Dendritic Cell Development in Haematological Malignancies and Neoplasia

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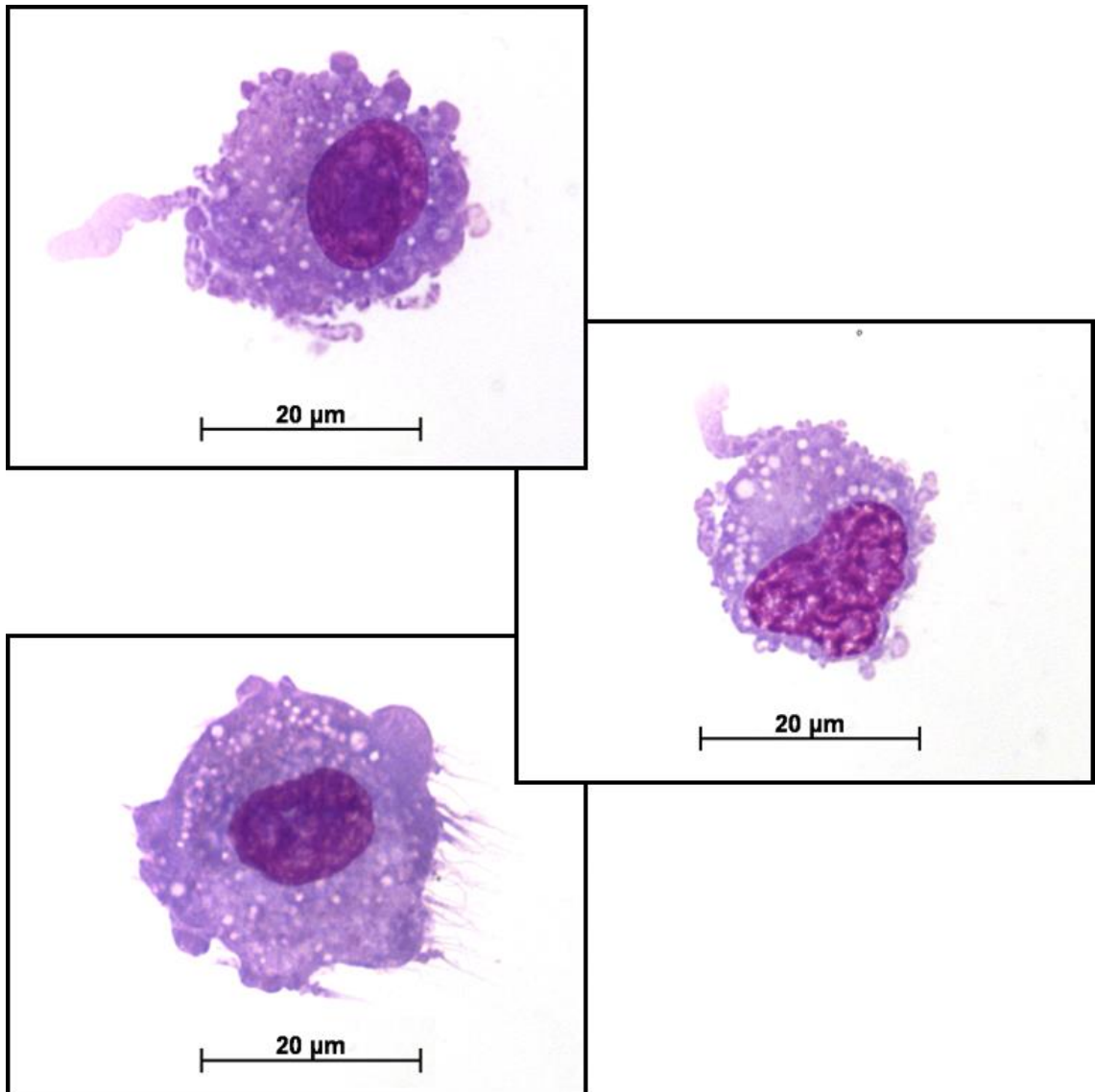**Re-Submitted - July 2015**
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All is for the best, in the best of all possible worlds.

François-Marie Arouet as 'Voltaire'. *Candide*, 1759

Langerhans Cell Histiocytosis Cells

Cells flow cytometrically sorted by expression of CD1a and Langerin from a skin lesion of a patient with Langerhans Cell Histiocytosis (A2712). Cytospin slides were prepared from sorted cells and stained with May-Grünwald-Giemsa (pH 6.8) on a Sysmex SP-1000i. Photographs were taken on a Zeiss Axioimager Z2 microscope with AxioVision 4.8 software at 100 times magnification. Scale as indicated.

Abstract:

Dendritic cells (DC) play a major role in the detection of antigens, initiation of immunity and induction and regulation of tolerance. DCs are Bone Marrow (BM) derived and their development may be influenced by haematological malignancy in several ways. Firstly, myelodysplastic, myeloproliferative or leukaemic transformation of bone marrow progenitors may involve DC precursors directly, when they become part of a malignant clone, or indirectly when neoplastic expansion of other lineages compromises the development of DCs. Secondly, neoplasia of the dendritic cell lineage itself may occur in a heterogeneous group of histiocytic disorders including Langerhans cell histiocytosis (LCH) and Erdheim Chester disease (ECD).

The first part of this thesis concerns the effect of haematological malignancies on the generation of DCs; in particular the relationship between DC, monocyte, B and NK lymphoid cell (DCML) deficiency, caused by GATA2 gene mutations, sporadic myelodysplastic syndrome (MDS) and acute myeloid leukaemia (AML). Comprehensive flow cytometric methods for profiling of the DCs and monocytes in the peripheral blood (PB) and peripheral tissues, and the CD34+ progenitors of the BM have been developed. Using these methods, it was possible to see that patients with the three diseases differed on several grounds. DCML deficiencies associated with a younger age of presentation, better preserved haemoglobin, neutrophils and platelets and much more severe defects of DCs, monocytes, and lymphoid cells. In patients with MDS, deficiencies are more moderate and mononuclear cell generation in AML patients is surprisingly preserved. Serum levels of Fms-like tyrosine kinase 3 ligand (FLT3L) also differed, with massively increased levels in DCML deficiency, stable levels in MDS and deficiencies in AML. Finally, strategies of grouping of AML patients have proven to be possible by using progenitor cell or DC related phenotypic markers which may correlate to known cytogenetic abnormalities.

The second part of this thesis explores the origin of the cells of LCH and ECD, both neoplasia involving DC-like cells. RT-PCR assays were developed to detect the $BRAF^{V600E}$ mutation, which is present in ~60% of LCH and ECD

cases. Surprisingly, it was seen that LCH and ECD express $BRAF^{V600E}$ in differing peripheral blood fractions. In LCH, 78-94% of all $BRAF^{V600E}$ alleles were found in HLA-DR+Lineage- cells, localised to the monocytes and CD1c+ myeloid DCs (mDCs). However in ECD, 80-82% of mutated alleles were recovered from the HLA-DR-Lineage- quadrant, localised to CD33+ early myeloid cells, and no mutation was found in the monocytes or mDCs.

The final part of the thesis examines whether the circulating cells which carry the $BRAF^{V600E}$ mutation in LCH and ECD can actually develop into LCH-like cells. In order to do this, culture systems were developed with a variety of cytokines and conditions. These experiments showed that CD14+ and CD16+ monocytes and CD1c+ mDCs could all induce the Langerhans Cell (LC) markers CD1a and Langerin in response to GM-CSF, TGFβ and BMP7, however only the CD1c+ mDCs could express Langerin at the high levels seen in LCs, along with EpCam and Birbeck granules.

Several techniques have been developed to study DCs in haematological malignancies. Throughout this thesis, these techniques have provided valuable data on the development and homeostasis of human DCs.

Acknowledgements:

x

Candidate's Declaration:

The material within this thesis was entirely produced by the candidate, other than the data listed below, which was generated in collaboration with colleagues and affiliates of the Human Dendritic Cell Lab (HuDC). However, the initial processing of all samples was performed by the candidate.

1. Around half of the GATA2 mutation patient and Control DC profiling panels were run by Dr Venetia Bigley (section 3.3.3, pg.97).

2. FLT3L ELISAs were performed by Sarah Pagan (section 3.3.4, pg.100).

3. Karyotyping analysis of cell line A375 was accomplished by Gavin Cuthbert, from cells prepared by the candidate (section 4.7.1, 140).

4. Lesional$BRAF^{V600E}$ mutation testing, unless otherwise specified, was performed by NewGene (www.newgene.org.uk) (section 5.2.1, pg.152).

5. DNA amplification for $NRAS^{Q61R}$ mutation detection was completed by Dr Rachel Dickinson. $NRAS^{Q61R}$Sanger sequencing was performed by SourceBioscience (www.sourcebioscience.com) (section 5.3.5.1, pg. 175).

6. Cells were processed for electron microscopy by Tracey Davey. Pre-preparation and EM imaging were performed by the candidate (6.3.5, pg. 199).

Publications Arising From This Thesis:

Some of the data in this thesis has been previously published in the following:

Papers:

1. **Milne, P**; Bigley, V; McGovern, N; Windebank, K; Cheung, B; Bacon, C; Tooze, Reuben; Allen, C; McClain, K; Haniffa, M and Collin, M. (In Revision) Cellular Localisation of *BRAF^V600E* in the Blood and Bone Marrow of Adults with Multi-System Langerhans Cell Histiocytosis and Erdheim Chester Disease, *Blood*.

2. **Milne, P**., Bigley, V., Gunawan, M., Haniffa, M. and Collin, M. (2015) CD1c+ Blood Dendritic Cells have Langerhans Cell Potential, *Blood*, 125(3)470-3. (Appendix 2).

3. Bigley, V., McGovern, N., **Milne, P**., Dickinson, R., Pagan, S., Cookson, S., Haniffa, M. and Collin, M. (2014) Langerin-Expressing Dendritic Cells in Human Tissues are Related to CD1c+ Dendritic Cells and Distinct from Langerhans Cells and CD141high XCR1+ Dendritic Cells, *Journal of Leukocyte Biology*, 97(4):627-34.

4. McGovern, N., Schlitzer, A., Gunawan, M., Jardine, L., Shin, A., Poyner, E., Green, K., Dickinson, R., Wang, X.N., Low, D., Best, K., Covins, S., **Milne, P**., Pagan, S., Aljefri, K., Windebank, M., Saavedra, D.M., Larbi, A., Wasan, P.S., Duan, K., Poidinger, M., Bigley, V., Ginhoux, F., Collin, M. and Haniffa, M. (2014) Human Dermal CD14(+) Cells are a Transient Population of Monocyte-Derived Macrophages, *Immunity*, 41(3), pp. 465-77.

5. Dickinson, R.E; **Milne, P**; Jardine, L; Zandi, S; Swierczek, S.I; McGovern, N; Cookson, S; Ferozepurwalla, Z; Langridge, A; Pagan, A; Gennery, A; Heiskanen-Kosma, T; Hämäläinen, S; Seppänen, M; Helbert, M; Tholouli, E; Gambineri, E; Reykdal, S; Gottfreðsson, M; Thaventhiran, J.E; Morris, E; Hirschfield, G; Richter, A.G; Jolles, S; Bacon, C.M; Hambleton, S; Haniffa, M; Bryceson, Y; Allen, C; Prchal, J.T; Dick, J.E; Bigley, V and Collin, M. (2013) The Evolution of Cellular Deficiency in *GATA2* Mutation, *Blood*, 123(6)863-74. (Appendix 1).

Presentation Abstracts:

1. **Milne, P**; Bigley, V; Dickinson, R; McGovern, N and Collin, M. (2014) Mapping the *BRAF^{V600E}* Hematopoietic Clones of Adult LCH and ECD Histiocytic Disorders, *30th Annual Meeting of the Histiocytosis Society*, Toronto, Canada

2. **Milne, P**; Bigley, V; Dickinson, R; McGovern, N and Collin, M. (2014) Mapping the *BRAF^{V600E}* Haematopoietic Clone of Adult Multi-System LCH, *Nikolas Symposium on Histiocytoses*, Athens, Greece. **Dr Jon Prichard Fellowship**.

3. **Milne, P**; Bigley, V; Dickinson, R; McGovern, N and Collin, M. (2014) Total Mononuclear Cell Profiling in Haematological Disorders, *Institute of Cellular Medicine Seminar Series*, Newcastle, UK.

4. **Milne, P**; Bigley, V; McGovern, N and Collin, M. (2013) Revisiting the Origin of Langerhans Cell Histiocytosis: Mutated *BRAF* Identifies Potential Precursor and Progenitor Cells, *North East Post Graduate Conference*, Newcastle, UK.

5. **Milne, P**; Bigley, V; McGovern, N and Collin, M. (2013) Revisiting the Origin of Langerhans Cell Histiocytosis: Mutated *BRAF* Identifies Circulating Precursor Cells, *Institute of Cellular Medicine Research Day*, Newcastle, UK.

6. **Milne, P**; Bigley, V; Dickinson, R; McGovern, N and Collin, M. (2013) Total Mononuclear Cell Profiling in Haematological Malignancies, *Institute of Cellular Medicine Seminar Series*, Newcastle, UK.

Posters:

1. **Milne, P**; V. Bigley; N. McGovern; M.A. Haniffa & M. Collin. (2015) CD1c+ Blood Dendritic Cells have Langerhans Cell Potential, Immunology Winter School, Singapore (Appendix 7)

2. **Milne, P**; N. McGovern; O.I. Abdel-Wahab; S. Pagan; P. Lovat; M.A. Haniffa; V. Bigley; & M. Collin. (2014) Mapping the $BRAF^{V600E}$ Haematopoietic Clone of Histiocytic Disorders, 13th International Symposium on Dendritic Cells, Tours, France.(Appendix 6).

