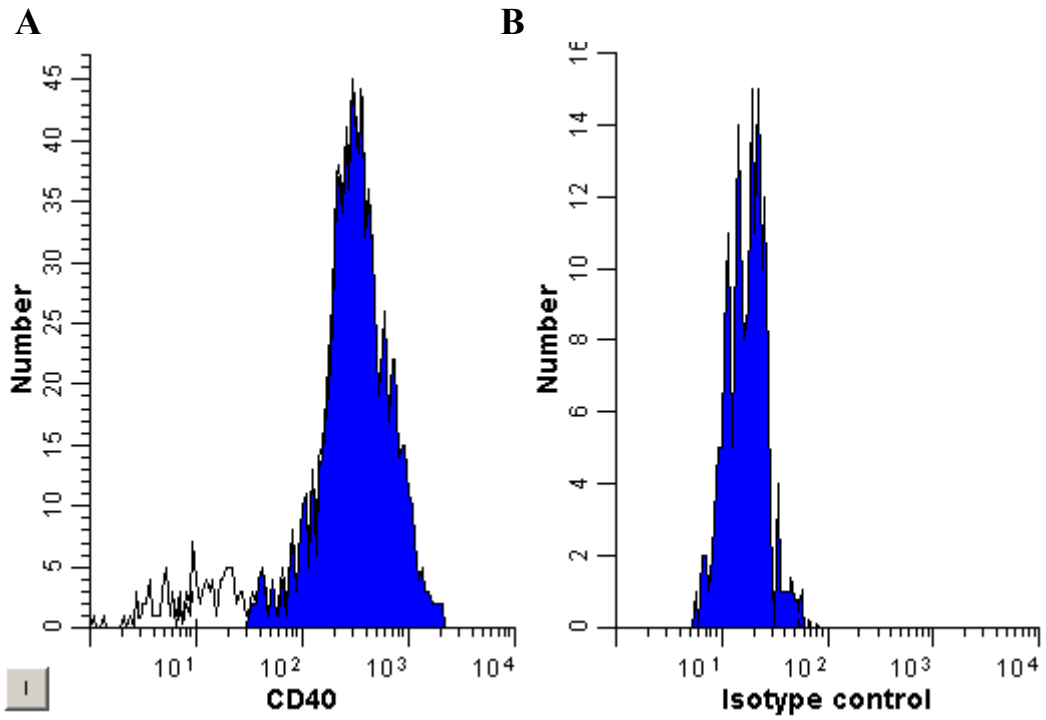


Figure 6.1 Subtraction method for analysis of expression of costimulatory markers

Isotype controls were used to delineate the negative expression of the markers to be identified (Figure 6.1B). The costimulatory marker expression was determined by subtracting staining with an isotype matched control antibody from staining with the marker (Figure 6.1A). Representative histograms shown from 21 experiments

The amount of costimulatory marker expression was analysed using the subtraction method as described above in Figure 6.1 (see **chapter 2, section 2.2.3.2.2** for a detailed explanation).

6.4.1.2 Differential expression of costimulatory markers by the CBDCs

As evidence in literature points to a reduced immunocompetence in the fetus, the expression of costimulatory markers on fetal CBDCs was analysed prior to assessing whether upregulation takes place with advancing age and on exposure to stressors.

Table 6.1 Data on expression of CD40 and CD86 on CBDC subsets

Origin of sample	Mean+/-SD			
	Absolute numbers			No:of samples (*n*)
	CD40	CD86	*p* values	
Myeloid subset	5.44+/-5.38	12.20+/-8.28	0.002	21
Plasmacytoid subset	13.24+/-10.92	6.30+/-5.01	0.003	21
$CD11c^{-}CD45^{inm}$ subset	4.90+/-4.51	6.19+/-4.21	0.197	21
	% positive			
Myeloid subset	32.83+/-20.99	79.52+/-21.82	<0.001	21
Plasmacytoid subset	62.30+/-22.39	32.61+/-20.37	<0.001	21
$CD11c^{-}CD45^{inm}$ subset	52.68+/-23.10	67.15+/-18.81	0.032	21
	PCI ratio			
Myeloid subset	4.04+/-2.02	6.68+/-5.19	0.048	21
Plasmacytoid subset	10.57+/-9.36	2.49+/-1.37	<0.001	21
$CD11c^{-}CD45^{inm}$ subset	3.01+/-3.22	2.90+/-2.54	0.481	21

Absolute numbers of DCs expressing CD40 and CD86, percentage of positive DCs and their PCI ratios are shown. *p* values shown are for comparisons between expressions of CD40 and CD86 by each subset

6.4.1.2.1 Expression of CD40 and CD86 on the CBDC subsets

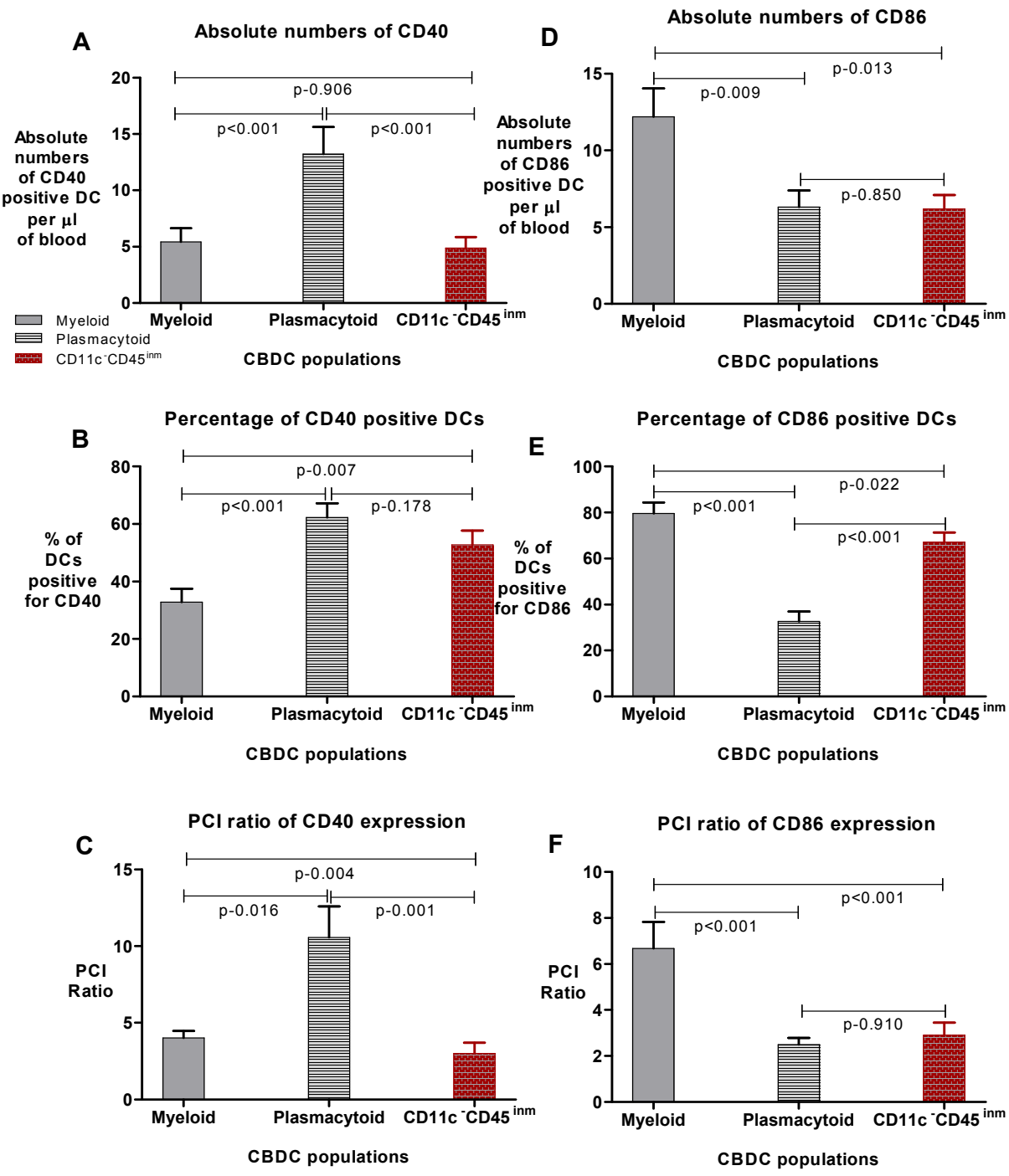


Figure 6.2 Comparison of CD40 and CD86 expression by CBDC subsets

The above multigraph with column and error bars shows the comparison of expressions of the CD40 and CD86 on the various CBDC populations

Costimulatory marker expression was analysed by comparison of the absolute numbers and the percentage of DCs expressing the marker (Figures 6.2A, 6.2B, 6.2D and 6.2E). As these values could be misleading if significant numbers of DCs expressed the marker but with low intensity, the PCI ratio was also compared (Figures 6.2C and 6.2F).

Comparison of CD40 expression between subsets showed it to be highest on the plasmacytoid subset on analysis of absolute numbers, percentage positive and PCI ratio. The CD86 expression was highest on the myeloid subset on analysis of absolute numbers, percentage positive and PCI ratio (*p* values on Figure 6.2).

Analysis of the markers on each subset revealed that all the CBDC subsets expressed CD40 and CD86 but the levels varied. The $CD11c^{-}CD45^{inm}$ subset expressed equal amounts of CD40 and CD86 on at least more than 50% of the DCs in this subset. The myeloid DC subset expressed significantly more CD86 than CD40 and this finding was reversed in the plasmacytoid subset (Table 6.1).

6.4.2 Change in costimulatory marker expression on CBDC populations with age

The major DC subsets were analysed for the expression of the CD40 and CD86 costimulatory markers in adult PB and additionally, the $CD11c^{-}CD45^{inm}$ subset in CB. Differences in the expression of the CD40 and CD86 markers between adult and $CD11c^{-}CD45^{inm}$ CBDCs could not be ascertained as the number of DCs in the adult exhibiting the $CD11c^{-}CD45^{inm}$ phenotype was found to be negligible, ie: 0.64 DCs per µl of blood.

6.4.2.1 The CD11c^{+}CD45^{hi} DC population increased CD40 expression with age

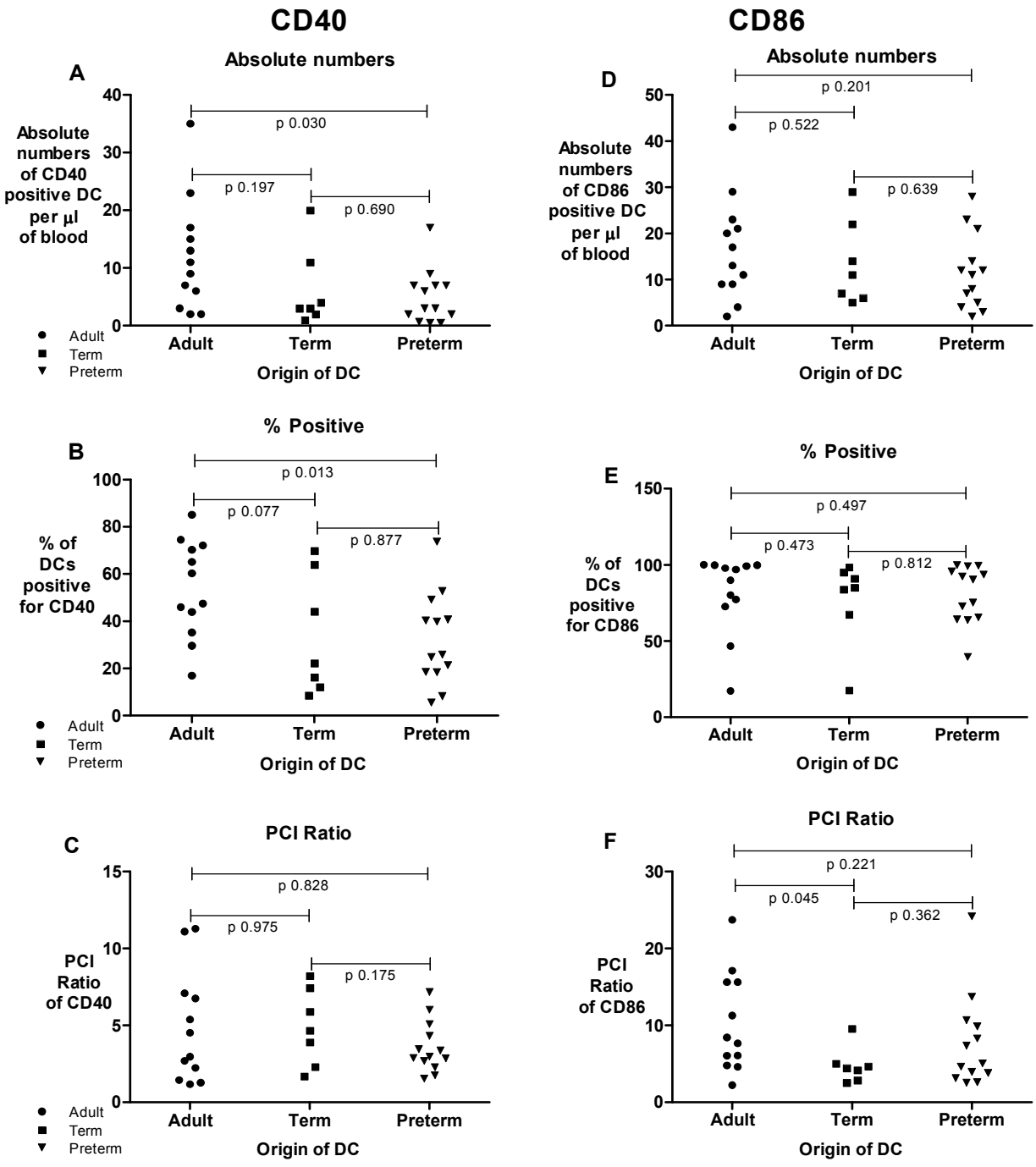


Figure 6.3 The expression of CD40 and CD86 in the CD11c^{+}CD45^{hi} DC subset

Multigraph with point plots and *p* values shown. Numbers of CD40 and CD86 positive DCs, percentage positive and PCI ratio shown per µl of blood. The fetal samples were analysed based on their gestational age alone into term and preterm

Table 6.2 Data on change in CD40 and CD86 expression on myeloid DCs with age

Origin of sample	Mean+/-SD						No : of samples (*n*)
	Absolute numbers		% positive		PCI ratio		
	CD40	CD86	CD40	CD86	CD40	CD86	
Adult	11.92+/-9.71	16.75+/-11.5	53.88+/-20.6	81.46+/-25.83	4.83+/-3.6	10.28+/-6.46	14
Term fetus	6.29+/-6.87	13.43+/-9.03	33.86+/-25.37	76.91+/-28.04	4.88+/-2.47	6.68+/-5.19	7
Preterm fetus	4.98+/-4.64	11.54+/-8.14	32.27+/-19.36	80.92+/-18.82	3.58+/-1.66	7.7+/-6.05	14
Fetus (term & preterm)	5.44+/-5.38	12.2+/-8.28	32.83+/-20.99	79.52+/-21.82	4.04+/-2.02	4.77+/-2.3	21

The expressions of CD40 and CD86 on the myeloid subset is shown as absolute numbers, percentage positive and PCI ratio

6.4.2.1.1 CD40 expression

Absolute numbers (Figure 6.3A) and percentage positive DCs (Figure 6.3B) increased significantly from preterm to adult.

On comparison between adult and fetus the absolute numbers (*p* 0.031) and percentage of positive DCs (*p* 0.037) showed a significant increase in the adult.

The PCI ratio showed no significant change between preterm and term CBDCs (Figure 6.3C) and between adult and fetus (*p* 0.426).

6.4.2.1.2 CD86 expression

Comparison of the subgroups of preterm and term did not reveal any change in numbers, percentage of positive DCs and PCI ratios (Figures 6.3D, 6.3E and 6.3F).

There was no statistically significant change between adult and fetal DCs in numbers (p 0.203), percentage of positive DCs (p 0.403) and PCI ratios (p 0.059).

Taken together the data above shows that the $CD11c^{+}CD45^{hi}$ subset significantly increased expression of the CD40 marker with increasing age from fetus to adult but did not change with advancing gestational age. The expression of CD86 showed no change with advancing age and gestation.

6.4.2.2 CD11c^{-}CD45^{hi} DCs expressing CD40 decreased with advancing age

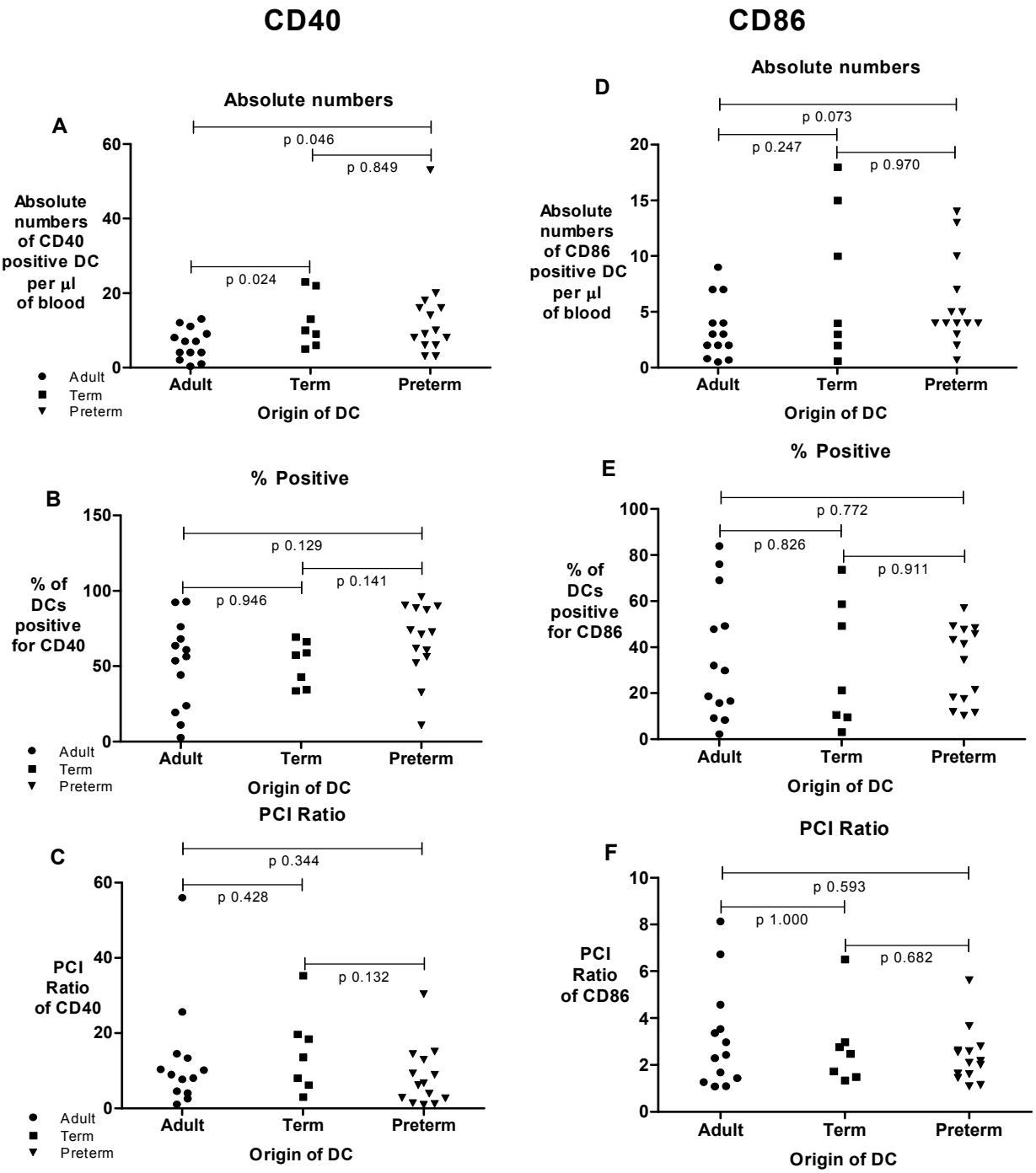


Figure 6.4 The expression of CD40 and CD86 in the CD11c^{-}CD45^{hi} DC subset

Multigraph with point plots and *p* values shown. Numbers of CD40 and CD86 positive DC, percentage positive and PCI ratio shown per µl of blood. The fetal samples were analysed based on their gestational age alone into term and preterm

Table 6.3 Data on change in CD40 and CD86 expression on plasmacytoid DCs with age

Origin of sample	Mean+/-SD						No : of samples (*n*)
	Absolute numbers		% positive		PCI ratio		
	CD40	CD86	CD40	CD86	CD40	CD86	
Adult	6.33+/-4.17	3.46+/-2.69	51.19+/-29.43	35.26+/-27.48	12.85+/-14.42	3.12+/-2.21	14
Term fetus	12.57+/-7.28	7.51+/-6.87	52.02+/-14.75	32.38+/-27.86	14.96+/-10.9	2.76+/-1.77	7
Preterm fetus	13.57+/-12.59	5.7+/-4	67.44+/-24.2	32.72+/-16.74	8.37+/-8.02	2.36+/-1.17	14
Fetus (term & preterm)	13.24+/-10.92	6.3+/-5.01	62.3+/-22.39	32.61+/-20.37	10.57+/-9.36	2.49+/-1.37	21

The expressions of CD40 and CD86 on the plasmacytoid subset is shown as absolute numbers, percentage positive and PCI ratio

6.4.2.2.1 CD40 expression

There was a statistically significant decrease in the numbers of $CD11c^{-}CD45^{hi}$ DCs expressing CD40 with advancing age from fetus to adult (*p* 0.037). On comparison within the subgroups of preterm and term the reduction in numbers was significant between term CB and adult PB and between preterm CB and adult PB (Figure 6.4A).

There was no significant difference in the percentage of DCs positive for CD40 (p 0.222) and in the PCI ratios (p 0.750) between adult PB and CB. Comparison of the subgroups of term and preterm also showed no difference in the percentage positive (Figure 6.4B) and PCI ratios (Figure 6.4C).

6.4.2.2.2 CD86 expression

The $CD11c^{-}CD45^{hi}$ CBDCs did not exhibit any change in the expression of CD86 with advancing age and gestation. No change was seen in absolute numbers (Figure 6.4D), percentage of DCs positive (Figure 6.4E) and PCI ratios (Figure 6.4F).

Taken together the data above shows that the plasmacytoid CBDC subset significantly decreased the numbers of DCs expressing CD40 with advancing age from fetus to adult, but there was no significant change in CD86 expression.