3. **Milne, P**; McGovern, N; Pagan, S; Lovat, Penny; Haniffa, M; Bigley, V and Collin M. (2013) Revisiting the Origin of Langerhans Cell Histiocytosis: Mutated *BRAF* Identifies Circulating Precursor Cells and Bone Marrow Progenitor Cells, 13th International Workshop on Langerhans Cells, Amsterdam, Netherlands. **Best Poster Award.** (Appendix 5).

4. **Milne, P**; Bigley, V; McGovern, N and Collin, M. (2013) Mutated *BRAF* Identifies Circulating Precursor Cells, British Society of Immunology Summer School, Newcastle, UK. (Appendix 4).

5. Bigley, V; Dickinson, D; **Milne, P**; McGovern, N; Hambleton, S; Gennery, A &Collin, M (2012) Dendritic Cell Haematopoiesis: Insights from Human Dendritic Cell Deficiency Syndromes, International Symposium on Dendritic Cells, Korea (7-11/10/2012) (Appendix 3).

List of Abbreviations:

95% CI	95% Confidence Interval
aCGH	Array-Based Comparative Genomic Hybridization
AL	Acute Leukaemia
ALL	Acute Lymphocytic Leukaemia
AML	Acute Myeloid Leukaemia
AMML	Acute Myelomonocytic Leukaemia
APC	Antigen Presenting Cell(s)
APML	Acute Promyelocytic Leukaemia
BM	Bone Marrow
BMT	Bone Marrow Transplantation
BMP	Bone Morphogenetic Protein
BPDCN	Blastic Plasmacytoid Dendritic Cell Neoplasm
BRAF	V-raf Murine Sarcoma Viral Oncogene Homolog B
$BRAF^{V600E}$	A thymine to adenine (T to A) substitution at nucleotide 1799, causing a valine (V) to glutamine (E) substitution at codon 600 of the BRAF gene
$BRAF^{WT}$	BRAF wild-type gene
CD (number)	Cluster of Differentiation (number)
CFU	Colony Forming Unit(s)
CFU-S	Colony Forming Unit(s) - Spleen
CLEC4K	C-Type Lectin Domain Family 4, Member K (Langerin)
CNS	Central Nervous System
DC	Dendritic Cell(s)
DCML	Dendritic Cell, Monocyte, B and NK Cell Deficiency
EBV	Epstein–Barr Virus
ECD	Erdheim Chester Disease
ELISA	Enzyme-Linked Immunosorbent Assay
EM	Electron Microscopy
FAB (Type)	French-American-British classifications of AML
FFPE	Formalin Fixed Paraffin Embedded
FLT3	FMS-Like Tyrosine Kinase 3
FLT3L	FMS-Like Tyrosine Kinase 3 Ligand
GATA2	GATA Binding Protein 2
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
Hb	Haemoglobin
HCL	Hairy Cell Leukaemia
HHV	Human Herpes Virus

HLH	Hemophagocytic Lymphohistiocytosis
HSCT	Haematopoietic Stem Cell Transplantation
HuDC Lab	Human Dendritic Cell Lab
IL	Interleukin
JXG	Juvenile Xanthogranuloma
LAP	Leukaemia Associated Phenotype
LCH	Langerhans Cell Histiocytosis
LC	Langerhans Cell(s)
LN	Lymph Nodes
LSC	Leukaemic Stem Cell(s)
mDC	Myeloid Dendritic Cell(s)
MDS	Myelodysplastic Syndrome
MF	Multi-Focal
MFI	Mean Fluorescence Intensity
MHC class I	class 1 major histocompatibility complex
mmLCH	Multifocal Multisystem LCH
MS	Multi-System
MSKCC	Memorial Sloan Kettering Cancer Centre (USA)
muLCH	Multifocal Unisystem LCH
NCCC	Northern Centre for Cancer Care (UK)
NGS	Next Generation Sequencing
NTC	No Template Control
OS	Overall Survival
PBMC	Peripheral Blood Mononuclear Cell(s)
PBSC	Peripheral Blood Stem Cell(s)
pDC	Plasmacytoid Dendritic Cell(s)
Plts	Platelets
POQR	Positive Outside of the Quantitative Range
RDD	Rosai Dorfman Disease
RP	Retroperitoneum
RT-PCR	Real-Time Polymerase Chain Reaction
SNP	Single Nucleotide Polymorphism
SS	Single-System
TLR	Toll-Like Receptor
TGFβ	Transforming Growth Factor Beta
WHO	World Health Organisation

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Chapter 1. Introduction

This introduction will focus on haematopoietic progenitor subsets, dendritic cells (DCs) and their roles in the healthy state, haematological malignancies and immunodeficiencies. The introduction is split into five parts:

A. Steady-State Haematopoiesis
B. Dendritic Cell and Monocyte Subtypes
C. Dendritic Cells in Acute Leukaemia, Myelodysplasia and Immunodeficiency
D. Dendritic Cells in Histiocytic Disorders
E. The Origins of Histiocytic Disorder Cells

1.1. Steady-State Haematopoiesis

Haematopoiesis is the process of formation and development of mature blood cells. This process needs to be tightly controlled as many blood cells are short lived, and thus need to be replaced continuously in order to maintain the circulatory and immune systems. However, this production also needs to be limited, in order to prevent an over accumulation of cells which can interrupt normal physiological processes.

An overview diagram of the locations of human haematopoiesis is given in Figure 1.1. In developing human embryos, blood cell formation first occurs in the blood islands of the foetal yolk sac (umbilical vesicle), around three weeks after fertilization. During gestation, the haematopoietic processes are taken on by the liver, spleen, lymph nodes (LN) and bone marrow (BM). Finally, by the time of birth, the BM has assumed all haematopoiesis, though maturation of lymphocytes still occurs in the lymphoid organs. In order to maintain the steady-state, 10^{11}-10^{12} new blood cells must be produced each day in adult human bone marrow(Parslow *et al.*, 2001; Doulatov *et al.*, 2012). In some pathological disorders, such as myelofibrosis, extramedullary haematopoiesis can occur to compensate for bone marrow failure (BMF).
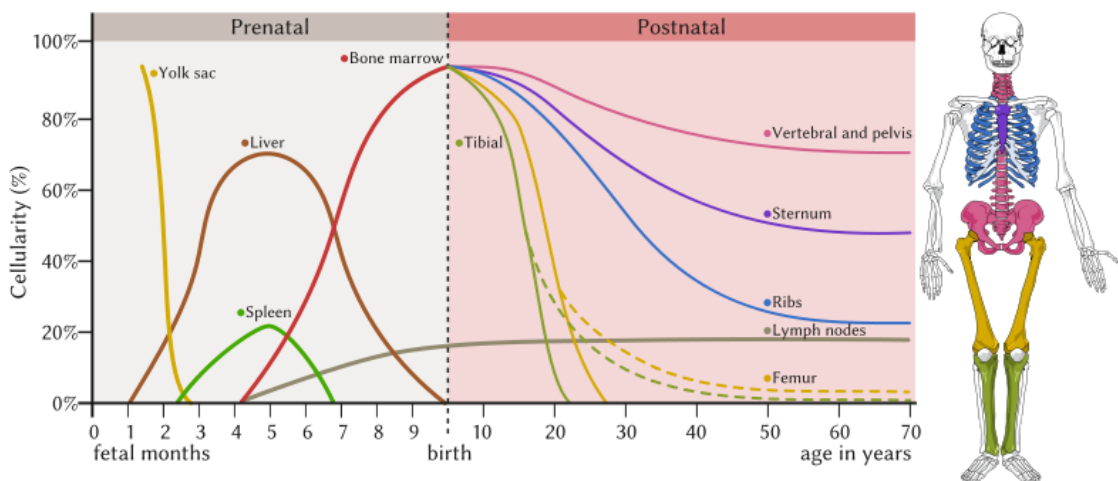
Figure 1.1. Timeline of Haematopoiesis in Humans

Overview diagram of the sites of human haematopoiesis from fertilisation through to adulthood. Line colours in the postnatal section relate to specific bones in the skeletal diagram. (Komorniczak, 2011)

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1.1.1. *Haematopoietic Stem Cells*

At the apex of the haematopoietic hierarchy is the haematopoietic stem cell (HSC). These cells have capacities for self-renewal and multilineage differentiation, which allow them to support the entire haematopoietic system in healthy individuals.

The concept of haematopoietic stem cells arose from observations of the aftermath of the Hiroshima and Nagasaki nuclear bombings. It was observed that the haematopoietic systems of patients with low dose radiation poisoning were unable to produce enough leukocytes to ward off opportunistic infections, or platelets to form blood clots.

It was discovered that these effects could be abated in rodents by shielding either the spleen or a single bone from radiation(Jacobsen *et al.*, 1949), or by repopulating the radiation-abolished haematopoietic system with injected BM cells (Jacobson *et al.*, 1951; Lorenz *et al.*, 1952). The injected donor-derived BM cells were able to form colonies of clones of maturing haematopoietic cells in the spleen (CFU-S), which were able to self-replicate and were multipotent, with the ability to generate multiple types of haematopoietic cells (Becker *et al.*, 1963; Siminovitch *et al.*, 1963).

It has since been found that these cells can also form colonies *in vitro* (Johnson *et al.*, 1977; Humphries *et al.*, 1979), and this has been the basis of much human HSC work, laying the foundations for clinical applications such as modern bone marrow transplantations.

HSCs can be identified by their small size and low side population staining with vital dyes (rhodamine 123 or Hoechst 33342) or their surface markers. CD34, which is single-pass transmembrane glycoprotein adhesion factor found on <5% of blood cells was the first surface marker used to enrich human HSCs and progenitor cells (Civin *et al.*, 1984). Human HSCs are more specifically identified as $Lin^-CD34^+CD38^{-/low}CD90^+CD59^+CD117^+$ (Baum *et al.*, 1992). As the cells differentiate into

multi-potent progenitors (MPP) expression of CD90 is lost, and when they differentiate to mature effector cells, expression of CD34 is gradually lost. It is important to note that at least a small proportion of human HSCs may be CD34⁻(Bhatia *et al.*, 1998). Doulatov *et al*, estimated this CD34⁻ population to be <1% of total HSCs (Doulatov *et al.*, 2012).

As HSCs differentiate, they follow a series of lineage restriction milestones before they eventually reach an endpoint as a mature cell. Further down the hierarchy are progenitor cells, which have a limited potential for proliferation and differentiation but cannot self-renew indefinitely. The final step before fully differentiated cells are precursor cells which have lost most or all of their differentiation capacity and cannot self-renew. All circulating blood cells and most tissue leukocytes are believed to originate from, and be continuously replenished by HSCs in the bone marrow, giving rise to all myeloid (erythrocytes, monocytes, macrophages, neutrophils, basophils, eosinophils, dendritic cells, megakaryocytes and platelets) and lymphoid (T-cells, B-cells, NK-cells) cell types.

1.1.2. *Multi-Potent Progenitors*

HSCs differentiate into multi-potent progenitors (MPPs), losing the ability to self-renew and gaining lineage bias but maintaining the ability to differentiate into any haematopoietic cell. Majeti *et al*, showed through the use of *in vivo* xenotransplantation as well as complementary *in vitro* assays, that the human cord blood HSC/MPP compartment can be split based on CD90 and CD45RA expression(Majeti *et al.*, 2007). They showed that HSCs are CD90⁺CD45RA⁻ and that there is a potential transiently engrafting MPP population which is CD90⁻CD45RA⁻. They also showed that further downstream of the MPP, there is a unipotent cell population which has acquired CD45RA expression. Doulatov *et al* have more recently improved the classification of MPPs as CD34⁺CD38⁻CD90⁻CD45RA⁻CD135⁺CD7⁻CD10⁻ by clonal mapping of single bone marrow and cord blood progenitors(Doulatov *et al.*, 2010).

CD38 (cyclic ADP ribose hydrolase) is a multifunctional transmembrane ectoenzyme glycoprotein which functions in signal transduction, calcium signalling and as an adhesion factor. CD38 is expressed in more than 90% of human $CD34^+$ cells (Carvalho *et al.*, 2009), however multipotent cells are $CD38^{low/-}$, gaining CD38 as they differentiate (Huang and Terstappen, 1994).

1.1.3. *Oligo-Potent Progenitors*

Downstream of the MPPs in the hierarchy is the oligo-potent progenitor (at least according to the classical haematopoiesis model, Figure 1.2). These cells have developed a level of lineage commitment and so their ability to differentiate has been reduced to a few cell types. These oligo-potent progenitors are defined by their function/lineage potential; however specific markers are required by which to isolate them.

CD45 (Protein tyrosine phosphatase, receptor type, C; PTPRC) is a transmembrane enzyme. It has several isoforms, which when taken together are presented on all differentiated haematopoietic cells apart from erythrocytes and plasma cells. In humans, progenitor cells are $CD45^{low}$, but the isoform CD45RA is seen on Multi-Lymphoid Progenitors (MLPs) and Granulocyte Macrophage Progenitors (GMPs) (Manz *et al.*, 2002). CD45RA is also seen on naive T cells, while more mature memory T cells express the shorter CD45R0 isoform (Shah *et al.*, 1988; Lansdorp *et al.*, 1990).