6.4.2.3 CD11c^{-}CD45^{inm} CBDCs expressing CD40 and CD86 increased with advancing gestational age

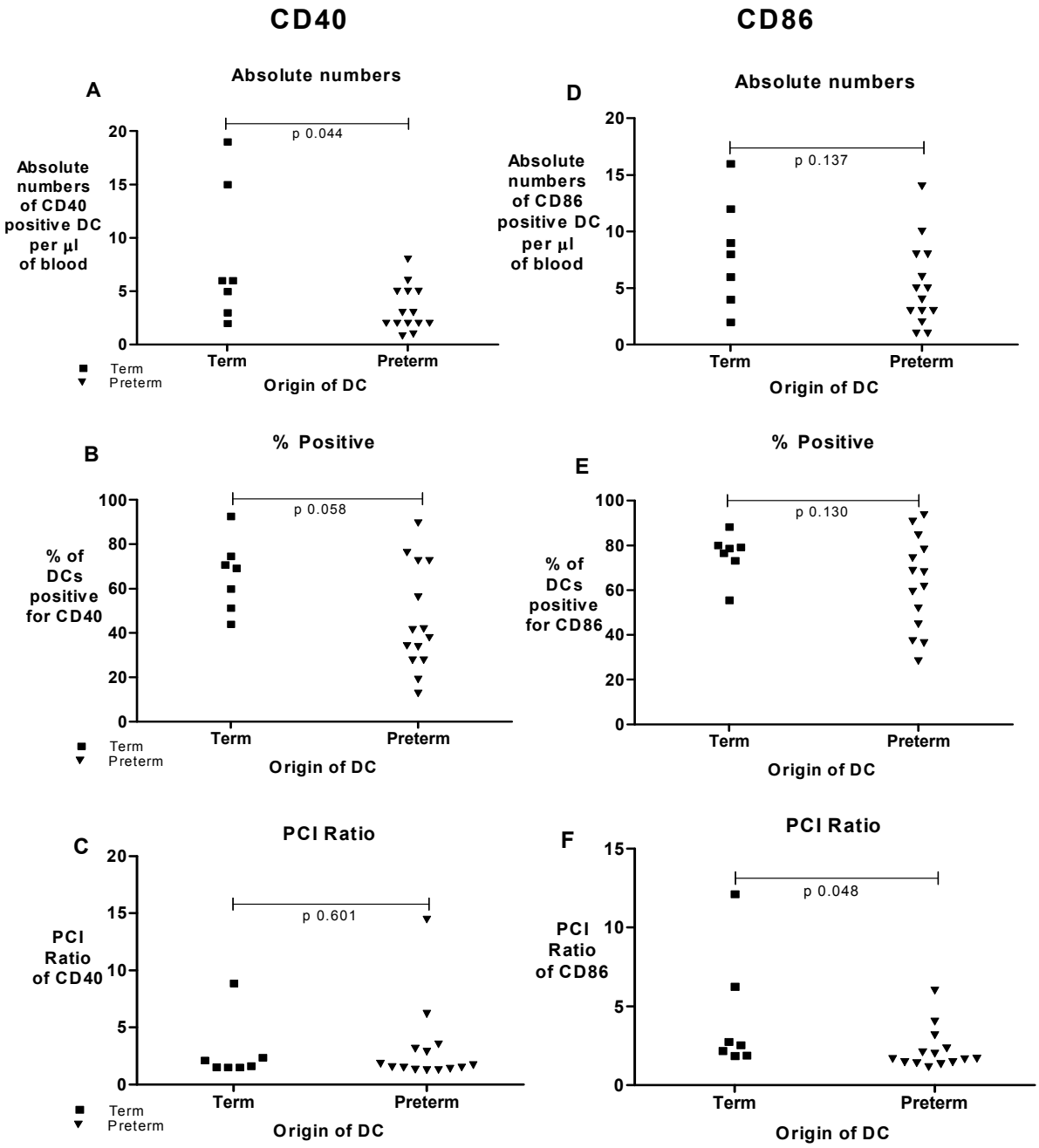


Figure 6.5 The expression of CD40 and CD86 in the CD11c^{-}CD45^{inm} CBDC subset

Multigraph with point plots and *p* values shown. Numbers of CD40 and CD86 positive

DCs, percentage positive and PCI ratio shown per µl of blood. The fetal samples were analysed based on their gestational age alone into term and preterm

Table 6.4 Data on change in CD40 and CD86 expression on $CD11c^{-}CD45^{inm}$ CBDCs with gestational age

Origin of sample	Mean+/-SD						No : of samples (*n*)
	Absolute numbers		% positive		PCI ratio		
	CD40	CD86	CD40	CD86	CD40	CD86	
Term fetus	8+/-6.43	8.14+/-4.78	66.08+/-16.11	76.01+/-10.14	2.80+/-2.71	4.23+/-3.81	7
Preterm fetus	3.34+/-2.11	5.21+/-3.7	45.98+/-23.59	62.72+/-20.82	3.11+/-3.54	2.24+/-1.34	14

The expressions of CD40 and CD86 on the $CD11c^{-}CD45^{inm}$ CBDC subset is shown as absolute numbers, percentage positive and PCI ratio

6.4.2.3.1 CD40 expression

A significant upregulation was seen in the absolute numbers in term CB compared with preterm CB (Figure 6.5A).

The percentage of DCs positive for CD40 in term CB did not reach statistical significance when compared with preterm CB (Figure 6.5B). The PCI Ratios were similar in term and preterm CB (Figure 6.5C).

6.4.2.3.2 CD86 expression

A statistically significant upregulation in the PCI ratio was seen in term when compared to preterm CB (Figure 6.5F).

No statistically significant increase was seen in the absolute numbers (Figure 6.5D) and in the percentage positive DCs (Figure 6.5E).

Taken together the data above indicates that the $CD11c^{-}CD45^{inm}$ CBDC subset significantly increased the numbers of DCs expressing CD40 with advancing gestational age. There was also a significant increase in the expression of CD86 with advancing gestational age.

6.4.3 Change in costimulatory marker expression on CBDC populations on exposure to stressors

It was postulated that increasing gestational age as well as fetal/intrauterine stressors could lead to increased expression of costimulatory markers. To distinguish between the upregulation effects of stressors and of increasing gestational age all the preterm CB samples (*n*-14) were analysed in two groups; samples from fetuses with (*n*-7) and without stress stimuli (*n*-7). All the term CB analysed did not have any exposure to stress stimuli (*n*-7).

6.4.3.1 $CD11c^{+}CD45^{hi}$ CBDCs did not change CD40 and CD86 expression with stressors

Table 6.5 Data on CD40 and CD86 expression on myeloid CBDCs with stressors

Origin of sample	Mean+/-SD						No : of samples (*n*)
	Absolute numbers		% positive		PCI ratio		
	CD40	CD86	CD40	CD86	CD40	CD86	
Term non stressed	6.29+/-6.87	13.43+/-9.03	33.86+/-25.37	76.91+/-28.04	4.88+/-2.47	4.77+/-2.3	7
Preterm non stressed	2.75+/-1.84	8.17+/-3.76	29.99+/-12.89	88.31+/-11.36	3.86+/-2.37	10.24+/-7.73	7
Preterm stressed	6.89+/-5.57	14.43+/-9.98	34.23+/-24.52	74.59+/-22.35	3.34+/-0.82	5.53+/-3.40	7

Numbers of myeloid DC positive for CD40 and CD86 expression, percentage positive and PCI ratio shown per µl of blood

The myeloid subset did not change on exposure to stressors in the expressions of CD40 and CD86 in the numbers of positive DCs, percentage of positive DCs and PCI ratios.

6.4.3.2 $CD11c^{-}CD45^{hi}$ CBDCs did not change CD40 and CD86 expression with stressors

Table 6.6 Data on CD40 and CD86 expression on plasmacytoid CBDCs with stressors

Origin of sample	Mean+/-SD						No : of samples (*n*)
	Absolute numbers		% positive		PCI ratio		
	CD40	CD86	CD40	CD86	CD40	CD86	
Term non stressed	12.57+/-7.28	7.51+/-6.87	67.48+/-32.42	32.38+/-27.86	14.96+/-10.9	2.76+/-1.77	7
Preterm non stressed	9+/-4.97	3.67+/-2.05	52.02+/-14.75	28.34+/-16.84	11.5+/-10.08	2.51+/-1.47	7
Preterm stressed	18.14+/-16.44	7.71+/-4.5	67.4+/-14.77	37.11+/-16.69	5.26+/-3.9	2.21+/-0.88	7

Numbers of plasmacytoid DC positive for CD40 and CD86 expression, percentage positive and PCI ratio shown per µl of blood

The plasmacytoid subset did not change on exposure to stressors in the expressions of CD40 and of CD86 in the numbers of positive DCs, percentage of positive DCs and PCI ratios.

6.4.3.3 Numbers of CD11c^{-}CD45^{inm} CBDCs expressing CD40 and CD86 increased with stressors

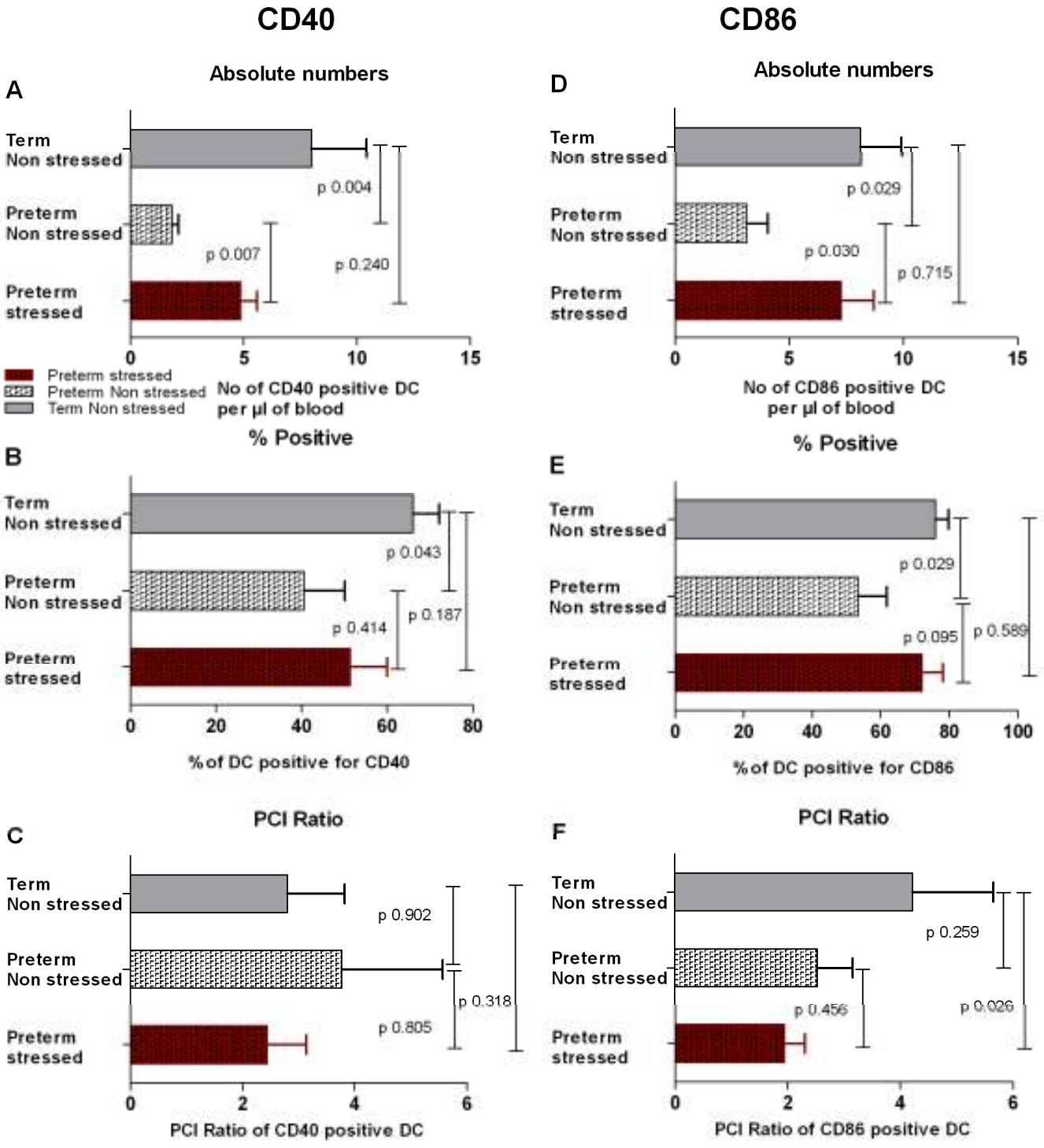


Figure 6.6 CD40 and CD86 expression on the CD11c$^-$CD45^{inm} DCs with stressors

Multigraph with column bars and *p* values shown. Numbers of CD40 and CD86 positive DCs, percentage positive and PCI ratio of positive DCs shown per µl of blood

Table 6.7 Data on CD40 and CD86 expression on CD11c$^-$CD45^{inm} CBDCs with stressors

Origin of sample	Mean+/-SD						No : of samples (*n*)
	Absolute numbers		% positive		PCI ratio		
	CD40	CD86	CD40	CD86	CD40	CD86	
Term non stressed	8+/-6.43	8.14+/-4.78	66.08+/-16.11	76.01+/-10.14	2.8+/-2.7	4.23+/-3.81	7
Preterm non stressed	1.83+/-0.73	3.14+/-2.41	40.59+/-25.02	53.42+/-21.92	3.78+/-4.77	2.54+/-1.66	7
Preterm stressed	4.86+/-1.95	7.29+/-3.79	51.38+/-22.62	72.03+/-16.02	2.44+/-1.83	1.95+/-0.95	7

Numbers of CD11c$^-$CD45^{inm} DC positive for CD40 and CD86 expression, percentage positive and PCI ratio shown per µl of blood

6.4.3.3.1 CD40 expression

A significant stress related increase in numbers was seen in the CB of preterm fetuses with stress compared to the preterm fetuses without stress. An age related significant increase in numbers was also seen in the term CB compared with preterm non stressed CB (Figure 6.6A).