1.1.3.1. *Lymphoid Progenitors*

In humans, the closest homologue to the murine Common Lymphoid Progenitors (CLPs), are the CD34+38-CD90-45RA+ Multi-Lymphoid Progenitors (MLPs), which can give rise to all lymphoid lineages (T, B and NK cells), but still may have some myeloid potential (Doulatov *et al.*, 2010). The MLP fraction of cord blood may also contain a small CD7+ fraction which gives rise solely to T, B and NK cells (Hao *et al.*, 2001; Hoebeke *et al.*, 2007).

After the MLP step, the progenitors can become either thymic progenitors, which mature into T cells in the thymus and tonsils, or CD34+CD38+CD10+ B/NK cell progenitors (B/NK), which still have some CD33+ myeloid DC potential (Galy *et al.*, 1995).

CD10 (Neprilysin) is a metalloprotease enzyme involved in the breakdown of peptides. Work on human BM has shown that CD34+10+ progenitor cells can produce T, B and NK lymphocytes (Galy *et al.*, 1995). The CD34+10+ progenitors can be split into CD38-MLPs and CD38+ B/NKs (Doulatov *et al.*, 2010). In patients with DCML deficiency (see section 1.3.1) who lack B and NK cells, there is also a complete deficiency of MLP and B/NK progenitors (Bigley *et al.*, 2011).

1.1.3.2. *Myeloid Progenitors*

Current insights into myeloid progenitors split the fraction into Common Myeloid Progenitors (CMP), Granulocyte/Macrophage Progenitors (GMP) and Megakaryocyte/Erythroid Progenitors (MEP). Manz *et al*, 2002 prospectively isolated human homologues to murine CMP, GMP and MEP using CD123 (IL-3Ra) and CD45RA and showed directly that CMPs are clonogenic progenitors of both GMPs and MEPs. They also phenotyped all three populations in humans: CMP are Lin-$CD34^{+}CD38^{+}CD123^{low}CD45RA^{-}$, GMP Lin^{-} $CD34^{+}CD38^{+}CD123^{low}$ $CD45RA^{+}$ and MEP Lin$^{-}CD34^{+}CD38^{+}CD123^{-}$ $CD45RA^{-}$ (Manz *et al.*, 2002).

CD123 is the cell surface receptor for interleukin-3 (IL-3R), which when activated, induces tyrosine phosphorylation, promoting proliferation and differentiation in some progenitor cells. CD123 is found to be positive on CMPs and GMPs (Manz *et al.*, 2002) and also has high expression on an ill-defined CD34+38+10-45RA+123^{high} cell subset.

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The differentiation of CMPs, GMPs and MEPs by CD123 has created controversy as the results do not appear to be easily reproducible. Doulatov *et al*, 2010 have attempted the separation with a different method, using single cell sorting with CD45RA with CD135 (FLT3) expression; MEPs are identified as $CD135^-CD45RA^-$ CMPs $CD135^+CD45RA^-$ and GMPs $CD135^+CD45RA^+$ (Doulatov *et al.*, 2010).

CD110, thrombopoietin (TPO) is a regulator of megakaryopoiesis and is also a potential stem cell factor, having been implicated in MEP and mast cell proliferation and differentiation (Sungaran *et al.*, 2000; Kirshenbaum *et al.*, 2005), therefore this could be another possible target for CMP, GMP, MEP separation.

In flow cytometric analysis of normal human BM samples, Carvalho *et al* have reported that early myeloid precursors in the CD34- compartment express CD33 and CD13, and more mature granulocytic cells express CD11b, CD11c and CD15 (Carvalho *et al.*, 2009). Therefore it is possible that some of these markers could be useful in the examination of the CMP/GMP/MEP phenotypic split.

As part of this thesis, a multi-colour flow cytometry panel was set up to examine the BM CD34+ mononuclear cell fractions, involving examination of some of the potential markers listed above.

1.1.4. *Models of Haematopoiesis*

An important step in our understanding of leukaemogenesis would be to understand the process of normal lineage commitment of HSCs, as this could help identify at what stage of the process the leukaemic aberration has occurred. Over the last twenty years there has been much progress in the understanding of HSC lineage commitment, with most evidence showing that commitment occurs as a stepwise process, however there have been several models presented demonstrating differing emphases on myeloid or lymphoid lineages and numerous alterations to progenitor subtype classifications.

The classical model of haematopoiesis was formulated after common lymphoid progenitors (CLPs) (Kondo *et al.*, 1997) and common myeloid progenitors (CMPs) (Akashi *et al.*, 2000) were isolated from mice. A diagrammatical representation of this model is given in Figure 1.2.
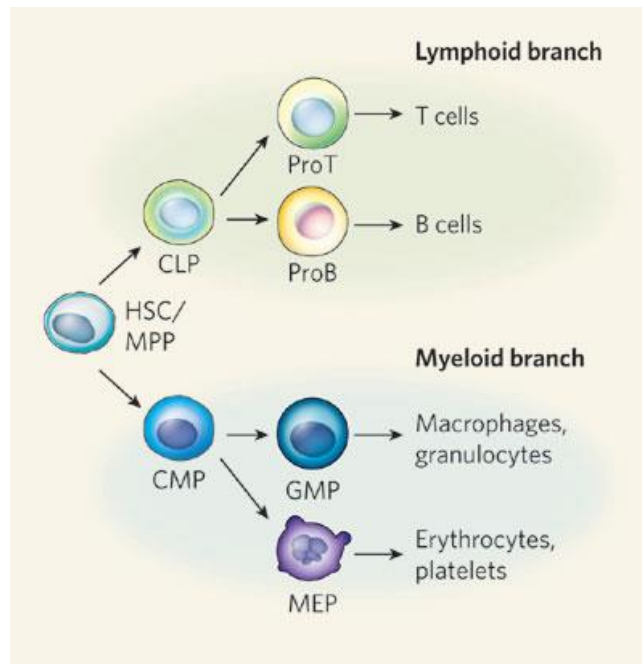
Figure 1.2. Classic Scheme of Haematopoiesis

Classic schema of haematopoiesis; showing an early bifurcation into lymphoid and myeloid branches.(Graf, 2008).

The three main tenets of this model are:

1. Cells lose the ability to self-renew prior to lineage commitment, becoming Multi-Potent Progenitors (MPPs).

2. Downstream of the MPP step, cells commit to either lymphoid (Common Lymphoid Progenitors; CLP) or myeloid (Common Myeloid Progenitors; CMP) lineages.

3. Further lineage commitments occur as a stepwise process.

It is starting to become apparent that the classical system is flawed in the human (and mouse) setting, at least for lymphoid lineage commitment. For example, studies have reported by clonally mapping the developmental potential of single cord blood and adult bone marrow progenitors that myeloid commitment follows the classical model, whereas

so called Multi-Lymphoid Progenitors (MLPs) were still capable of giving rise to macrophages and DCs (Galy *et al.*, 1995; Doulatov *et al.*, 2010; Doulatov *et al.*, 2012).

Current evidence strongly suggests a lineage differentiation from MPPs to CMPs and MLPs, however several groups examining the MPP compartment of mice have demonstrated a Lin$^-$Sca-1$^+$CD117$^+$FLT3^{high} cell fraction which shows full lineage potential, with only a very rare population with MkE potential (Adolfsson *et al.*, 2001; Adolfsson *et al.*, 2005; Yang *et al.*, 2005; Arinobu *et al.*, 2007), which they term lymphoid-primed multipotent progenitors (LMPPs). This would suggest in mice that either there is a lineage commitment step prior to CMP/CLP restriction where cells commit entirely to MkE or at least that for some reason MkE potential is lost early on in the commitment process or that LMPPs and CLPs both exist.

In human studies, it has been demonstrated that CD34+38+45RA+10+ MLPs do have MkE potential (Doulatov *et al.*, 2010), however a CD34+38+45RA+10- fraction has been seen to functionally relate to the murine LMPP, lacking granulocyte potential (Goardon *et al.*, 2011). The Goardon *et al* paper splits the multi-lymphoid progenitors (MLP) into CD10+ myeloid-lymphoid progenitors (MLP) and CD10- Lymphoid-Primed Multi-Potent Progenitors (LMPP).

For the purposes of this thesis, I used a model of haematopoiesis based on the work by Doulatov *et al*, earlier work demonstrating the isolation of CMPs using CD123 (Manz *et al.*, 2002) and work within the Human Dendritic Cell Lab (HuDC Lab) (Figure 1.3).
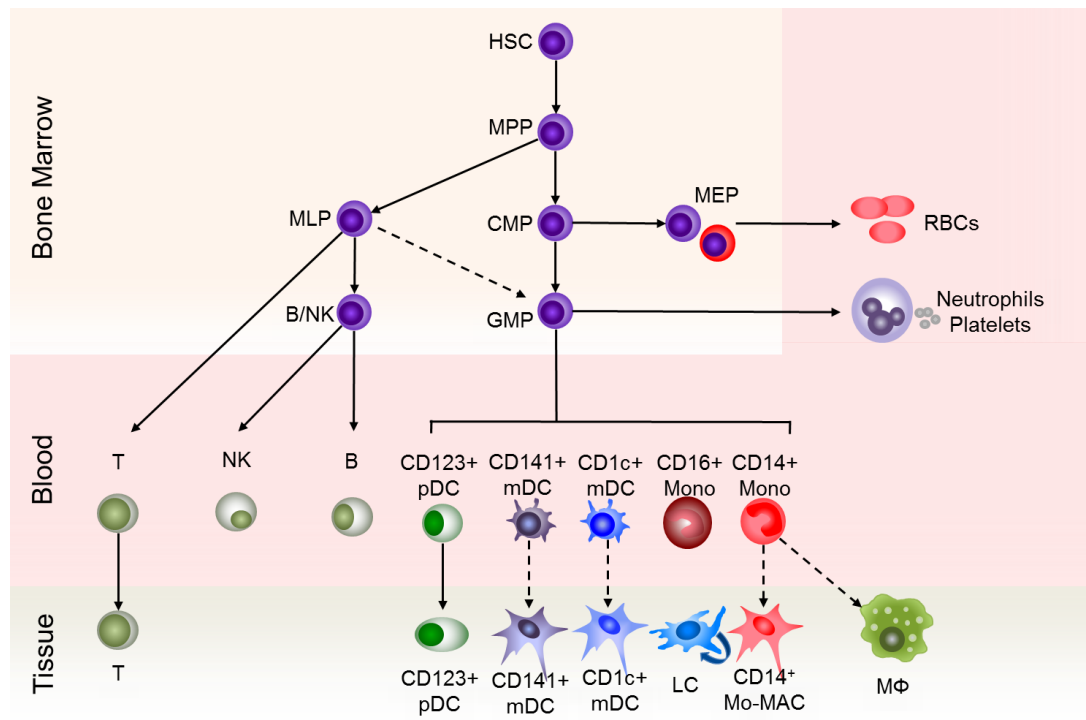
Figure 1.3. Basic Representation of Unidirectional Haematopoiesis

HSC = Haematopoietic Stem Cell; MPP = Multi-Potent Progenitor; MLP = Multi-Lymphoid Progenitor; B/NK = B/NK Cell Progenitor; CMP = Common Myeloid Progenitor; MEP = Megakaryocyte/Erythroid Progenitor; GMP = Granulocyte Macrophage Progenitor; RBC = Red Blood Cell; T = T Cell; B = B Cell; NK = Natural Killer Cell; mDC = Myeloid Dendritic Cell; pDC = Plasmacytoid Dendritic Cell; Mono = Monocyte; LC = Langerhans Cell; Mo-MAC = Monocyte/Macrophage; MΦ = Macrophage.

1.2. Dendritic Cell and Monocyte Cell Subsets

Dendritic cells and monocytes, as well as tissue macrophages constitute the mononuclear phagocytic system, though they may not be related between themselves. Dendritic cells have a unique function in the sensing of 'danger' to the host and initiation of appropriate immune responses (Banchereau and Steinman, 1998). Their role *in vivo* is to transport antigens to draining lymph nodes where they interact with T cells and are critical in stimulating immunity to infections, vaccines and tumours. It is now known in mice that DCs arise independently from monocytes in the steady-state, dependant on specific growth factors and genes (Liu and Nussenzweig, 2010; Collin *et al.*, 2013), however, monocytes may play a role in the origin of some inflammatory DCs (Collin *et al.*, 2013; Segura *et al.*, 2013). Monocytes have roles in pathogen challenge and inflammation, while macrophages are involved in the resolution of inflammation and tissue homeostasis.

1.2.1. *Blood Monocyte and Dendritic Cell Subsets*

In human peripheral blood there are three known monocyte subsets; CD14++16- classical monocytes, CD14+16+ non-classical monocytes and CD14++16+ intermediate monocytes, hereafter referred to as CD14+, CD16+ and CD14+16+ monocytes respectively. There are also at least three subtypes of dendritic cells including a CD123+ plasmacytoid DC (pDC) and two myeloid DCs (mDCs); CD11c+1c+ mDCs and CD141+ mDCs.

1.2.1.1. *Classical CD14+ Monocytes*

The CD14+ monocyte is the most common in human peripheral blood, making up ~90% of the total monocyte fraction. These monocytes are antigen presenting cells (APCs) which can phagocytose and kill microbes by means of opsonin mediated complement system activation, direct binding via toll-like receptors and/or by antibody dependant cell-mediated cytotoxicity.

1.2.1.2. *Non-Classical CD16+ Monocytes*

There is debate as to whether the non-classical CD16+ monocyte is most closely related to CD14+ monocytes or mDCs, as there are several differences from the classical monocytes. These differences include morphology, cytochemistry, function and immunophenotype (Almeida *et al.*, 2001), migratory properties and macrophage differentiation (Randolph *et al.*, 2002). However, the gene expression profile of CD16+ monocytes is most similar to that of the CD14+ monocytes (Robbins *et al.*, 2008).

1.2.1.3. *Intermediate CD14+16+ Monocytes*

The third subset of monocytes is CD14+16+, which can produce high levels of tumour necrosis factor and interleukin-12 (Zawada *et al.*, 2011). It is currently unknown whether this subset is a descendant of either the CD14+ or CD16+ monocytes, or a completely separate entity. A study by Ghattas *et al* has suggested that the CD14+16+ population is independent as it has markers representative of reparative processes at much higher levels than both the other monocyte subtypes (Ghattas *et al.*, 2013). Observations from the HuDC lab have shown that CD14+ monocytes are present in the BM where CD16+ and CD14+16+ monocytes are absent. Also, it has recently been reported that a small group of patients can have a specific immunodeficiency of CD16+ monocytes and very low numbers of CD14+16+ monocytes, where CD14+ monocytes are preserved (Frankenberger *et al.*, 2013).

1.2.1.4. *CD123+ Plasmacytoid DCs*

Plasmacytoid dendritic cells (pDCs) have been observed for many years in the T cell zones of lymphoid tissue and in the circulation as immature veiled cells, but it was only in 1999 that they were identified as dendritic cells rather than plasmacytoid T cells (Grouard *et al.*, 1997). In humans, pDCs can be distinguished by lack of Lineage markers, CD14, CD16 and CD11c, and positivity for HLA-DR, CD123, CD303 (BDCA-2) and CD304 (BDCA-4) and constitute just 0.2-0.4% of

all the mononuclear cells in the peripheral blood. pDCs are part of the innate immune system, which can internalise and detect pathogenic ssDNA and CpG DNA motifs via intracellular toll-like receptors (TLR) 7 and 9 (Sallusto and Lanzavecchia, 2002; Colonna *et al.*, 2004). When stimulated with viruses, pDCs produce large amounts of IFN-α and to a lesser extent IFN-β (Kadowaki and Liu, 2002). Finally, pDCs express Fc epsilon RI (FcεRIα), the high-affinity receptor for IgE (Foster *et al.*, 2003; Novak *et al.*, 2003), indicating that these cells are likely contributors to the inflammatory response in allergy. Currently it is unknown whether pDCs develop from the lymphoid or myeloid progenitors in the bone marrow.

1.2.1.5. CD11c+1c+ Myeloid DCs

CD11c+1c+ myeloid DCs are the most common type of DC, at 0.3-0.5% of normal peripheral blood mononuclear cells. Most studies refer to the entire CD14-11c+ fraction as myeloid DCs, as the majority of these cells express CD1c (BDCA-1), these cells are referred to herein as CD1+ DCs. The remainder of the CD14-11c+ fraction are CD141+ myeloid DCs which are discussed below. CD1c+ DCs are APCs which are in an immature state in the blood (Dzionek *et al.*, 2000; MacDonald *et al.*, 2002). Once stimulated, they have a high T cell stimulation capacity, with a tolerogenic Th1 immune response bias and chemokine production (Bachem *et al.*, 2010; Haniffa *et al.*, 2012).

1.2.1.5. *CD141+ Myeloid DCs*

CD141+ (BDCA-3) mDCs are the rarest form of DC, contributing only 0.01-0.02% of peripheral blood mononuclear cells, making them extremely difficult to study. These cells are phenotypically similar to the CD1c+ DCs, and also circulate in an immature state (Dzionek *et al.*, 2000; MacDonald *et al.*, 2002), but they do not express CD1c in blood, and express CD11c at a lower level.

Like CD1c+ mDCs, the CD141+ mDCs can promote a Th1 immune response by secretion of IL-12, and are involved in the

activation of CD8+ cytotoxic T cells by cross-presentation of extracellular antigens (Bachem *et al.*, 2010; Haniffa *et al.*, 2012). CD141+ mDCs are able to take up dead or necrotic cells via CLEC9A (Bachem *et al.*, 2010; Collin *et al.*, 2013) and they express TLR2 and TLR4 (Sallusto and Lanzavecchia, 2002), indicating a role in bacterial immunity.

1.2.2. *Dermal Monocyte and Dendritic Cell Subsets*

In the dermis of the skin there are two main dendritic cell populations, CD1c+ and CD14+, a smaller CD141+ DC population and also macrophages and T cells. Interestingly there are no skin pDCs in health.

The largest DC population is a CD1c+ DCs which, by gene expression analysis, is likely to be the tissue homologue of the circulating CD1c+ DC (Haniffa *et al.*, 2012). Tissue CD1c+ DCs have a wide range of antigen presentation capacity, including lipopolysaccharide, poly(IC), and flagellin (van der Aar *et al.*, 2007) and mycobacterial glycolipids (Van Rhijn *et al.*, 2013). Other functions, such as fungal recognition, Th1 and Th17 polarisation have also been suggested (Collin *et al.*, 2013).

The second dermal DC is the CD14+ dermal dendritic cell, which is now known to be from the monocyte-macrophage lineage rather than DC, by gene expression analysis (McGovern *et al.*, 2014). CD14$^+$ DCs secrete interleukin-10 (IL-10) and IL-6 and have been shown to induce regulatory T cells and helper follicular T cells (Chu *et al.*, 2012; Klechevsky *et al.*, 2008). A notable feature of CD14$^+$ DCs is their poor ability to stimulate allogeneic T cell proliferation (Klechevsky *et al.*, 2008; Morelli *et al.*, 2005; de Gruijl *et al.*, 2006), which is consistent with their status as a monocyte-derived macrophage.

CD14$^+$ DCs also express variable levels of CD141, so they had been presumed to be related to blood CD141$^+$ DCs (Chu *et al.*, 2012). However, recently, the tissue counterpart of the blood CD141+ mDC has

been found to be a small population of CD14-CD11c^{low}CD141^{high} DC in skin(Haniffa *et al.*, 2012). Unlike the blood counterpart, these cells express CD1c and have been seen in haematopoietic tissues such as the BM and lymph nodes, and non-lymphoid tissues such as the liver (Collin *et al.*, 2013). These cells have a superior cross-presentation capacity and they secrete TNF-α and CXCL10(Haniffa *et al.*, 2012), suggesting roles in inflammatory reactions.

Macrophages have long been thought of as tissue monocytes, derived from circulating monocytes (van Furth and Cohn, 1968), though recent work in mice has shown that most tissue macrophages are derived from embryonic precursors (Ginhoux and Jung, 2014). Macrophages have roles in phagocytosis, but also in adaptive immunity and wound healing. Unlike tissue DCs, macrophages are fixed within the tissue (Collin *et al.*, 2013).

1.2.3. *Epidermal Langerhans Cells*

Langerhans cells (LCs) were first described in 1868 by the German anatomist and physician Paul Langerhans, during an undergraduate project using gold chloride staining (developed by Julius Friedrich Cohnheim) to analyse human epidermal skin cells (Langerhans, 1868). The cells were thought to be neurons, due to their branched morphology, and positive staining with gold chloride, which at the time was thought to be a marker specific for nerve tissue. It wasn't until 1985 that the cells were characterised as antigen-presenting dendritic cells by the team of Ralph Steinman (Schuler, 1985).

LCs form a network of epidermal DCs which are able to proliferate in the skin, dispersed throughout the epidermis (2-5% of total cells), but mainly in the stratum spinosum (see Figure 1.4 and Figure 1.5) and associated with the hair follicles. It is important to note that LCs can also be found in the mucosal stratified squamous epithelium of the respiratory, digestive, oral and genital mucosa and in the papillary dermis around blood vessels.

The hallmarks of LCs are their high expression of Langerin and CD1a and the presence of Birbeck granules. LCs also express major histocompatibility complex (MHC) Class II and low levels of CD11c, which are characteristic of 'classical' myeloid dendritic cells (Ginhoux *et al.*, 2010). The tissue CD1c+ DC also expresses CD1a, but at a lower level than LCs (Collin *et al.*, 2013) and may also express a low level of langerin (Bigley *et al.*, 2014). However, they do not express other LC-specific markers such as EpCam and ECadherin. (Zaba *et al.*, 2007; Haniffa *et al.*, 2009).
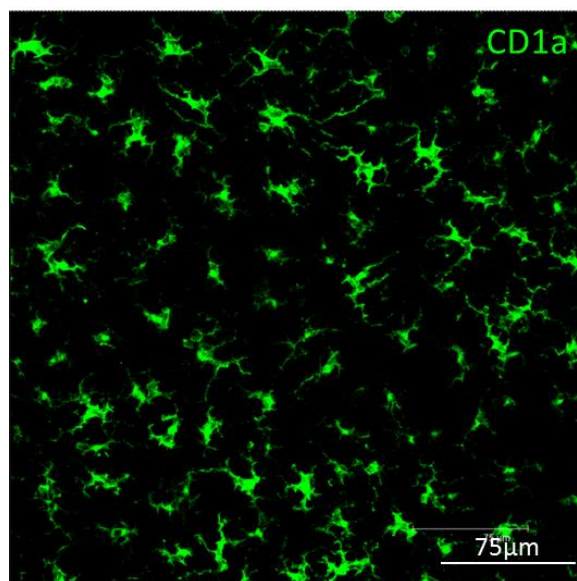
Figure 1.4. Langerhans Cells Forming a Network of DCs in the Skin

Electron microscopy image of Langerhans cells in the skin, with langerin showing in green. The spaces around the Langerhans cells are keratinocytes which take up DAPI staining. (Unpublished data).

16
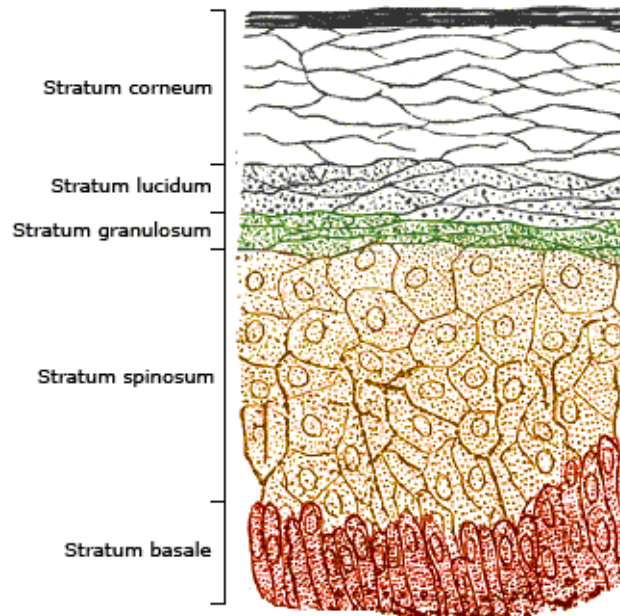
Figure 1.5. The Different Layers of the Epidermis

A diagrammatic representation of the layers in the epidermis (Serephine, 2006)

1.2.3.1. *Function*

In the simplest of terms, LCs are antigen presenting cells (APCs) which collect antigens in the peripheral tissues, internalise these and transport them to the lymph nodes, where the antigens can be displayed to naïve T cells. LCs take up bacteria by phagocytosis (phagocytic cup formation) (Reis e Sousa *et al.*, 1993), and are now known to have several more intricate functions such as the production of TGF-B, IL-1B IL-15 and IL-23 (Aliahmadi *et al.*, 2009).

1.2.3.2. *Langerin (CD207/CLEC4K)*

Langerin (CD207/CLEC4K, C-type lectin domain family 4 member K) is a type II transmembrane, calcium-dependant, carbohydrate-binding protein domain (See Figure 1.6), which is expressed on the surface of LCs and which, when internalised, associates with Birbeck granules (Valladeau *et al.*, 2000; Valladeau *et al.*, 2002). The extracellular region of langerin exists as a stable trimer held together by a coil of α-helices formed by the neck region (Takahara *et al.*, 2002; Stambach and Taylor, 2003).

Langerin has mannose, fructose and N-acetylglucosamine (part of bacterial cell wall) binding specificity (Takahara *et al.*, 2002; Stambach and Taylor, 2003), so it has been suggested that langerin accesses a non-classical antigen-processing pathway by the internalisation of these antigens into Birbeck granules. In HIV, langerin has been seen to co-localise with caveolin-1 (also present in BGs), leading to a reduction in infection rate, possibly by autophagy (Manches *et al.*, 2014).

It was at first thought that langerin was specific for skin Langerhans cells, however it has now been found on a small population of non-LC DCs (Bigley *et al.*, 2014). In mice, langerin is expressed by dermal CD103+ DCs and splenic CD8+ DCs (Nagao *et al.*, 2009). In humans, langerin can be expressed by a small proportion of tissue CD1c+ DCs (Romani *et al.*, 2010; Bigley *et al.*, 2011) and by DCs in the marginal zone of the spleen (Idoyaga *et al*, 2009), indicating that it is not solely an LC marker.
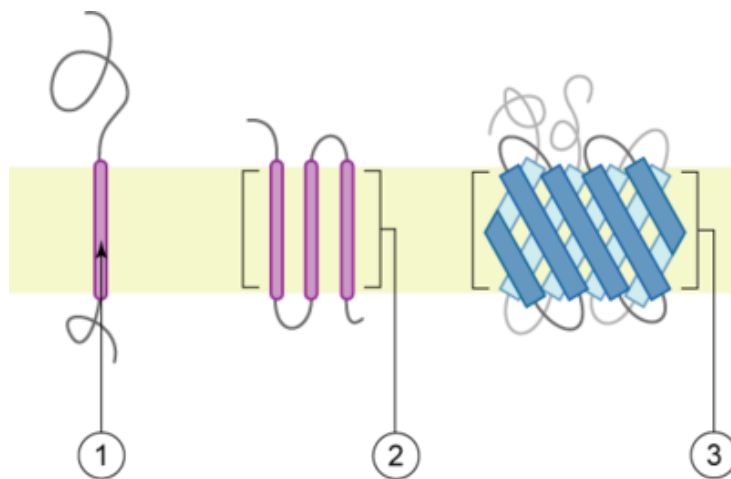
Figure 1.6 Types of Transmembrane Proteins

Schematic representation of transmembrane proteins: 1. a single transmembrane α-helix (bitopic membrane protein) 2. a polytopic transmembrane α-helical protein 3. a polytopic transmembrane β-sheet protein. The membrane is represented in light brown. (Foobar, 2006). Langerin is a type 2 membrane protein.