No stress related change was seen in the percentage of DCs positive for CD40 or in the PCI ratios (Figures 6.6B and 6.6C). There was an age related increase in the percentage of DCs expressing CD40 in the term non stressed CBDCs compared to the preterm non stressed CBDCs.

6.4.3.3.2 CD86 expression

An increase in CD86 expression was seen which was similar to the increase in CD40 expression. Thus a stress related as well as an age related increase was seen in the numbers of CBDCs positive for CD86 (Figure 6.6D).

No stress related change was seen in the percentage of DCs positive for CD86 or in the PCI ratios (Figures 6.6E and 6.6F). There was an age related increase in the percentage of DCs expressing CD86 in the term non stressed CBDCs compared to the preterm non stressed CBDCs (Figure 6.6E).

These findings postulate that advancing gestational age as well as exposure to stressors increased costimulatory marker expression in the $CD11c^{-}CD45^{inm}$ subset.

6.4.4 Ongoing IL-12 production by CBDC populations

6.4.4.1 Analysis of expression

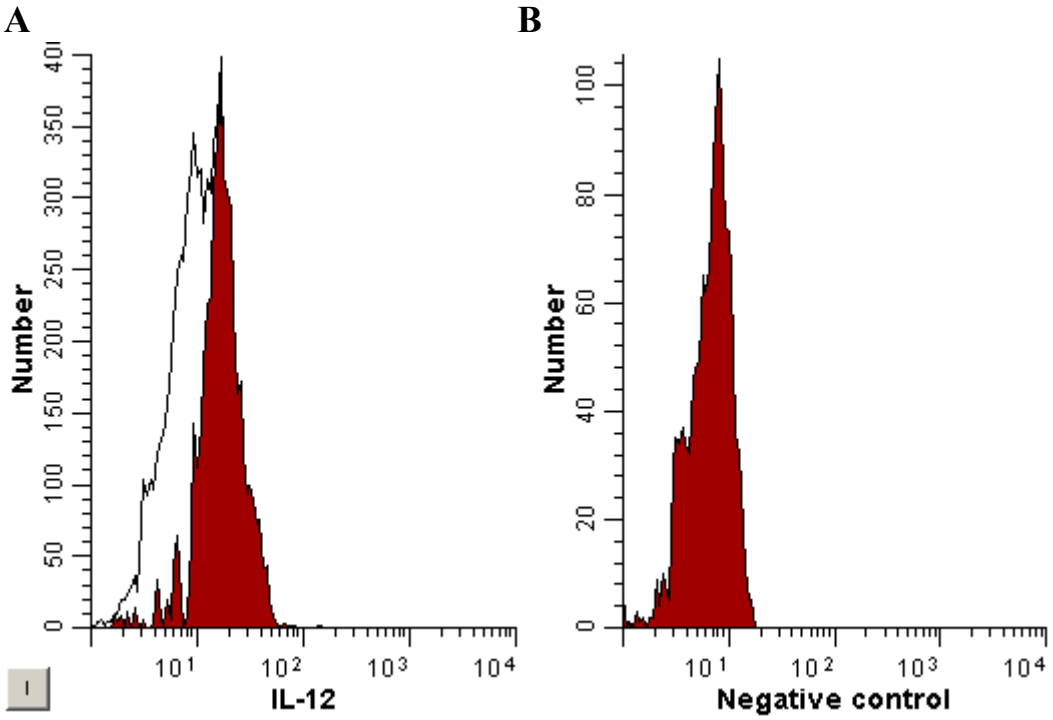


Figure 6.7 Subtraction method for analysis of production of IL-12

Accumulation of IL-12 within freshly-isolated cells during a 4hr incubation was measured. No monensin controls were used to delineate the negative expression of the marker to be identified (Figure 6.7B). The intracellular cytokine production was determined by subtracting a no monensin control staining with the same cytokine antibody from staining with the marker (Figure 6.7A). Histogram shown with unmarked region indicating negative fluorescence (Figure 6.7A). Representative histograms shown from 18 experiments

The amount of intracellular cytokine expression was analysed using the subtraction method as described in Figure 6.7. A no monensin control was used rather than an isotype control antibody as this avoided the potential complications from the labelling of preformed cytokine, and of the differences in the fluorochrome to antibody ratio on isotype controls. The given method provided us with an accurate assessment of the amount of IL-12 produced during the time window of 4 hours. Detailed discussion of the advantages of this method is given in **Chapter 7.**

6.4.5 Changes in IL-12 production by CBDC populations with age

6.4.5.1 IL-12 production by the $CD11c^{+}CD45^{hi}$ and $CD11c^{-}CD45^{hi}$ CBDC populations

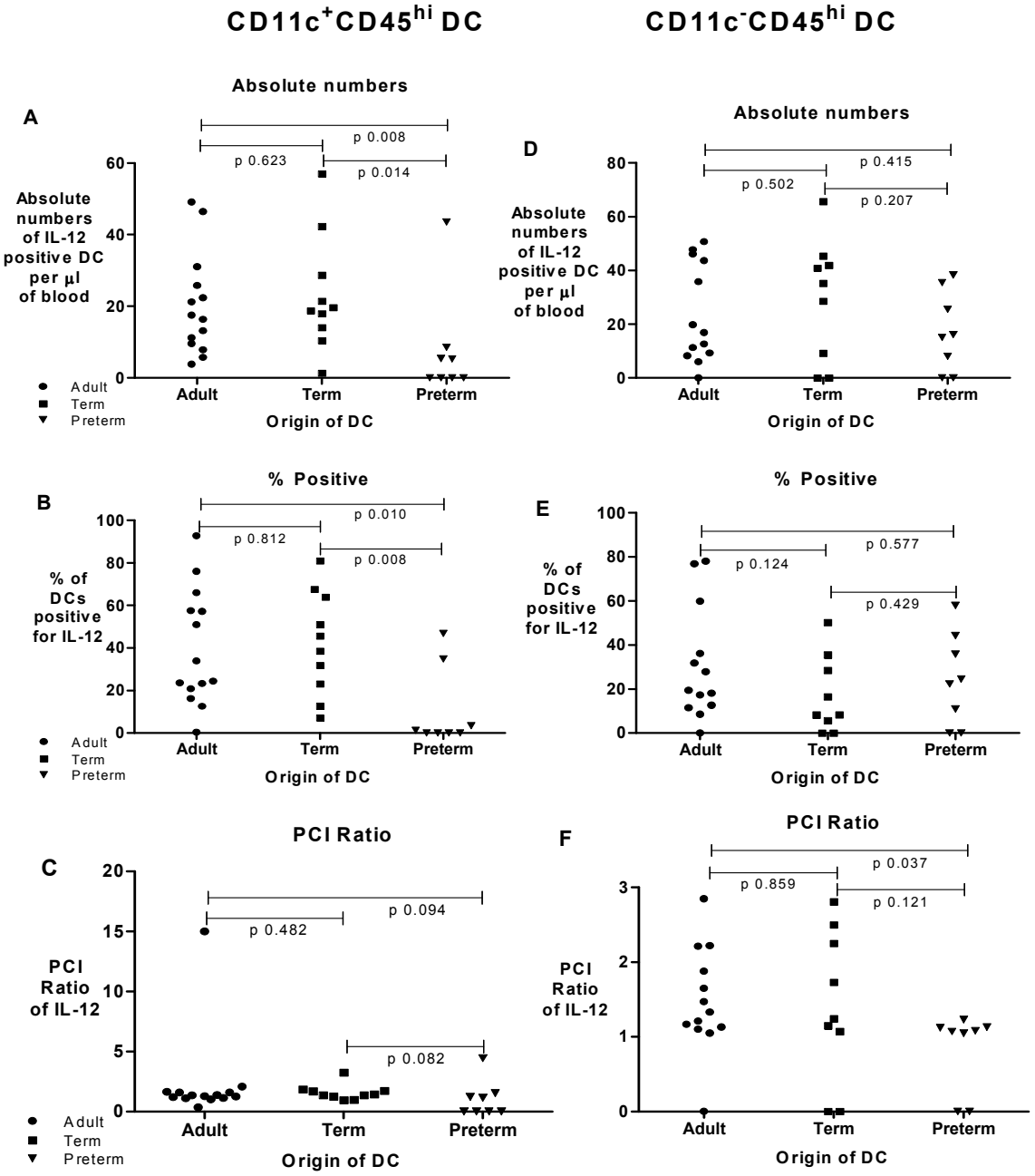


Figure 6.8 IL-12 production by the $CD11c^{+}CD45^{hi}$ and $CD11c^{-}CD45^{hi}$ CBDCs

Multigraph with point plots and *p* values shown. Numbers of IL-12 positive DCs,

percentage positive and PCI ratio of positive DCs shown per µl of blood. The fetal samples were analysed based on their gestational age alone into term and preterm

Table 6.8 Data on IL-12 production by the myeloid and plasmacytoid DCs with age

Origin of sample	Mean+/-SD						No:of samples (*n*)
	Absolute numbers		% positive		PCI ratio		
	Myeloid	Plasma cytoid	Myeloid	Plasma cytoid	Myeloid	Plasma cytoid	
Adult	20.08+/-14.07	23.69+/-18.29	39.7+/-27.08	30.58+/-25.58	2.28+/-3.69	1.48+/-0.71	14
Term fetus	23.15+/-16.06	29.63+/-22.43	42.29+/-24.21	16.95+/-17.42	1.59+/-0.65	1.42+/-1.01	10
Preterm fetus	7.84+/-14.79	17.3+/-14.76	10.82+/-18.83	24.48+/-20.79	1.04+/-1.52	0.84+/-0.52	8
Fetus (term & preterm)	16.35+/-16.97	23.83+/-19.67	28.3+/-26.74	20.5+/-18.87	1.35+/-1.12	1.14+/-0.85	18

Numbers of myeloid and plasmacytoid DC positive for IL-12, percentage positive and PCI ratio shown per µl of blood

6.4.5.1.1 IL-12 production by the $CD11c^{+}CD45^{hi}$ subset

Significant increase was seen in the numbers (Figure 6.8A) and in the percentage positive DCs (Figure 6.8B) from preterm to term and from fetus to adult showing increased production with advancing gestation as well as with age. No change was seen in the PCI ratios (Figure 6.8C).

6.4.5.1.2 IL-12 production by the $CD11c^{-}CD45^{hi}$ subset

There was a significant increase in PCI ratios between preterm and adult (Figure 6.8F). There was no significant change in the numbers of positive DCs and percentage positive DCs (Figure 6.8D and 6.8E).

Taken together the data above indicate that the myeloid DCs increased IL-12 production with age on comparison between adult and preterm CB and with advancing gestational age on comparison between preterm and term CB. The plasmacytoid DCs increased IL-12 expression with increasing age, but did not change with advancing gestational age.