1.2.3.3. *CD1a*

CD1a (Leu-6) is part of a family of transmembrane glycoproteins (CD1a-e), structurally similar to class 1 major histocompatibility complex (MHC class I) proteins. The role of CD1a is in the formation of heterodimers with beta-2-microglobulin and the antigen presentation of lipids, including mycobacterial cell wall components to CD1a-autoreactive T cells (Brigl and Brenner, 2004; de Jong *et al.*, 2010), otherwise known as invariant natural killer T cells.

CD1a is found on Langerhans cells and thymocyte precursors (Brigl and Brenner, 2004). In LCs, CD1a is expressed very brightly, while CD1c+ dermal DCs and moDCs have lower CD1a expression with higher CD1b expression.

In disease, CD1a is expressed by hairy cell leukaemia cells (De Panfilis *et al.*, 1988), the alveolar macrophages in bronchial asthma (Agea *et al.*, 1998) and the pathological cells in Langerhans cell histiocytosis (Harrist *et al.*, 1983; Rousseau-Merck *et al.*, 1983; Schuler *et al.*, 1983).

Interestingly, CD1a expression on monocyte derived DCs (moDCs) from normal donors is highly variable and CD1a deficiency caused by single nucleotide polymorphisms (SNPs) is fairly common (3/19 normal donors) preventing moDC mycobacterial lipid expression to T cells (Seshadri *et al.*, 2013). The authors did not examine CD1a expression in tissue, where CD1a deficiency is rarely, if ever, seen (unpublished data).

1.2.3.4. *Birbeck Granules*

Birbeck granules (BGs) are pentilaminar, cytoplasmic organelles thought to be found exclusively in Langerhans Cells (Birbeck *et al.*, 1961). In two dimensional electron microscopy sections, BGs are seen as rod or tennis-racquet shaped. However, when three dimensional modelling is performed from electron

microscopy images of single BGs from multiple serial sections, their structure is seen to be more complex (see Figure 1.7). BGs are actually likely to have a flattened or curved orthogonal net of particles, which is bounded externally by a limiting membrane and which may be disc-shaped, cup-shaped, or combinations of both shapes(Sagebiel and Reed, 1968), rather like a single sycamore seed or a single-bladed propeller.

During antigen phagocytosis, langerin localises to the BGs. After this point, the role of the BGs is unsure, they could possibly migrate to the periphery for exocytosis, or receptor mediated endocytosis. The formation of BGs is induced by langerin (Kissenpfennig *et al.*, 2005), however LCs can function even when BGs are abolished by disruption of the langerin gene.
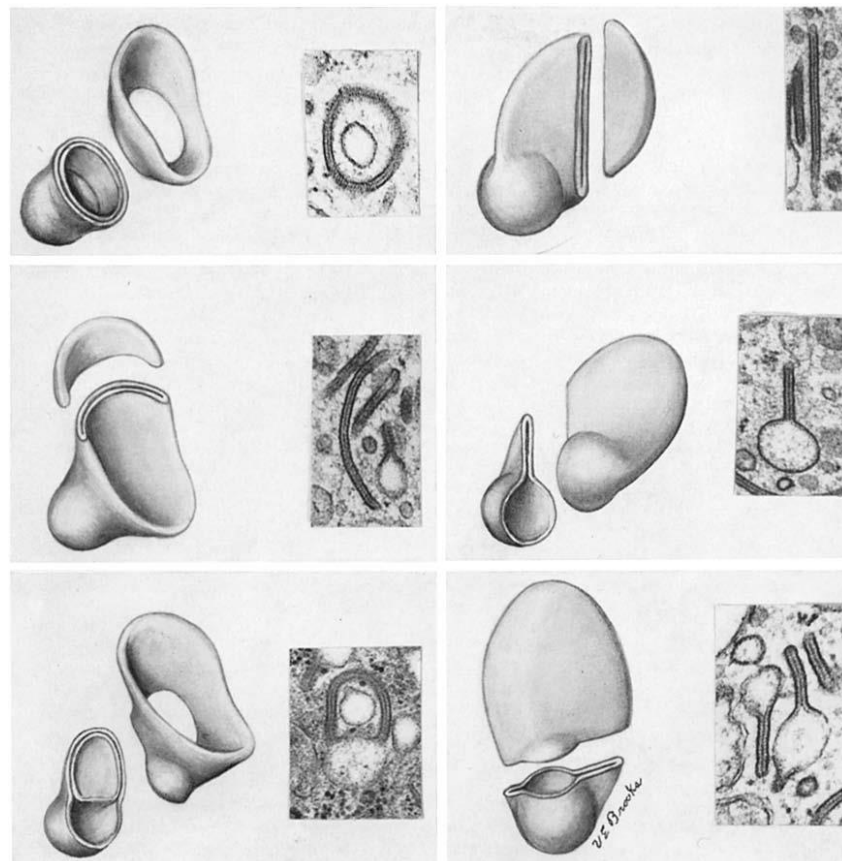
Figure 1.7. Models of Birbeck Granules

Models of the Birbeck granules found in Langerhans cells based upon three dimensional modelling of multiple electron microscopy serial sections (Sagebiel and Reed, 1968).

1.2.3.5. *Origin of Langerhans Cells*

In mice, Langerhans cells (LCs) are thought to be originally derived from early foetal progenitors (Nezelof *et al.*, 1973; Hoeffel *et al.*, 2012; Schulz *et al.*, 2012), and self-renew in the skin in the steady-state independently from circulating cells (Merad *et al.*, 2002; Bogunovic *et al.*, 2006). Some evidence suggests that there may actually be more than one LC type in murine skin, some self-renewing and some renewed from the bone marrow (Ginhoux *et al.*, 2006), and that these cells may appear in waves, a steady-state population and an inflammatory population (Seré *et al.*, 2012).

The true origin of LCs in humans is unclear, though it is known that they can proliferate *in situ* within cutaneous hair follicles (Czernielewski and Demarchez, 1987; Gilliam *et al.*, 1998; Chorro *et al.*, 2009). It has also been seen in skin allografts that LCs can remain of donor origin for several years (Kanitakis *et al.*, 2011) and recipient LCs can persist in bone marrow transplantation (Perreault *et al.*, 1985; Collin *et al.*, 2006).

Homeostatic mechanisms of LCs are distinct from those of myeloid DCs (Ginhoux and Merad, 2010) and patients without monocyte and DC populations such as those with DCML deficiency (see section 1.3.1, page 23) still maintain LCs (Bigley *et al.*, 2011), indicating that LCs are able to at least partially maintain themselves for some time by local proliferation.

Conversely, after bone marrow transplantation (BMT), LCs in the skin are eventually replaced by bone marrow derived cells, although at a slower rate than dermal DCs (Collin *et al.*, 2006; Mielcarek *et al.*, 2014). Studies have reported cells with a phenotype similar to LCs in monocyte colonies derived from human bone marrow (Goordyal and Isaacson, 1985; Gothelf *et al.*, 1986) and in several studies LC-like cells have been derived from CD34+ BM progenitor cells and from circulating mononuclear cells (See Table 1.5, page 64,

for a summary of LC generation studies to date), suggesting that LCs are likely to have an alternate origin from a circulating precursor.

1.3. Dendritic Cells in Immunodeficiencies

There are currently few known specific deficiencies of any of the dendritic cell or monocyte subsets; Pitt-Hopkins syndrome has a specific dysfunction of pDCs caused by mutations in the E2.2 gene (Pitt and Hopkins, 1978; Amiel *et al.*, 2007; Cisse *et al.*, 2008) and more recently patients have been seen with a specific CD16+ monocyte deficiency (Frankenberger *et al.*, 2013). Dendritic cells are found to be low or completely missing in patients with IRF8 mutations (Hambleton *et al.*, 2011), and in the recently described disease Dendritic Cell, Monocyte, B and NK Cell (DCML) Deficiency(Bigley *et al.*, 2011).

1.3.1. Dendritic Cell, Monocyte, B and NK Cell (DCML) Deficiency

DCML deficiency patients have diminished numbers of myeloid and plasmacytoid DCs, CD14+ and CD16+ monocytes and B and NK lymphocytes, though T cells and circulating CD34+ progenitors are preserved (Bigley *et al.*, 2011). It has been seen that children with GATA2 mutation can have normal responses to vaccines, and relatives of some patients have been found to carry the GATA2 mutation but are asymptomatic, suggesting that the patients are likely to have normal counts in their younger life (Dickinson *et al.*, 2014). The deficiency can be caused by a number of different mutations of the GATA2 gene (Dickinson *et al.*, 2011). GATA2 is a transcription factor required for stem cell homeostasis and contains 2 highly conserved zinc fingers which are critical for the survival of HSCs through mediating protein-DNA binding.

All symptomatic patients with GATA2 mutations feature the same pattern of DCML cellular loss (Dickinson *et al.*, 2011; Dickinson *et al.*, 2014), but there are a number of associated clinical syndromes. These features include: monocytopenia with Mycobacterium avium complex (monoMAC) (Vinh *et al.*, 2010; Hsu *et al.*, 2011; Hsu *et al.*, 2013); lymphedema, deafness and myelodysplasia (Emberger syndrome) (Mansour *et al.*, 2010; Ostergaard *et al.*, 2011); and familial myelodysplastic syndrome (MDS) or acute myeloid leukaemia (AML)(Hahn *et al.*, 2011; Holme *et al.*, 2012; Kazenwadel *et al.*, 2012). Recent work suggests that monoMAC, lymphedema and familial MDS/AML are all

facets of GATA2 mutation that may occur heterogeneously (Ishida *et al.*, 2012; Kazenwadel *et al.*, 2012), even within a single pedigree (Mutsaers *et al.*, 2013). Some historical cases of familial AML, and NK cell deficiency, are now also known to be due to GATA2 mutation (Kaur *et al.*, 1972; Robinson *et al.*, 1983; Horwitz *et al.*, 1996). It is not clear which of these clinical features are the direct consequences of GATA2 mutation and which are related to the loss of mononuclear cells.

Up to 50% of individuals with GATA2 mutation develop myelodysplasia (MDS) associated with fibrosis and megakaryocyte dysplasia (Calvo *et al.*, 2011; Hsu *et al.*, 2011; Bodor *et al.*, 2012; Holme *et al.*, 2012; Hsu *et al.*, 2013). However, many patients present with clinical problems prior to their meeting the standard WHO (World Health Organisation) criteria for MDS, despite the mononuclear cell failure. The striking loss of mononuclear cells with initially well preserved BM histology and trilineage haematopoiesis seen in DCML deficiency leads to the question of whether there is a similar loss of mononuclear cells in sporadic MDS.

It has previously been reported that fms-like tyrosine kinase 3 ligand (FLT3L) is elevated in patients with DCML deficiency (Bigley *et al.*, 2011). FLT3L is an important growth factor in DC development(Shortman and Naik, 2007; Rahman *et al.*, 2011), but elevated levels have also been reported in Fanconi anaemia and aplastic anaemia suggesting that hematopoietic stress is a trigger (Lyman *et al.*, 1995; Haidar *et al.*, 2002). Further evaluation may indicate whether this is a useful marker for diagnosis and monitoring of GATA2 mutation.

Later in this thesis is presented an analysis of a European cohort of DCML deficiency patients with GATA2 mutation from a range of clinical backgrounds, alongside classical MDS patients and *de novo* AML patients. This work describes in detail the evolution of cellular deficiency, the utility of FLT3L and specific deficiencies in differential diagnosis and the effects of failing mononuclear cell development upon peripheral mononuclear cell homeostasis.

1.4. Dendritic Cells in Haematological Malignancies

The roles of DCs in haematological malignancies such as myelodysplastic syndrome (MDS) and acute myeloid leukaemia (AML) are uncertain, though it has been reported that DC-like cells can be derived from blast cells *in vitro*(Robinson *et al.*, 1998; Harrison *et al.*, 2001; Panoskaltsis *et al.*, 2002).

One study shows a decrease of mDCs and pDCs in the peripheral blood of MDS patients (Ma *et al.*, 2004) and another showed a reduction in DC precursors in the BM, associated with higher risk patients (Saft *et al.*, 2013). In AML, levels of DCs have been seen to be vastly variable, some patients with expansions and others with absolute losses (Mohty *et al.*, 2001). All of these studies were performed using limited flow cytometric analysis. As part of this thesis, a number of different multiparameter flow cytometry panels were set up to analyse in detail the mononuclear components of the peripheral blood and bone marrow of MDS and AML patients for DC numbers.