6.4.5.2 The CD11c^{-}CD45^{inm} CBDC population did not change IL-12 production with advancing gestational age

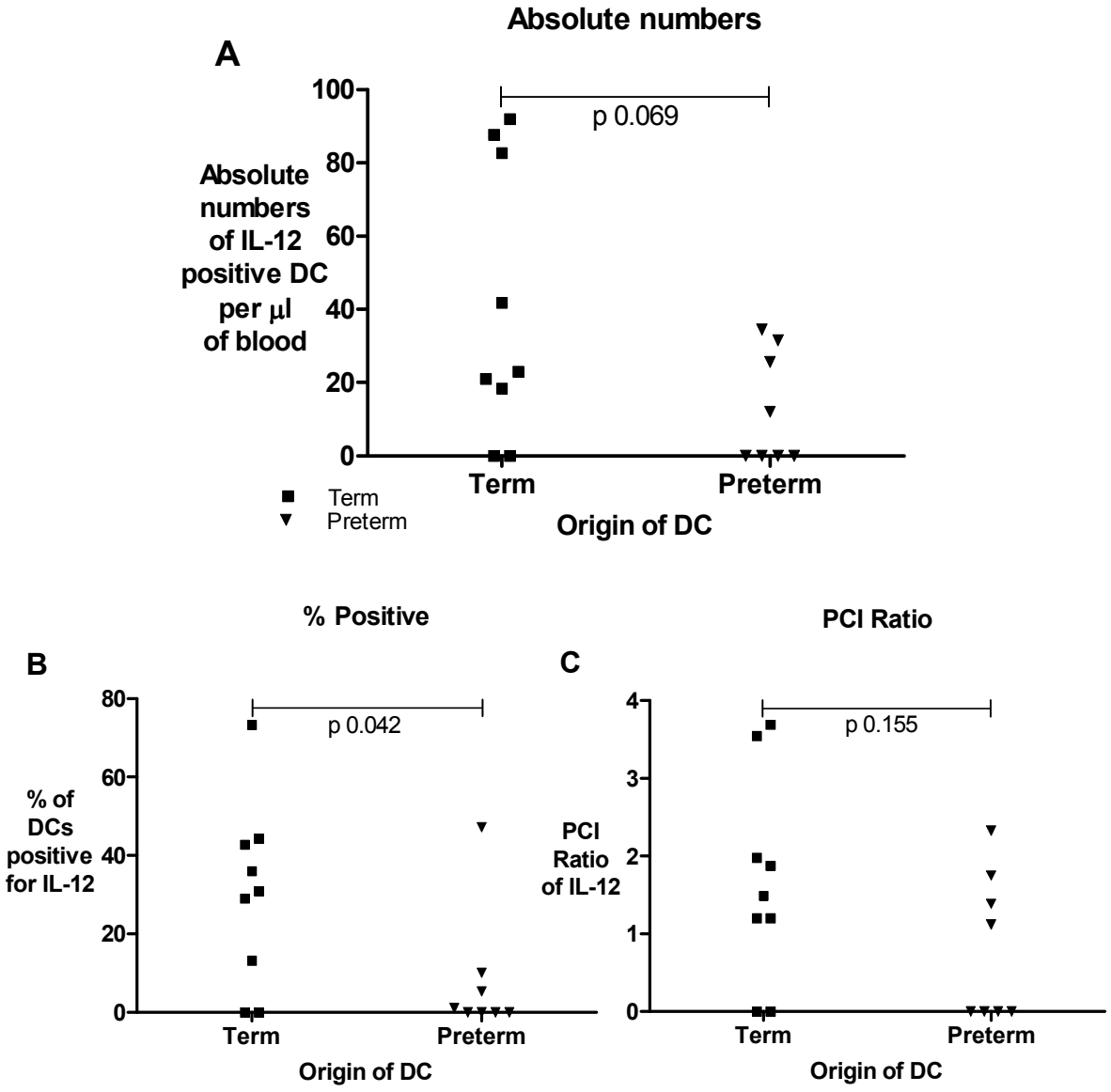


Figure 6.9 IL-12 production by CD11c^{-}CD45^{inm} CBDCs with gestational age

Multigraph with point plots and *p* values shown. Numbers of IL-12 positive DCs, percentage positive and PCI ratio of positive DCs shown per µl of blood

Table 6.9 Data on IL-12 production by $CD11c^{-}CD45^{inm}$ CBDCs with gestational age

Origin of sample	Mean+/-SD			No : of samples (*n*)
	Absolute numbers	% positive	PCI ratio	
Term fetus	40.75+/-37.26	29.97+/-23.35	1.67+/-1.31	10
Preterm fetus	12.96+/-15.31	7.97+/-16.27	0.82+/-0.95	8

Numbers of $CD11c^{-}CD45^{inm}$ DC positive for IL-12, percentage positive and PCI ratio shown per µl of blood

The $CD11c^{-}CD45^{inm}$ CBDCs did not change IL-12 production in the numbers of positive DCs (Figure 6.9A) and PCI ratios (Figure 6.9C).

There was a significant increase in the percentage of IL-12 positive DCs with advancing gestational age (Figure 6.9B). As this measure was of the percentage of positive DCs, this could be affected by the change in the actual numbers of DCs occurring with the change in gestation. And this may not be a real reflection of increased IL-12 production.

6.4.6 Changes in IL-12 production by CBDC populations on exposure to stressors

It was postulated that increasing gestational age as well as fetal/intrauterine stressors could lead to increased production of IL-12. To distinguish between the upregulation effects of stressors and of increasing gestational age all the preterm CB samples (*n*-8) were analysed in two groups; fetuses with (*n*-3) and without (*n*-5) stress stimuli. The term CB samples (*n*-10) were analysed in two groups; fetuses with stress (*n*-5) and without stress (*n*-5) stimuli.

6.4.6.1 IL-12 production by the $CD11c^{+}CD45^{hi}$ and $CD11c^{-}CD45^{hi}$ CBDCs with stressors

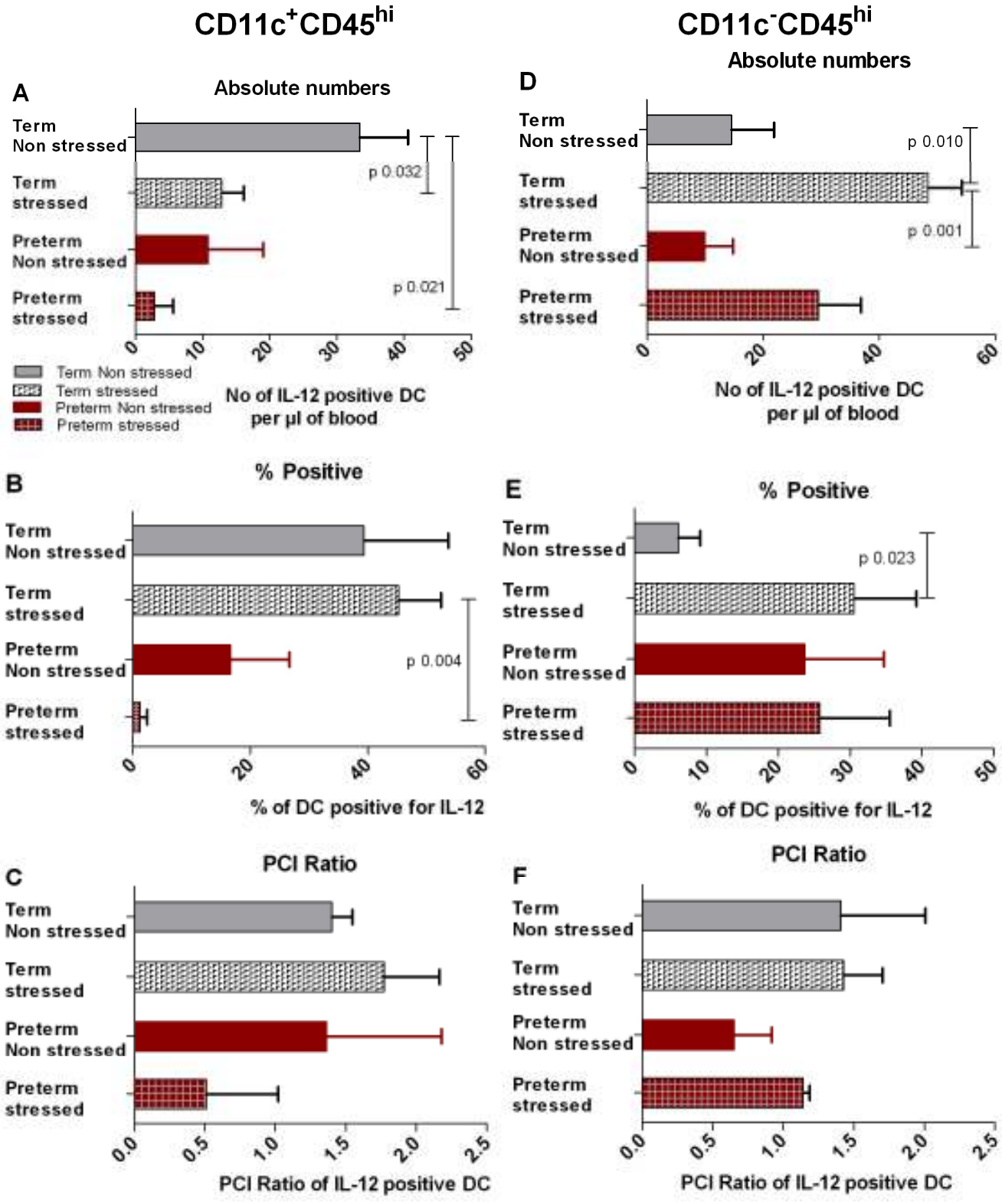


Figure 6.10 IL-12 production by myeloid and plasmacytoid CBDCs with stressors

Multigraph with column bars and significant *p* values shown. Numbers of IL-12 positive DCs, percentage positive and PCI ratio of positive DCs shown per µl of blood

Table 6.10 Data on IL-12 production by myeloid and plasmacytoid DC subsets with stressors

Origin of sample	Mean+/-SD						No : of samples (*n*)
	Absolute numbers		% positive		PCI ratio		
	Myeloid	Plasma cytoid	Myeloid	Plasma cytoid	Myeloid	Plasma cytoid	
Term non stressed	33.46+/-16.12	14.59+/-16.4	39.37+/-32.12	6.09+/-6.84	1.4+/-0.32	1.41+/-1.34	5
Term stressed	12.85+/-7.42	48.43+/-11.62	45.21+/-16.3	30.53+/-17.44	1.77+/-0.88	1.43+/-0.55	5
Preterm non stressed	10.84+/-18.46	9.92+/-10.96	16.6+/-22.52	23.69+/-24.74	1.36+/-1.82	0.65+/-0.6	5
Preterm stressed	2.84+/-4.92	29.59+/-12.64	1.19+/-2.07	25.81+/-16.89	0.51+/-0.88	1.14+/-0.08	3

Numbers of myeloid and plasmacytoid DC positive for IL-12, percentage positive and PCI ratio shown per µl of blood

6.4.6.1.1 IL-12 production by the $CD11c^{+}CD45^{hi}$ CBDCs

The $CD11c^{+}CD45^{hi}$ CBDC subset significantly decreased the numbers of IL-12 producing DCs in the term stressed group when compared to the term non stressed group. In the preterm stressed group the numbers did not reach statistical significance. Both the stressed groups (term and

preterm) were not different and hence this decrease in production was independent of the gestational age (Figure 6.10A).

The above change was not reflected in the percentage of positive DCs and PCI ratios (Figures 6.10B and 6.10C).

6.4.6.1.2 IL-12 production by the $CD11c^{-}CD45^{hi}$ CBDCs

The $CD11c^{-}CD45^{hi}$ CBDC subset significantly increased the numbers of IL-12 producing and percentage positive DCs in the term stressed group when compared to the term non stressed group. In the preterm stressed group the numbers did not reach statistical significance. Both the stressed groups were not different and hence this increase in production was independent of the gestational age (Figures 6.10D and 6.10E).

The above change was not reflected in the PCI ratios (Figure 6.10F).

Taken together the data given above shows that myeloid DCs decreased and plasmacytoid DCs increased IL-12 production on exposure to stressors. The above changes were not reflected in the PCI ratios which could be the effect of the small number of samples in each group.

6.4.6.2 The numbers of CD11c^{-}CD45^{inm} CBDC producing IL-12 increased with stressors

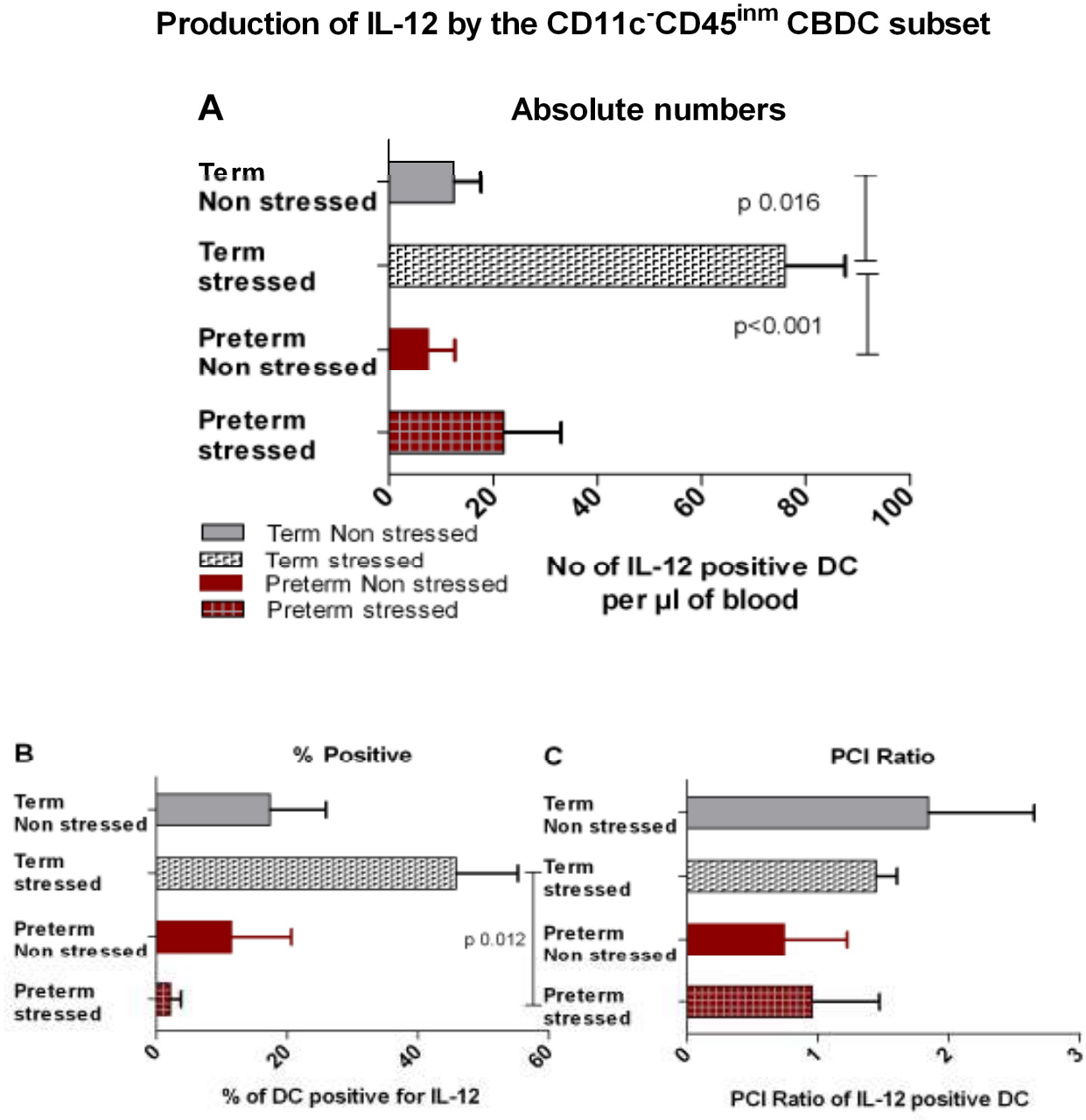


Figure 6.11 IL-12 production by the CD11c^{-}CD45^{inm} CBDCs with stressors

The above multigraph with column bars show the numbers, percentage positive DCs and PCI ratios for IL-12 production. Only significant *p* values are shown on the graph

Table 6.11 Data on IL-12 production by $CD11c^{-}CD45^{inm}$ CBDCs with stressors

Origin of sample	Mean+/-SD			No : of samples (*n*)
	Absolute numbers	% positive	PCI ratio	
Term non stressed	12.49+/-11.52	17.34+/-19.28	1.84+/-1.81	5
Term stressed	76.06+/-23.08	45.77+/-18.97	1.44+/-0.32	5
Preterm non stressed	7.54+/-11.39	11.46+/-20.46	0.74+/-1.07	5
Preterm stressed	22+/-19.11	2.14+/-2.83	0.96+/-0.89	3

The change in $CD11c^{-}CD45^{inm}$ CBDCs with stressors is shown as absolute numbers, percentage positive DCs and PCI ratios

Exposure to stressors resulted in an increase in the numbers of $CD11c^{-}CD45^{inm}$ CBDCs producing IL-12 in term. The term stressed CBDCs produced significantly more IL-12 than the term non stressed group. The preterm stressed group were not significantly different to the preterm non stressed group. Thus the numbers of CBDCs producing IL-12 was increased on exposure to stressors and this increased production was seen irrespective of the gestation (Figure 6.11A).

The above increase was not reflected in the percentage of positive DCs, and the PCI ratios which could be a reflection of the small numbers of samples in each group (Figure 6.11B and 6.11C).

6.5 DISCUSSION

This study analysed the proinflammatory changes on CBDC populations on exposure to stressor stimuli. The findings confirmed increased numbers of CD40 and CD86 expressing DCs and increased production of IL-12 by the $CD11c^{-}CD45^{inm}$ DC subset in response to infection or stress in the CB samples. Both the myeloid and the plasmacytoid CBDC subset did not alter the expression of costimulatory markers on exposure to stressors in the preterm subset. The myeloid subset decreased production of IL-12 while the plasmacytoid subset increased IL-12 production on exposure to stressors. The previous findings of change in these DC subsets with increasing gestation lead us to investigate whether an infectious or immunogenic stimuli has the same potential to trigger proinflammatory responses in the CBDC populations.

As CBDCs have long been labelled as tolerogenic, their expression of costimulatory markers was assessed initially prior to analysing the change seen with stressors. The myeloid subset expressed more CD86 than CD40 and this finding was reversed in the plasmacytoid subset. Comparison showed CD40 expression to be highest in the plasmacytoid subset and CD86 expression to be highest in the myeloid subset. The $CD11c^{-}CD45^{inm}$ subset expressed equal amounts of both CD40 and CD86 with at least more than 50 percentage of DCs in this subset expressing the markers. These findings add credence to an immunogenic capacity of fetal DCs. The role and mechanism of action of CD40 is different from that of CD86 and as myelod DC and pDC are different DC types, it would be immunologically advantageous that they have different levels of such co-stimulatory markers.

Any change in the expression of costimulatory markers with advancing gestational age was analysed. CBDCs regulated CD40 expression more than CD86. The myeloid CBDC subset

increased and the plasmacytoid CBDC subset decreased CD40 expression with advancing age to adult. Both subsets did not change with advancing gestational age from preterm to term. CD40 and CD86 were upregulated with advancing gestational age from preterm to term by the $CD11c^{-}CD45^{inm}$ subset.

Costimulatory marker expression of CD40 and CD86 was analysed on the CBDC subsets on exposure to stressors. The integration of antigenic stimuli through the costimulatory molecules is critical for the activation of immune responses and hence the upregulation of these molecules indicate an immune system which has or is being activated. A stressor related increase was seen in the $CD11c^{-}CD45^{inm}$ CBDC with preterm CBs exposed to stressors upregulating both CD40 and CD86. Upregulation by preterm CBs exposed to stressors were similar to the non stressed term samples which lead to the hypothesis that exposure to stressors stimuli could be maturing the premature CBDCs to a gestationally mature profile in readiness for delivery. An age related increase was also seen between the non stressed preterm and term samples. The ability of DCs to express costimulatory molecules indicates the maturation of the APC (Hellstrom, *et al.,* 1996). These findings confirm the hypothesis that the $CD11c^{-}CD45^{inm}$ DC subset appear to be an immunologically immature (decreased levels of CD45), changing population, which is matured with increasing gestation and exposure to stressors.

Significantly the myeloid and plasmacytoid subsets did not change on exposure to stressors in the expression of these costimulatory markers. Only the $CD11c^{-}CD45^{inm}$ subset DCs increased the expressions of CD40 and CD86 markers in response to stressors. This finding enhances the importance of this DC subset as the pivotal APC within the fetal immune system capable of mounting an immune response and thus the measure of the maturation of this DC subset could be a measure of the immunocompetence of the fetus.

IL-12 is a proinflammatory cytokine produced by DCs on exposure to infectious stimuli, the analyses of which would give a lucid depiction of the immunogenic capacity of the fetal DCs. Hence the production of IL-12 by all of the CBDC subsets was analysed. As previous findings have indicated that increasing gestational age alone has the potential to alter the immunogenicity of the CBDC, the changes with age were analysed initially. The $CD11c^{+}CD45^{hi}$ and $CD11c^{-}CD45^{hi}$ DC subsets increased IL-12 production with advancing age. This increase of IL-12 production was consistent with a shift by the DCs towards preparation for birth and therefore perhaps a Th1 generating capacity. Conversely the $CD11c^{-}CD45^{inm}$ DCs did not change production of IL-12 with advancing gestational age.

Alterations of IL-12 production on exposure to stressors were analysed. Exposure to stressors increased the production of IL-12 by the $CD11c^{-}CD45^{inm}$ CBDCs in the term fetuses. The myeloid CBDC subset decreased and the plasmacytoid CBDC subset increased IL-12 production on exposure to stressors.

Preterm neonates born as a result of an infectious aetiology are more likely to have long term sequelae such as bronchopulmonary dysplasia (Yoon, *et al*., 1997b), PVL and an increased incidence of CP (Yoon, *et al*., 1997a). The Th2 bias of the fetal immune system with its attendant immunodeficiency has been implicated in the increased susceptibility of neonates to infection (Kalinski, *et al*., 1990; Wegmann, *et al*., 1993; Naderi, *et al*., 2009). Infections in a decreased immunocompetent environment are potentially disastrous, with long term sequelae especially neurologically (Yoon, *et al*., 1997a). Identification of preterm infants who are at the greater risk of short and long term infectious morbidity will have an impact on further management and prognosis.

The ability of DC to alter their phenotype and function in response to microbial exposure suggests that changes of CBDC populations might be useful markers of intrauterine infection and neonatal sequelae (Kadowaki, *et al*., 2001). The sensitivity of DCs to changes *in utero* distinguishes this APC as ideal to be used in identifying such changes. The observation that out of the three subsets of DCs identified in the fetus only the $CD11c^{-}CD45^{inm}$ subset increased the costimulatory markers expression is significant in understanding the importance of this DC subset in the modulation of the fetal immune system. This finding emphasises the sensitivity of this DC subset to antigenic stimuli as opposed to the other DC subsets. The implications of these findings in relation to the known infectious aetiology of preterm births are vital to the understanding of the pathophysiology of prematurity.