1.4.1. *Myelodysplastic Syndromes*

The myelodysplastic syndromes are conditions of clonal stem cells in the marrow which cause an ineffective production of one or more myeloid cell types and can lead to bleeding, anaemia and infection. MDS is usually a disease of the elderly, with a median age of diagnosis of ~70 years (Corey *et al.*, 2007; Germing *et al.*, 2008). The prognosis is fairly poor, with worsening progression over time. Mean survival rates are between 6-24 months dependant on risk factors such as cytogenetics, BM blast counts and cytopenias (Greenberg *et al.*, 1997), and allogeneic stem cell transplantation is the only potential curative therapy available (Zeidan *et al.*, 2013). Diagnosis is based on the observation of a dysplastic hypercellular bone marrow with or without blast cells and peripheral blood cytopenia. Cytogenetic abnormalities are present in around 50% of MDS patients, quite often including trisomy 5, 7 or 8 (Haase, 2008). One third of MDS patients later progress to acute myeloid leukaemia (AML) (Adès *et al.*). The cut-off point for blast counts between a diagnosis of MDS or AML

is 20% by World Health Organisation (WHO) criteria (Vardiman *et al.*, 2009).

1.4.2. *Acute leukaemia*

Acute leukaemia (AL) is an umbrella term for a cancer which arises from the haematopoietic system of the bone marrow. AL is a life threatening condition where abnormal, immature leukocytes accumulate in the bone marrow and interfere with normal blood cell production. Whilst commonly confined to bone marrow and blood, leukaemic cells can invade other tissues including the central nervous system, skin, lung and gum.

Patients often present with clinical features of BMF (bleeding, anaemia, infection) and malignancy (weight loss, sweats, tiredness etc.), which is reflected in a full blood count showing anaemia, thrombocytopenia and neutropenia. Circulating blasts can often be seen on a blood film, though diagnosis is confirmed by finding >20% leukaemic blast infiltration in a BM smear or trephine. Leukaemic blasts, (and hence the leukaemia) are classified according to their morphology, cytochemical stains, cell surface antigen expression assessed by flow cytometry, and genetic abnormalities assessed by cytogenetics and fluorescent *in situ* hybridization.

Two to three thousand new cases of adult AL are diagnosed in the UK each year (Milligan *et al.*, 2006), which is up to ten times higher than the incidence rates seen in children (see Figure 1.8). In the Northern region of the UK, there are approximately 100 new cases of MDS and 50 new cases of AML per annum. About half of these present to the Newcastle Hospitals NHS Foundation Trust. In the UK, there are ~10,000 adults living with AL, ~50% of who will likely die from this disease, 43% of women and 57% of men (CancerResearchUK, 2014b). Hence, it can be seen that AL has a significant impact on the UK population and the high mortality rate indicates that further research into this disease is required.

A.
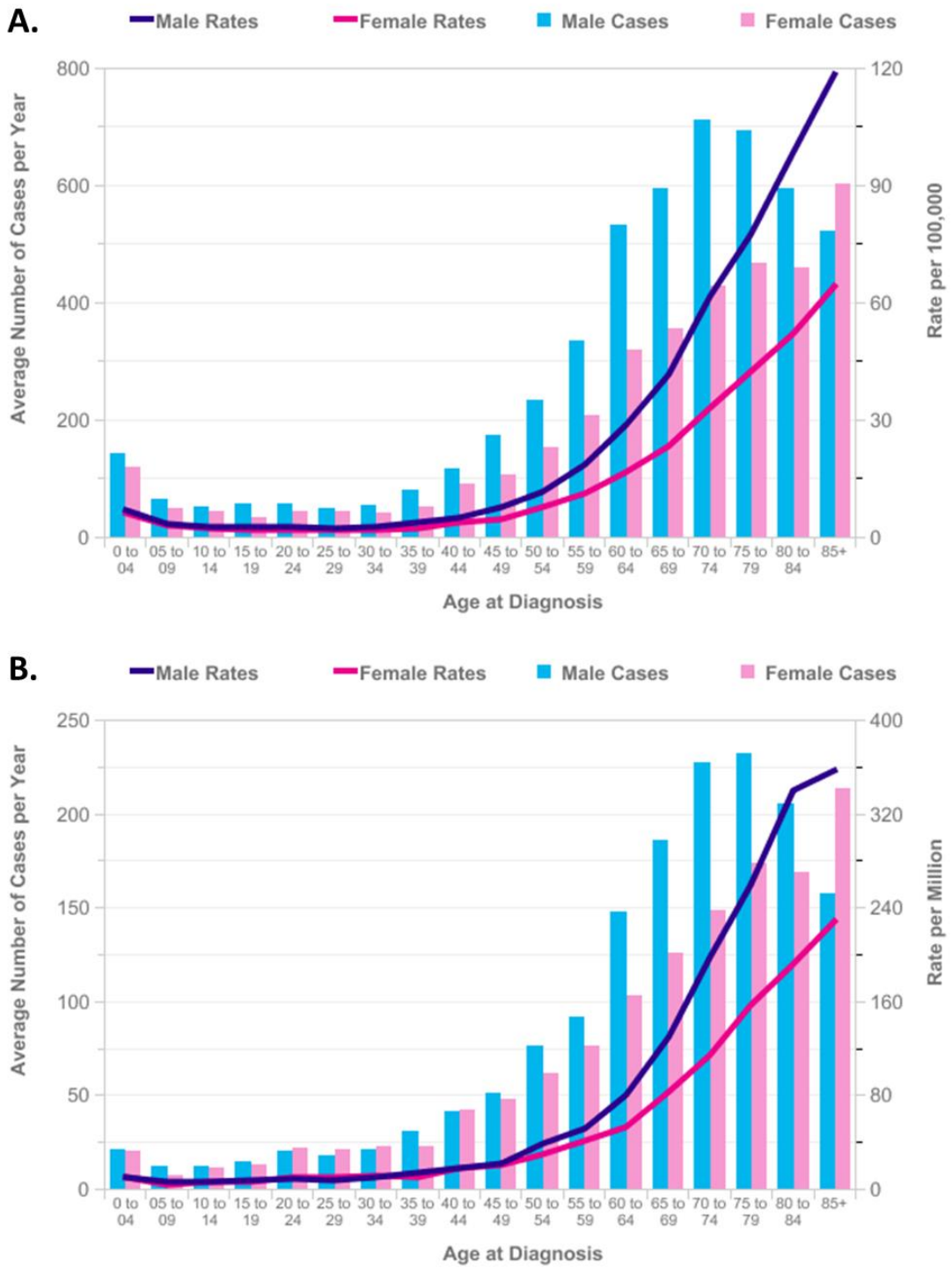
Figure 1.8. Leukaemia Incidence Rates, UK

Average Number of New Cases per Year and Age-Specific Incidence Rates per 100,000 Population, UK. (CancerResearchUK, 2014a)

1.4.2.1. *Leukaemia Classifications*

Leukaemia has several different but complementary forms of classifications. The leukaemia can be of the lymphocytic or myeloid (or biphenotypic) lineage and can be acute; a rapid accumulation of immature blood cells which prevents normal blood cell production, or chronic; where the accumulation of cells is of a more mature, but still abnormal phenotype.

Acute leukaemia can be classified using the differentiation morphology of the blasts (FAB Classification; Table 1.1), cytogenetics (Grimwade *et al*., 2010);Table 1.2), or a combination of both (WHO Classification; Table 1.3). It is important to note that not all leukaemias, such as the rare acute basophilic leukaemia are included in the FAB classifications. This study is focused on myeloid cells and their roles in acute myeloid leukaemia.

Acute leukemia: morphological classification

Acute Myeloid (AML)

M_0: minimally differentiated
M_1: without maturation
M_2: with maturation
M_3: hypergranular promyelocytic
M_4: myelomonocytic
M_5: (a) monoblastic, (b) monocytic
M_6: erythroleukemia
M_7: megakaryoblastic
Rare types (e.g. eosinophilic, natural killer)

Acute Lymphoblastic (ALL)

L_1: small, monomorphic
L_2: large, heterogeneous
L_3: Burkitt-cell type

Table 1.1.French-American-British (FAB) Leukaemia Classifications

The myeloid leukaemias are divided into eight types (M0-7) and the lymphoblastic leukaemias into three (L1-3). These classifications are based on examining the differentiation state appearance of the leukaemic cells (light microscopy and cytogenetics) and which normal progenitors they most likely resemble.(Bennett et al., 1976)

Cytogenetic abnormality	Comments
Favorable	
t(15;17)(q22;q21)	
t(8;21)(q22;q22)	Irrespective of additional cytogenetic abnormalities*
inv(16)(p13q22)/t(16;16)(p13;q22)	
Intermediate	
Entities not classified as favorable or adverse	
Adverse	
abn(3q) [excluding t(3;5)(q21~25;q31~35)],	
inv(3)(q21q26)/t(3;3)(q21;q26),	
add(5q), del(5q), −5,	
−7, add(7q)/del(7q),	Excluding cases with favorable karyotype†
t(6;11)(q27;q23),	
t(10;11)(p11~13;q23),	
t(11q23) [excluding t(9;11)(p21~22;q23) and t(11;19)(q23;p13)]	
t(9;22)(q34;q11),	
−17/abn(17p),	
Complex (≥ 4 unrelated abnormalities)	

*All favorable-risk abnormalities.
†All adverse-risk abnormalities.

Table 1.2. AML Cytogenetic Risk Status Classifications

AML Cytogenetic Risk Status Classifications based on multivariable analyses of cytogenetic results seen in 5876 acute myeloid leukaemia patients (16-59 years of age) (Grimwade et al., 2010)

AML with recurrent genetic abnormalities

AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*
AML with inv(16)(p13,1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
AML with t(15;17)(q22;q12); *PML-RARA*
AML with t(9;11)(p22;q23); *MLLT3-MLL*
AML with t(6;9)(p23;q34); *DEK-NUP214*
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*
AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM15-MKL1*
Provisional entity: AML with mutated *NPM1*
Provisional entity: AML with mutated *CEBPA*

AML with myelodysplasia-related changes
Therapy-related myeloid neoplasms
AML, not otherwise specified

AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Acute erythroid leukemias
    Pure erythroid leukemia
    Erythroleukemia, erythroid/myeloid
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis

Myeloid sarcoma

Precursor lymphoid neoplasms

B-cell lymphoblastic leukemia/lymphoma, not otherwise specified
B-cell lymphoblastic leukemia/lymphoma with recurrent genetic abnormalities

B-cell lymphoblastic leukemia/lymphoma with t(9;22)(q34;q11.2); *BCR-ABL1*
B-cell lymphoblastic leukemia/lymphoma with t(v;11q23); *MLL* rearranged
B-cell lymphoblastic leukemia/lymphoma with t(12;21)(p13;q22); *TEL-AML1 (ETV6-RUNX1)*
B-cell lymphoblastic leukemia/lymphoma with hyperploidy
B-cell lymphoblastic leukemia/lymphoma with hypoploidy (hypodiploid ALL)
B-cell lymphoblastic leukemia/lymphoma with t(5;14)(q31;q32); *IL3-IGH*
B-cell lymphoblastic leukemia/lymphoma with t(1;19)(q23;p13.3); *E2A-PBX1 (TCF3-PBX1)*

T-cell lymphoblastic leukemia/lymphoma

Table 1.3. World Health Organisation (WHO) AML/ALL Classifications

Based on cytogenetics and cellular morphology (Falini et al., 2010)

1.4.3. *Acute Myeloid Leukaemia (AML)*

Acute myeloid leukaemia is a haematopoietic disorder of the bone marrow where precursors of myeloid cells are arrested early in development and accumulate at a high rate, which may cause BMF. AML is the most commonly diagnosed form of AL in adults and has a similar median age of diagnosis as MDS, at 69 years (Juliusson *et al.*, 2009). In the UK, AML has a poor average 5 year survival rate of around 25%, compared to ALL with a 5 year survival rate of around 40% (CancerResearchUK, 2013b; CancerResearchUK, 2013a). Cytogenetics in AML are highly heterogeneous (Grimwade *et al.*, 2010).

It is becoming apparent that it can be possible to define leukaemia based on the similarity of the clonal cells to progenitor subsets. Most experimentation of this type has employed transplanting AML cells into immunodeficient mouse models, such as NOD/SCID mice, to observe *in vivo* differentiation ability based on cell markers. Some markers appear to be expressed at corresponding levels in HSCs and AML cells (e.g. CD34, CD38, HLA-DR and CD71 (Bonnet and Dick, 1997) whereas others are aberrantly expressed in AML cells (CD33 (Appelbaum *et al.*, 2001), CD96 (Hosen *et al.*, 2007), CD123 (Lapidot *et al.*, 1994; Bonnet and Dick, 1997), or absent in LSCs (CD66 (Appelbaum *et al.*, 2001), CD90 (Blair *et al.*, 1997). Also, some AML cells show markers consistent with a more mature phenotype (Goardon *et al.*, 2011).

These investigations have shown interesting preliminary results; however there are several issues with employing mouse models for the study of human AML, such as low levels of engraftment of human leukaemic cells into xenograft mouse models (50-70%), and the debatable relevance of induced murine AML. Cook and Pardee recently published a review of the available animal models for the study of AML, with detailed descriptions of their strengths and weaknesses (Cook and Pardee, 2013).

1.4.3.1. *Classifying Leukaemic Clones*

An interesting recent study attempted to divide AML based on the progenitor immunophenotype of the blast cells (Goardon *et al.*, 2011). Looking specifically in CD34+ AML patients, they found that two distinct progenitor immunophenotypes were apparent. In the larger group of patients (87.8%, 65/74), most CD34+38+ cells were CD123+CD45RA+ (similar to normal granulocyte macrophage progenitors; GMPs) and most CD34+38- cells were CD90-CD45RA+ (similar to normal multi-lymphoid progenitors; MLPs) and thus are referred to as GMP/MLP type patients in this thesis. In the smaller group of patients (13.8%, 10/74), most CD34+38+ cells were CD123+CD45RA- (similar to normal common myeloid progenitors; CMPs) and most CD34+38- cells were CD90-CD45RA- (similar to normal multi-potent progenitors) and thus are referred to as CMP/MPP type patients in this thesis.

1.4.4. *Blastic Plasmacytoid Dendritic Cell Neoplasm*

Blastic Plasmacytoid Dendritic Cell Neoplasm (BPDCN) is a subtype of AML defined as a CD4+CD56+ tumour with a high expression of HLA-DR and CD123 (IL-3 receptor) and a lack of T and B cell lineage specific markers (e.g. CD3, CD19, CD20). The first reported case in 1995, was thought to be an aggressive cutaneous immature cell lymphoma (Brody *et al.*, 1995), due to the markers CD4+56+2-. This was redefined as Blastic Natural Killer Cell Lymphoma or CD4+/CD56+ NK-Cell Lymphoma, in the 2001 WHO reviews, (Jaffe, 2001) due toCD56 positivity and lymphoid like morphology indicating NK cells.

The first connection between the tumour cells and pDCs was highlighted in 2001, where it was seen that when stimulated with IL-2 and CD40-ligand, the leukaemic cells matured to DCs with functional capabilities similar to pDCs, such as IFN-α production and TH2 polarisation(Pilichowska *et al.*, 2007). Therefore the disease was re-classified as BPDCN in the 2008 WHO reviews (Swerdlow *et al.*, 2008).

BPDCN is most often a leukaemia of the elderly, with a mean diagnosis age of 67, and a gender bias to males (2M/1F). BPDCN can occur in children, where there is a better prognosis to adults(Jegalian *et al.*, 2010), who have an aggressive disease with a very poor survival of median 13 months (Lucioni *et al.*, 2011).

The most frequent symptom (85%) at presentation is cutaneous lesions. These can vary from one or two nodules to disseminated cutaneous lesions associated with superficial lymphadenopathy and occasionally systemic symptoms, such as weakness and weight loss (Jegalian *et al.*, 2010; Borchiellini *et al.*, 2012). This is followed by a leukaemic transformation of CD4+56+ cells in almost all cases (Borchiellini *et al.*, 2012).

The pathogenic cells seen in the cutaneous lesions are medium sized with lack of granules and little cytoplasm (see Figure 1.9). The nucleus tends to be regular within distinct or absent nucleoli and fine chromatin (Petrella, 2010; Borchiellini *et al.*, 2012). A diagnostic criterion for BPDCN has been set out by Tsagarakis *et al* (see Table 1.4), including the markers CD4+56+HLA-DR+123^{bright}to differentially diagnose from other CD56+ leukaemias.

The most effective treatment for BPDCN is ALL type chemotherapy regimens followed by haematopoietic stem cell transplantation (HSCT), which gives the patients a median survival of 60 months (Tsagarakis *et al.*, 2010; Lucioni *et al.*, 2011). However, relapses post-chemotherapy are frequent at median 9-11 months (Jegalian *et al.*, 2010), and due to the late age of onset of this disease, most patients are not eligible to receive HSCT.10-20% of BPDCN patients have a co-existence of AML and/or MDS (Herling and Jones, 2007), which is strong evidence of a myeloid origin of the disease.
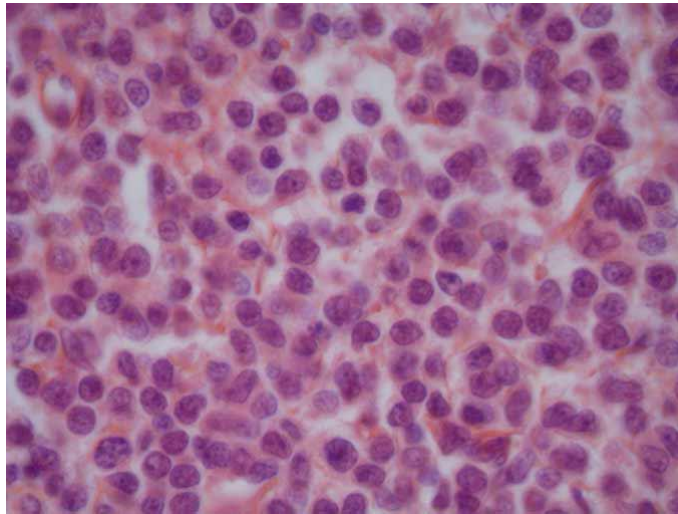
Figure 1.9. Blastic pDC in a patient with BPDCN

Haematoxylin and Eosin staining. X400 magnification (Petrella, 2010)

FCM algorithmic approach for BPDCN diagnosis			
	Blast cluster identification and characterization by CD45/RT-SC gating		
Immunophenotype	**CD45/RT-SC low cluster (compatible with BPDCN)**	**CD45/RT-SC high cluster (indicative of leukemia of myelomonocytic origin)**	**CD45/RT-SC with two blast populations one of them DC2 (indicative of leukemia of ambiguous lineage, LAL)**
Important positives	• CD123+ (high intensity) • HLA-DR+ • CD4+/dimCD56+/dim	• CD64+, • lysozyme+ • MPO+	CD56 negative, DC2 population coexistent with another leukemic population of different lineage myeloid or lymphoid
Important negatives	• MPO-, CD3-, CD19- • CD14-, CD64-, lysozyme- • cCD3- or cCD79a- (dim?)	• BDCA-2+ • CD45RA+	
DC2 associated	• BDCA-2+ (specific but non-sensitive) • BDCA-4+ • CD45RA+ • NG2+ • TCL1+, ILT3+	• CD123+ • HLA-DR+ • CD4+ • CD56+ • NG2+ • ILT3+	

Table 1.4. BPDCN Diagnosis Algorithm (Tsagarakis et al., 2010)

1.4.5. *Acute Leukaemia Treatment Related Mortality Risk Markers*

AML treatment related mortality during induction chemotherapy has substantially decreased over the last two decades (~17% in 1991; ~3.5% in 2009), most likely due to improved supportive clinical practices (Othus *et al.*, 2014). However, complications related to sustained disease and treatment still cause significant adverse event and mortality rates in AML and MDS patients, due to prolonged general cytopenias leading to infections and uncontrolled bleeding (Gaydos *et al.*, 1962; Bodey *et al.*, 1966; Bodey *et al.*, 1978; Pagano *et al.*, 2012; Buckley *et al.*, 2014; Buckley *et al.*, 2015). These studies are limited to analysis of bulk cell populations such as 'lymphocytes' and 'monocytes', and focus on post-chemotherapy markers to ascertain patient prognosis and outcome. There is a surprising scarcity of data documenting pre-chemotherapy markers or the role of specific immunodeficiencies in leukaemia. Clinical trials have shown that routine use of prophylactic anti-infection drugs such as colony stimulating factors do not increase overall survival or decrease infection rates in AML patients (Gurion *et al.*, 2012), although these trials were inclusive of all AML patients, and so may not have been able to identify small groups of specific patients who would benefit from such treatment. Identification of markers which could be used to stratify patients for likelihood of treatment-related mortality and/or infection risk, before the induction of chemotherapy would be a major step forward in the clinical management of patients. Such markers could be used to tailor prophylactic treatments against likely infections, strategies to reduce the severity of other treatment-related adverse events and treatment regimens for haematopoietic system recovery post-chemotherapy. They could also be used to inform patients of the likelihood of such events. One such marker could be the extended phenotype of leukaemia at diagnosis.

1.4.6. *Acute Leukaemia and the Extended Phenotype*

Leukaemia is directly linked to bone marrow failure in some cases. Part of this thesis investigates leukaemogenesis by examining not only the leukaemic clone cells but also the specific cellular deficiency that arises with the leukaemia; it's 'extended phenotype' of cellular immunodeficiency.

This information is almost always neglected by current analyses, although it is clear that leukaemia phenotyping is capable of identifying 'residual normal cells'. The extent of mononuclear cell differentiation in AL has not previously been examined; however it is possible that the frequency of each mononuclear subtype, and in particular any deficiencies in specific subtypes, could have an important impact on prognosis in terms of infection risk at presentation and during early treatment. Immunodeficiency may increase the incidence of severe sepsis or fungal infection, responsible for the majority of induction deaths (Lech-Maranda *et al.*, 2010).

An extended phenotype of Immunodeficiency could potentially be caused by the blast cells blocking the generation of specific cell subsets or causing a global reduction in mononuclear cells by BMF. Alternatively, there could be an expansion of specific cellular subsets as part of the leukaemic clone, which may or may not have aberrant functionality (See Figure 1.10).
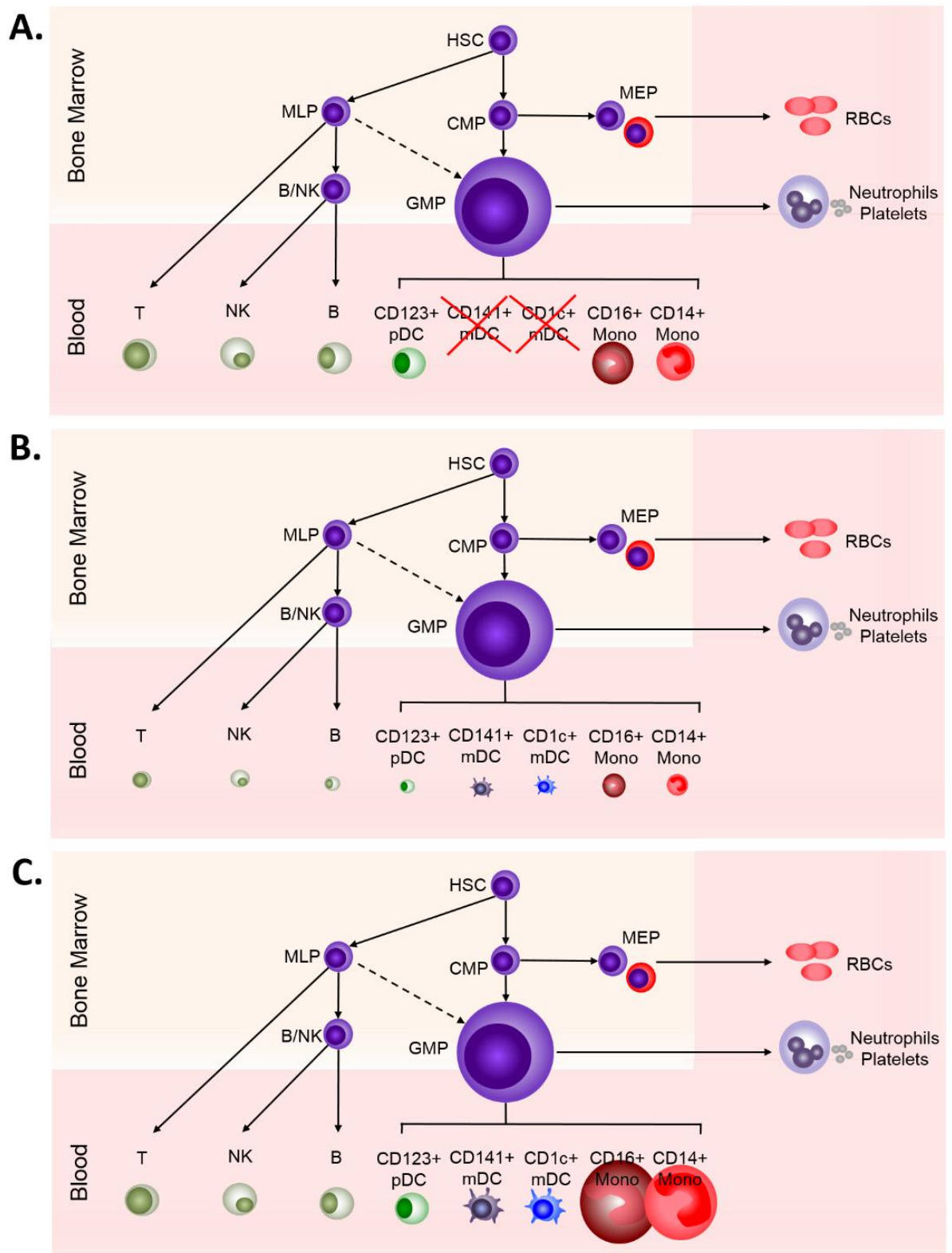
Figure 1.10. Potential Extended Phenotypes of Leukaemia

A) *Generation of specific subsets blocked by the leukaemic clone. The idea that the expansion of the malignant cells may block specific downstream progeny related to the blast cell progenitor immunophenotype, while progeny from other progenitor types remain unaffected. In this example, an expansion of GMPs has blocked the maturation of mDCs.*

B) *Global reduction of mononuclear cells caused by bone marrow failure. The idea that the expansion of any malignant cell type will cause a universal reduction on all haematopoietic progeny due to the blast cells occupying the majority of the haematopoietic niche of the bone marrow.*

C) *Expansion of specific subsets which are part of the leukaemic clone. The idea that the expansion of malignant cells with a specific blast cell immunophenotype will lead to an expansion of the downstream progeny of that progenitor type. In this example, an expansion of GMPs has led to an expansion of monocytes.*

HSC = Haematopoietic Stem Cell; MPP = Multi-Potent Progenitor; MLP = Multi-Lymphoid Progenitor; B/NK = B/NK Cell Progenitor; CMP = Common Myeloid Progenitor; MEP = Megakaryocyte/Erythroid Progenitor; GMP = Granulocyte Macrophage Progenitor; RBC = Red Blood Cell; T = T Cell; B = B Cell; NK = Natural Killer Cell; mDC = Myeloid Dendritic Cell; pDC = Plasmacytoid Dendritic Cell; Mono = Monocyte; LC = Langerhans Cell; Mo-MAC = Monocyte/Macrophage; MΦ = Macrophage.