The observed upregulation of costimulatory markers with infection raises the question whether these changes on the $CD11c^{-}CD45^{inm}$ DC subset have the potential to be used as biomarkers to identify neonates exposed to infection or stress *in utero.* Monitoring of the phenotype and functional changes of the $CD11c^{-}CD45^{inm}$ DC subset could provide a diagnostic marker of infection/stress and identify the immunologically challenged fetus. Developing the technology to analyse the CB provides us with a sample obtained at no discomfort or pain to the baby and as it is the earliest possible neonatal sample available, will provide us with the earliest clue to the infection risk and prognosis. It would also act as a prognostic factor to predict long term neurological outcome as increased levels of cytokines are associated with cerebral palsy (Yoon, *et al*., 1997a). Flow cytometer facilities are available in most laboratories and thus this analysis could be a valuable aid in the management of prematurity in decreasing the infectious sequelae, both neonatal and maternal.

CHAPTER 7

DISCUSSION

7.1 General Discussion

Major findings

By the very definition the primary function of an APC is orchestrating the defence of the human body against infections, which the DC does so admirably. Many studies catalogue the central role played by the DC in infectious states, e.g.: HIV. But to date there is no evidence in published literature of the role of the DC in preterm labour. Research in preterm labour has so far focussed on the microbiological and preventative aspects. The annals of history have taught us that understanding the molecular pathogenesis of a disease is the key to formulating preventative and curative strategies. Thus as professional APCs, the DC would be fundamental to the immune response mounted during preterm labour and it seems logical that studying the immune modulation by the DC would provide us with many answers regarding the pathogenesis of preterm labour and delivery.

This body of work presented has successfully identified DC populations in CB comparable to the populations seen in adult PB. Additionally analyses of the DC subsets in CB lead to identification of a hitherto unreported putative $CD11c^{-}CD45^{inm}$ DC population with phenotypic and functional properties conforming to the characteristics of DC in established literature. We have shown that the $CD11c^{-}CD45^{inm}$ CBDCs have the capacity to mount an immune response on exposure to intrauterine stressor stimuli by their upregulation of costimulatory markers and increased production of IL-12. Of the three CBDC populations, the novel $CD11c^{-}CD45^{inm}$ population proved to be the most kinetic, changing with gestational age as well as on exposure to stressors.

As expected, phenotypic analysis of plasmacytoid markers was negative on the myeloid subset and positive on the plasmacytoid subsets in adult and fetus. The $CD11c^{-}CD45^{inm}$ subset was negative for the expression of CD123, BDCA2, CD45RA, CD33 and CD13, showing these DCs to be distinct from the plasmacytoid DCs and not to be of myeloid lineage. In literature to date, previous reports of increased $CD11c^{-}$ DC in CB have not distinguished plasmacytoid DCs from the distinct $CD11c^{-}CD45^{inm}$ population described here. The phenotypic analyses show this novel population to be a distinct subset of CBDCs, and not a subset of or form a part of the $CD11c^{-}$ plasmacytoid CBDCs.

The functional studies showed that all CBDC subsets endocytosed Dextran particles. The $CD11c^{-}CD45^{inm}$ subset exhibited endocytosis comparable to the myeloid DC subset in the adult. CBDCs stimulated MLR and the capacity was equivalent to adult DCs. It would have been pertinent to assess the stimulatory capacity of the various CBDC subsets separately after sorting. But unfortunately labelling and sorting which required handling of the cells negated the stimulatory capacity.

The fetal immune system exhibit changes in all its components and function with advancing gestational age. Hence the effect of gestational *per se* on the composition and maturation of these CBDC subsets was analysed. The myeloid and plasmacytoid CBDC subsets did not change with advancing gestation. The $CD11c^{-}CD45^{inm}$ subset decreased significantly with increasing gestational age. This subset represented 31.33% of the total number of DCs in the preterm CB, but represented 21.26% in the term CB samples and only 1.54% of the total number of DCs in adult PB. It was interesting to note that whilst the myeloid subset did not change with advancing gestational age from preterm to adult it predominated in the adult. Does the increase in numbers

occur during the neonatal or childhood periods is a question not answerable to date as no longitudinal comparative studies from fetus to adult have been published.

Gestational age itself affected the total numbers of DC. There was a significant decrease seen from preterm CB to adult PB. Why does the fetus situated especially intrauterine and protected from the environment need more numbers of DCs than the adult? Are the increased numbers in the fetus required for immune modulation at the materno-fetal interface to protect the fetus from immune rejection by the mother? Additionally although the numbers are increased, fetal DCs are tolerogenic promoting a Th2 polarisation (Klein and Remington, 2001; Adkins, *et al.*, 2004). The impaired immune activity may be partly due to increased fetal production of IL-6 which exhibit Th2 polarising functions by inhibiting neutrophil migration to sites of infection (Marchini, *et al*., 2000; Schultz, *et al*., 2002) and also leads to low levels of secretory IgA (Markel, *et al*., 2006). Thus the increased numbers could be a requirement to ensure effectiveness of a Th1 response if necessary.

The maturation process in the DC is associated with increased expressions of HLA-DR and CD45. The surface expressions of HLA-DR and CD45 on the myeloid and plasmacytoid CBDCs were comparable to the adult contradicting the various literature reports of immature CBDCs. Hunt *et al* reported in their study that umbilical CBDCs had lower levels of ICAM-1 and MHC classes I and II than PB DCs from adults (Hunt, *et al.,* 1994). The $CD11c^{-}CD45^{inm}$ DCs exhibited lower levels of CD45 and HLA-DR indicating an immature phenotype. The immaturity of this novel subset could be a major factor in the immaturity of the fetal immune system as significantly increased numbers were seen in the preterm CB compared to the term CB. A lower functional capacity has been attributed to CBDCs when compared to adult DCs. They have a reduced ability to attain a fully mature adult phenotype and to activate CD4+ T cells to produce

IFN-α (Langrish, *et al*., 2002). Theoretically the expressions of HLA-DR and CD45 should increase with advancing gestational age and birth reflecting a maturing immune system, but no such change was identified in our study. This could be a reflection of the small sample size as the changes in expressions could be subtle requiring large numbers of samples in order to identify a significant change.

The microenvironment in which antigen uptake occurs impacts the manner in which DCs process antigens and can determine whether a tolerogenic or an immunogenic response should be mounted. Thus maturation of DCs by an inflammatory cytokine filled milieu result in an immunogenic response and immature DCs in a steady state environment may present antigens that promote tolerance and tissue growth. Evidence to date shows that, in the fetus as both tolerogenic and immunogenic responses are needed, most immune responses are modulated and executed differently from the adult. It was of interest to ascertain the immune responses mounted by the fetus on exposure to stressor stimuli as this in turn would impact on the pathogenesis and initiation of preterm labour. Hence we decided to analyse the changes seen on the identified CBDC populations in response to exposure to intrauterine stressor stimuli. All CBDC subsets increased in numbers on exposure to stressors. This increase in numbers was reflected in the increase in the total numbers of CBDCs on exposure to stressors. Thus the CBDCs in our study exhibited capacity to mount a response to immunogenic stimuli.

But as evidence shows this response has to be tempered with a need to mask an overt expression of the fetal antigens and hence the tolerogenic expressions of fetal DCs predominate. Thus although they increased in numbers of DCs on exposure to stressor stimuli, the myeloid and plasmacytoid CBDC subsets did not upregulate the costimulatory markers; CD40 and CD86 on exposure to stressor stimuli. This immature functioning of the DCs conforms to literature

evidence of immunotolerance in the fetus. Conversely the $CD11c^{-}CD45^{inm}$ CBDC subset upregulated the above costimulatory markers on exposure to stressor stimuli. Antigen presentation in the feto-maternal interface is unique and presentation on 'non-classical' MHC class I proteins, such as HLA-E and HLA-G may inhibit maternal responses to fetal antigen and induce a 'tolerogenic' state (Holt and Jones, 2000; Petty and Hunt, 1998). Thus the upregulation of costimulatory markers by the $CD11c^{-}CD45^{inm}$ CBDCs contrary to the established tolerogenic immune capacity of fetal DCs confer an important role on these DCs in the capacity of the fetus to mount an immune response.

Advancing age was seen to have an impact on the expressions of the costimulatory markers. Holloway *et al* has shown the percentage of CBDCs expressing CD40, CD86 and CD54 are lower than adults throughout gestation with a reduced capacity for costimulation and the capacity increases with advancing gestation (Holloway, *et al*., 2009). The level of expressions of the costimulatory markers varied between the various CBDC subsets. On comparison between the subsets the myeloid subset expressed the highest levels of CD86 and the plasmacytoid subset expressed the highest levels of CD40. This was in line with literature evidence of differences in the expressions of CD86 by the myeloid and plasmacytoid DCs. Myeloid DCs expressed CD86 in basal conditions but not plasmacytoid DCs (Della Bella, *et al*., 2008). With advancing age from fetus to adult, both these subsets varied the expressions of CD40 and showed no change of CD86 expression. The myeloid subset increased while the plasmacytoid subset decreased CD40 expression with age. This finding of decreasing CD40 expression by the plasmacytoid DC is in accordance with the tolerogenic function ascribed to plasmacytoid DCs in their immature state (Liu, 2005; Merad, *et al*., 2007; Steinman and Banchereau, 2007).

Advancing gestational age from preterm to term increased the expressions of CD40 and CD86 on $CD11c^{-}CD45^{inm}$ DCs, while the myeloid and plasmacytoid subsets showed no change. The above finding again displays $CD11c^{-}CD45^{inm}$ DCs to be the most kinetic of all the CBDC subsets.

A similar enhanced activity was seen on analysis of the production of the proinflammatory cytokine, IL-12. The plasmacytoid and the $CD11c^{-}CD45^{inm}$ subset showed increased production of IL-12 on exposure to stressors. Conversely, the myeloid subset decreased the IL-12 production with stressors. Neonatal myeloid DCs are deficient in IFN-b and IL-12 synthesis in response to TLR triggering (Renneson, *et al*., 2009). Functionally neonatal increased susceptibility to infection has been related to their defect in synthesis of interleukins, especially IL-12 in response to both LPS and CD40L (Goreily, *et al*., 2001; Aksoy, *et al*., 2007) and this IL-12 defect has been hypothesised to stem from alterations in the expression of the p35 chain (Goreily, *et al*., 2004).

Advancing age affected the capacity of the CBDCs to produce IL-12. Increased production of IL-12 by the myeloid and plasmacytoid subsets was seen on comparison between fetus and adult. The $CD11c^{-}CD45^{inm}$ subset showed no change in IL-12 production with advancing gestational age.

The above analyses of the response of CBDC subsets to advancing gestational age and exposure to stressor stimuli focussed our attention on the novel $CD11c^{-}CD45^{inm}$ subset as these DCs were more dynamic and changed with gestational age as well as stressor stimuli as opposed to the myeloid and plasmacytoid subsets.

DCs do not function in isolation during an immune response and many cellular elements of the immune system interact to facilitate defence against pathogen attack. Accordingly we analysed the neutrophils in CB samples classified as exposed to stressor stimuli to ascertain if they

exhibited the established neutrophilic changes in response to exposure to infectious stimuli. We were able to validate the clinical classification of CB samples by showing a correlation between the changes on DCs and on neutrophils in response to stressor stimuli.

Technical challenges

We selected CB as the sample for conducting the experiments as it was freely available without additional procedural steps and at no risk to the mother or infant. We analysed the CB as whole blood as analysis of whole blood without manipulation avoids selective loss of cell population during mononuclear cell separation. The separation process itself can result in cell activation, maturation and skew cytokine production and thus invalidate the results. Whole blood analyses also allows for cell populations to be studied in a state mimicking the *in vivo* state as much as possible with host serum and plasma factors present. The addition of the prenumbered bead solution to the cell sample has enabled accurate counts of cell numbers per μl of blood. The fluorescent beads were added into the samples prior to acquisition of the cells, and thus the beads were acquired from an unmanipulated sample. Absolute counts distinguish between absolute loss and gain of cell populations with certainty. As physiological processes itself such as advancing gestational age can change CBDC populations, comparisons between percentage positive cells alone was thought to be not accurate enough.

Adult PB samples were used as controls and labelled contemporaneously with the CB samples. All samples were analysed within 1-2 hours of delivery to prevent *de novo* activation of cells and also to reflect the *in vivo* environment as closely as possible.

The Flow cytometer used had four channels and an additional channel was required to study the costimulatory marker expression and production of IL-12 by the $CD11c^-$ CBDC subsets. To this purpose, cychrome conjugated CD11c was added to the lineage cocktail in the third channel which removed the $CD11c^+$ DCs from the DC gate staining thus freeing up a channel. Freeing of one channel enabled the further delineation of CD11c- subsets with respect to their phenotype and function. Concurrent cell samples were stained with CD123 and CD45RA to ensure no loss of CD11c- DCs occurred due to the addition of CD11c to the lineage cocktail (see **chapter 2, section 2.3.1**).

The methodology of the experiments was tested to check the integrity and reproducibility. DC sorting and labelling experiment was conducted to check the integrity of flow cytometer sorting. Purity of the flow cytometer sorting was confirmed by pre and post sorting labelling experiments (see **chapter 2, section 2.3.2**). Potential loss of DC populations due to the metrizamide separation was also checked and found not to occur (see **chapter 2, section 2.3.2**).

Surface phenotyping by flow cytometry does not provide functional information and assessing of cytokine production by the CBDCs was felt to be necessary. ELISA measurements of cytokine production give limited information as it does not measure functionally active cytokine binding onto receptors and internalisation. Measurement of cytokine production intracellularly is a more accurate reflection of cytokine activity, thus intracellular detection of cytokine production by flow cytometry gives accurate functional data. As we have used whole blood without manipulation the results of the analyses may characterise *in vivo* cytokine production.

CBDCs were exposed for 4 hours to medium alone, or to the intracellular transport vesicle blocking agent monensin, such that active production of the cytokine within the cell in that window period of time could be measured. The cytokine expression of the sample without added

monensin was subtracted from the cytokine expression of that with monensin to determine the cytokine accumulation by production within the cell in the above time period. We did not use an intracellular isotype control for cytokine expression as a no-monensin sample was felt to provide more accurate results due to the following reasons. Isotype control comparison may also detect the cytokine stored already inside the cell and not just the amount produced in the 4 hours. Cytokine bound to surface receptors depends on the affinity and avidity of the antibody which may vary and also may not be equal to that exhibited by the anti-cytokine antibody. Additionally some antibodies have a high staining intensity at baseline due to a high flourochrome:antibody ratio and can give false positive results.

Although we have established ongoing intracellular cytokine production, further work needs to be done. Experiments using excess non-specific antibodies to show non blockage of cytokine staining and thereby indicating the specificity of the technique for the detection of intracellular cytokines should be done.

Enhanced normalised subtraction gives an accurate measurement of the staining intensity and was used throughout the study to derive the positive control intensity ratios. Non specific binding represented by the isotype staining was subtracted from the phonotype labelling using the WinList^{TM} programme. This calculation identifies low positive events occurring within the distribution of the control histogram and is more accurate than the geometric mean fluorescence of a gated population.

Subtraction was not used for the CD45, HLA-DR and CD16 analyses as an isotype control labelled tube for these markers was not included in the experiments. For analyses of these markers geometric mean fluorescence of the positive population was compared with the

geometric mean fluorescence of the corresponding isotype. It is submitted that any error would be negated as the same comparisons were done within the same sample in each experiment.

Significance of the work

The most significant area of research described in this study was the identification of a hitherto unreported putative $CD11c^{-}CD45^{inm}$ DC population with phenotypic and functional properties conforming to the characteristics of DC in established literature. Of the three CBDC populations, the novel $CD11c^{-}CD45^{inm}$ population proved to be the most kinetic, changing with gestational age as well as on exposure to stressors. Furthermore we have highlighted the possibility that these identifiable changes, with further studies could lead to identification of the systemically compromised infant immediately post delivery.

Spontaneous preterm labour and delivery is a syndrome caused by multiple pathologic processes that activate the common pathway of parturition and is a diagnostic conundrum due to the multifactorial aetiology (Romero, *et al*., 1993b; Romero, *et al*., 1994). Mechanisms of disease involved in the "preterm labor syndrome" include infection/inflammation (Minkoff, 1983; Gibbs, *et al*., 1992; Fidel, *et al*., 1994; Gomez, *et al*., 1995; Fidel, *et al*., 1998; Lamont, 1998; Goldenberg, *et al*., 2000; Hirsch and Wang, 2005; Romero, *et al*., 2007; DiGiulio, *et al*., 2008; DiGiulio, *et al*., 2010), vascular disease (Arias, *et al*., 1993; Kim, *et al*., 2002; Kim, *et al*., 2003), uterine over distension (Manabe, *et al*., 1981; Manabe, *et al*., 1994; Many, *et al*., 1995; Leguizamon, *et al*., 2001), abnormal allograft reaction (eg, rejection) (Kim, *et al*., 2008; Kim, *et al*., 2009; Kim, *et al*., 2010), an allergic-like phenomenon (Romero, *et al*., 1991; Romero, *et al*., 2010; Bytautiene, *et al*., 2002; Bytautiene, *et al*., 2004), a progesterone deficiency (Mesiano,

2001; Pieber, *et al*., 2001; Mesiano, 2004; Hassan, et al., 2011) and cervical disorders (Smith, *et al*., 1992; Iams, *et al*., 1995; Heath, *et al*., 1998; Hassan, *et al*., 2000).

The first mechanism of disease responsible for preterm labour and delivery for which a causal link was well established is infection (Romero and Mazor, 1988; Fidel, *et al*., 1994; Gravett, *et al*., 1994; Hirsch, *et al*., 1995; Hirsch and Wang, 2005; Romero, *et al*., 2007). Moreover, the mechanisms responsible for this process have been identified and involve PRRs (Elovitz, *et al*., 2003; Abrahams, *et al*., 2004; Kim, *et al*., 2004; Abrahams and Mor, 2005; Mor, et al., 2005), chemokines (Kelly, *et al*., 1992; Hamill, *et al*., 2008; Nhan-Chang, *et al*., 2008), or inflammatory cytokines (Romero, *et al*., 1989a; Romero, *et al*., 1990). In spite of this wealth of information on aetiology robust tests to identify the mother and fetus at risk of preterm labour sequelae have not yet been formulated. The Wilson criteria for a screening test advocates specificity, sensitivity, acceptability to the patient, reliability, good positive and negative predictive values and should be subject to quality assurance (Wilson and Jungner, 1968). Acceptability to the patient and feasibility has always been issues in fetal tests as obtaining samples from the fetus itself are associated with risks. Tests using placental tissue and CB which are currently perceived as 'waste products' of labour are ideal as they are available immediately post delivery and without additional procedures or risks. Thus the study of DCs in CB and their changes with stressor exposure could lead to identification of the high risk infant immediately after birth without any further tests on the neonate. Our work has focussed attention on the novel CBDC population $CD11c^{-}CD45^{inm}$, as being the most kinetic and responsive to intrauterine stressors compared to the myeloid and plasmacytoid CBDC subsets. Analyses of this subset could offer a more accurate depiction of the immune status of the fetus. Additionally DC changes reflect the immune modulations by the fetus in response to intrauterine stressors, studying which would

contribute to our understanding of the pathogenesis of preterm labour and delivery. This in turn could lead to formulation of more effective preventative and curative strategies.

Non invasive diagnosis using maternal or cord blood is the future direction as obtaining fetal samples by amniocentesis and chorionic villus sampling have an approximately 0.5-1% risk for fetal loss. Recent development in the field of non-invasive prenatal diagnosis through the use of cell-free fetal nucleic acids in maternal circulation during pregnancy is a successful example of the same. Thus extrapolating similar non invasive diagnostic techniques using cord blood will be the realistic future and should be cost neutral on comparison with invasive tests.

7.2 Future work

The results discussed in this body of work may form the basis of future studies assessing the role of CBDC populations in spontaneous preterm labour. This body of work has raised many questions which could be answered by further studies especially regarding the role of the novel $CD11c^{-}CD45^{inm}$ DCs in the fetal immune system. What is the significance of this immature DC subset? Does this subset contribute to the general haemopoietic immaturity of CB which inhibits immune responses and maintains a tolerogenic environment? The $CD11c^{-}CD45^{inm}$ immature DC population appeared similar to the immature DC population identified in haemalogical malignancies (Panoskaltsis, Ph.D. thesis, 2008). This could be due to a common mechanism through which the immature DC in the fetus as well as in malignancies provide tolerogenic signals to facilitate growth of the 'foreign' tissue. Blood and bone marrow $CD11c^{low}$ cells can behave as a precursor and give rise to all DC subsets. The ontogeny of this subset could be studied using tissue samples which would give a picture of very early development in the first

trimester. Tissue could be obtained after terminations of pregnancies in the first trimester and no data exists to date of the same. Similar techniques of immunohistochemistry, flow cytometry surface phenotype and intracellular cytokine labelling could be done to track the development of these DC subsets and their evolution. In addition to any condition leading to suspected intrauterine immunocompromise, adult conditions such as haematological malignancies and autoimmune disorders could be evaluated for this $CD45^{inm}$ DC subset.

The $CD11c^{-}CD45^{inm}$ DC might be a subset of DC in transition to another DC subset. The decreasing numbers with increasing age of the fetus indicated that this might be so. Does decreasing numbers with increasing age in the fetus indicate evolvement of the $CD11c^{-}CD45^{inm}$ DCs into the predominant myeloid DC subset in the adult? Longitudinal studies of CB at different gestations and from neonate to infant to adult should provide insight into the evolution of this subset. This subset expressed lower levels of CD45 and HLA-DR, but no significant increased in levels were seen with increasing gestation and on exposure to stressors. As discussed, this could be due to the small number of samples in the groups. Analyses of larger numbers of samples could show an increasing level of maturity on exposure to stressors thus adding credence to an evolving DC phenotype.

Further work needs to be done to establish the functional characteristics of the CBDC subsets. As manipulation and labelling of CBDCs have been shown to negate their lymphocytic stimulatory capacity other methods of separating the subpopulations has to be considered. Negative selection using microbeads where the cells of interest are not manipulated is one of the methods which could be used to sort the subpopulations.

Power calculations were not applied as practically the time limit of the project as well as the scarcity of samples resulted in few numbers. This does mean that results which could have been

statistically significant were not significant due to the small sample size. The present study could be considered as a feasibility/observational study of the differences in CBDCs. In future if these changes are to be developed as a test power calculations would need to be applied to ensure adequate number of samples are obtained.

The next steps would be to analyse larger numbers of samples, especially to provide comparisons between stressed and non stressed fetuses at various gestational ages. In our study we were not able to obtain samples from term fetuses exposed to intrauterine stressors except for the analyses of IL-12 production. The type and efficacy of the immune response mounted by the fetal DC populations could vary with the gestational age and hence comparisons would be valid only between the same gestational age groups. Further analyses could show if these CBDC phenotypic changes are consistent and reliable to be used as a test for diagnosis of the immunocompromised fetus.

Ex vivo models of infection could be set up including culturing the CBDCs with microbial antigens to ascertain whether the *in vivo* DC changes could be reproduced. These experiments may provide insight into the functioning of the innate and the establishment of the adaptive immune system in the fetus.

Furthermore the clinical course of the neonates identified as having been exposed to stressors by the CBDC changes should be monitored. The clinical information gathered would add to the reliability of the DC changes in identifying the compromised fetus. Long term follow up work would also establish whether the DC phenotypic changes are indicative of long term adverse sequelae of FIRS.

Understanding the function and role that the DC populations play in the activation of the fetal immune cells is crucial to understanding the differential functioning of the fetal immune system

in their ability to fight infections and their immuno protective capacity or Th2 bias which leads to a successful pregnancy. Also a greater understanding of the characteristics and function of DCs, in the differential response seen in the fetal immune system will help us understand the role of infections in the pathophysiology of preterm birth and in turn will lead to more effective preventive and curative measures.

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