The concept of an extended phenotype of acute leukaemia is a novel and potentially important idea. It is possible that total mononuclear cell profiling will identify a different profile of immunodeficiency associated with each biological type of acute leukaemia. This hypothesis stemmed from the following observations. Firstly, that human leukaemic blasts are phenotypically similar to specific progenitor subsets but are unable to differentiate into mature cells *in vivo* (Goardon *et al.*, 2011); so there is likely to be a deficiency of the normal progeny of the related progenitors. Secondly, that there is evidence of loss of MLP and GMP progenitors and mononuclear cells in the pre-leukaemic disease DCML deficiency prior to AML development (Vinh *et al.*, 2010; Dickinson *et al.*, 2014). The difference in DCML deficiency being that there is a loss of progenitor cells rather than an expansion of abnormal progenitors as seen in AML.

The caveat of this concept is that it relies on the clonality of the BM in AML patients before leukaemia develops. If the majority of the BM is not clonal, then the accessory phenotype is unlikely to be seen. However, if the majority of the BM is clonal before onset of AML, such as is the case in DCML deficiency (Dickinson *et al.*, 2014) and MDS (Koeffler and Golde, 1978; Raskind *et al.*, 1984), then I expect to see specific immunodeficiencies.

These immunodeficiencies can be observed using the flow cytometry panels set up previously and in this thesis (Bigley *et al.*, 2011). Testing of this hypothesis will increase our understanding of leukaemogenesis; test current models of human haematopoietic hierarchy and may be very useful in the prognosis of leukaemia and leukaemia biology itself.

1.5. Dendritic Cells in Histiocytic Disorders

Histiocytic Disorders (Histiocytosis) is a collective term for a group of diseases characterised by an abnormal accumulation of histiocytes, (or 'tissue cells'), a term usually encompassing tissue macrophages and mature activated DCs.

The most common histiocytosis is Langerhans Cell Histiocytosis (LCH), which is rare, but has an incidence in children similar to Hodgkin's Lymphoma and acute myelogenous leukaemia at around 5 cases/million. Other non-LCH histiocytoses include Erdheim Chester Disease (ECD), Hemophagocytic Lymphohistiocytosis (HLH), Juvenile Xanthogranuloma (JXG), Rosai-Dorfman Disease (RDD), and much rarer Sea-Blue Histiocytic Syndrome, Histiocytic Sarcomas and Interdigitating Dendritic Cell Sarcomas. The work in this thesis focuses on the histiocytoses with high levels of BRAF mutation; LCH, which is the most common histiocytosis, and ECD.

It is currently unclear how these diseases are related, as some can occur in the same patient at the same time. A common hematopoietic aetiology of LCH and ECD is suggested by the occasional coincidence of these diseases and the discovery of mutated BRAF in both diseases, even though ECD is clinically and pathologically distinct from LCH (Haroche *et al.*, 2012b; Yin *et al.*, 2013; Hervier *et al.*, 2014). In the study by Hervier *et al*, approximately 19% of the ECD patient cohort also had evidence of LCH. Histiocytoses may also occur concurrently with other pathologies including AML, lymphoma, or viral infection (Egeler *et al.*, 1994; Hwang *et al.*, 2013).

As with all rare diseases, there are inherent problems in the study of histiocytic disorders. There is a relative lack of clinical recognition of the disorder prior to biopsy, with common misdiagnosis as other skin diseases (e.g. dermatitis, eczema, burns) or other diseases such as osteomyelitis or malignancies. This makes attaining fresh material very difficult, with patients presenting to virtually any specialty (dermatology, haematology, respiratory, neurology, endocrinology, rheumatology, orthopaedics, oncology etc.).

1.5.1. *Disease Cells and Target Organs*

LCH cells are large (15-25 micrometres), express langerin and CD1a, contain Birbeck granules (Figure 1.14) and cause granulomatous lesions predominantly in the bone, epithelia, haematopoietic organs, lung, liver and CNS(Nezelof *et al.*, 1973; Laman *et al.*, 2003; Abla *et al.*, 2010; Allen *et al.*, 2010; Badalian-Very *et al.*, 2013). ECD lesions show abundant CD68+ foamy macrophages and affect the distal long bones, skin (xanthelasmata) and cardiovascular tissue (Haroche *et al.*, 2012b). See Figure 1.14, page 51 for a brief description of the main differences between LCH and ECD.

In LCH, there is an admixed infiltrate of T cells, activated macrophages, eosinophils, neutrophils, fibroblasts and osteoclast-like-multinucleate giant cells in the granulomas. This is similar to the infiltrate seen in Hodgkin's Lymphoma, but without the classic Reed-Sternberg cells and plasma cells. ECD lesions have a Th1-type lymphocyte infiltrate (Stoppacciaro *et al.*, 2006), variable fibrosis and the occasional Touton or Foreign body fatty multinucleated giant cells (Figure 1.14), which contain a central eosinophilic cytoplasmic area surrounded by a ring of nuclei and clear peripheral cytoplasm.

1.5.2. *Prevalence*

LCH is most commonly found in children aged 1-15 years old (Incidence of 5-15/million per year<10yrs old) and less common in adults, but may be underdiagnosed (Lipton and Arceci, 2008). ECD is less common, just over five hundred cases have been described since the first in 1930, and it mostly occurs in adult patients (age 40-60) (Haroche *et al.*, 2012b). There is a male to female ratio of 2:1 in LCH(Herring, 2013), 1:1 in ECD (Mazor *et al.*, 2013).

1.5.3. *Sub-Classifications*

ECD patients are heterogeneous, but due to the limited number, there are no sub-classifications. LCH has two main sub-classifications:

<u>Unifocal</u> – Single bone lesions containing a slowly expanding accumulation of abnormal cells. These lesions can most often be cured by curettage.

<u>Multifocal</u> – Multiple lesions in various organs of the body. Multifocal LCH can be further subdivided based on the organs of involvement.

<u>Low-Risk Organs</u>– Skin, bone, lung, gastrointestinal tract, pituitary gland and/or central nervous system. Patients with lesions confined to low/non-risk organs have an almost 100% survival rate (Gadner *et al.*, 2008).

<u>High-Risk Organs</u> – Bone marrow, liver, spleen. Paediatric patients with lesions in high-risk organs have a higher mortality rate of around 20% (Gadner *et al.*, 2008).

1.5.4. *LCH Symptoms, Prognosis and Treatment*

Symptoms and presentation of LCH can be wide-ranging from those related to the lesion such as bone swelling, pain or skin rashes (see Figure 1.11); a more general inflammatory response such as fever, lethargy or weight loss; or organ impairment such as pancytopenia or diabetes insipidus. LCH can be classified based on which 'risk' organs are involved.

The liver, spleen, haematopoietic system and lung are considered high risk organs; patients with LCH lesions in these organs have a worse overall prognosis (Gadner *et al.*, 2008). If LCH lesions are found in the central nervous system (CNS) risk organs such as the ears, eyes, oral cavity or craniofacial zones, or if the patient has diabetes insipidus, then there is higher risk of LCH involvement in the CNS (Grois *et al.*, 1998; Grois *et al.*, 2006; Mittheisz *et al.*, 2007). Finally there are 'special sites' where disease (and/or local treatment) will directly cause significant problems because they are located in anatomically and/or functionally critical sites e.g. vertebral and/or intraspinal lesions with or without soft

tissue extensions (Girschikofsky *et al.*, 2013). All other organs are considered low risk.

1.5.4.1. *Childhood LCH*

Treatment of childhood LCH depends on the location and number of lesions. Single lesions can often be surgically removed with a low chance of recurrence. Other treatments include steroid injections, chemotherapy and radiation therapy.

Hashimoto-Pritzker Disease (Congenital self-healing reticulocytosis) is a self-limited form of LCH restricted to skin involvement (Hashimoto and Pritzker, 1973). This disease is only seen in neonates, displaying reddish-brown papulovesicular lesions which spontaneously resolve within 3 months. The skin specificity and self-resolution of this disease has interesting connotations when thinking about cell of origin of these diseases.

1.5.4.2. *Adult LCH*

Adult LCH frequently causes unifocal or multifocal bone disease that responds to surgical treatment, anti-inflammatory drugs or bisphosphonates. A limited form of multisystem (MS) LCH involving the skin, bone and pituitary axis is recognized and smoking-related pulmonary LCH also occurs. A small number of adults have high risk MS LCH involving the hematopoietic system, spleen or liver that may be refractory to intensive chemotherapy and has a poor outcome (Teng *et al.*, 2005). Treatment algorithms are less well defined than in children, particularly for high risk MS disease (Aricò *et al.*, 2003; Girschikofsky *et al.*, 2013).

1.5.5. *ECD Symptoms, Prognosis and Treatment*

The most common symptom of ECD is mild bone pain, followed by retroperitoneal fibrosis, diabetes insipidus, exophthalmos, xanthomas and neurological, kidney, liver and cardiovascular problems (Veyssier-Belot *et al.*, 1996). Treatment options again depend on the extent of the disease

and location, but due to the rarity of the disease, no large scale clinical studies have been performed. The most common treatments for ECD are corticosteroids, chemotherapy, surgical debulking or immunotherapy with interferon-α, which has shown to improve prognosis (Haroche *et al.*, 2012b). More recently, and with the discovery of the *BRAF*[V600E]mutation in ~50-60% of ECD patients (Haroche *et al.*, 2012a), the BRAF inhibitor Vemurafenib has been employed for treatment of ECD, with dramatic and reproducible results (Haroche *et al.*, 2013; Haroche *et al.*, 2015). Some of the patients with ECD lesions in these studies also had coexisting LCH lesions (skin/lymph node), which likewise improved with vemurafenib treatment, indicating that this could be a useful treatment option for LCH patients. Therefore clinical trials are currently underway studying the efficacy of vemurafenib and other BRAF inhibitors such as dabrafenib in histiocytoses.
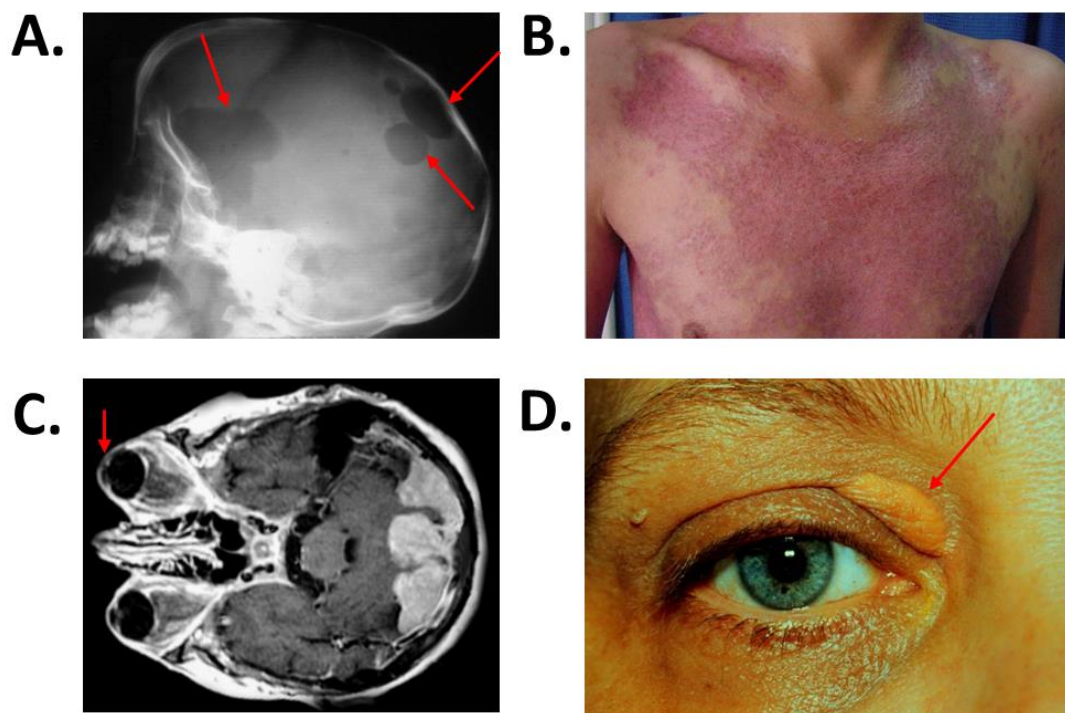
Figure 1.11. Example Images of Histiocytosis Symptoms

A. Cranial lesions in a patient with LCH(Sethi, 2004)

B. Extensive skin rash in a patient with LCH(Mortazavi et al., 2002)

C. Exophthalmos in a patient with ECD(Gaillard, 2007)

D. Xanthoma in a patient with ECD(Tayani, 1998)

1.5.6. *The MAPK Pathway in Cancer and Histiocytosis*

The MAPK (or RAS-RAF-MEK-ERK) pathway is involved in gene regulation; transcription and preventing apoptosis (See Figure 1.13). In mice, deficient MAPK signalling leads to a general anaemia, leukopenia and bone marrow aplasia (Warburg, 1924; Chan *et al.*, 2013). More specifically, in murine haematopoiesis, the MAPK pathway is known to play an essential role in the differentiation of thymocytes (Pages *et al.*, 1999), cytokine-induced myeloid cell differentiation (Miranda *et al.*, 2005) and EPO-induced erythroid differentiation (Matsuzaki *et al.*, 2000). Furthermore, MAPK signalling has been implicated in megakaryocyte differentiation in human leukaemia cell line models (Racke *et al.*, 1997; Whalen *et al.*, 1997).

A recent review concluded that the pathway is hyper-activated in ~30% of cancers (Roberts and Der, 2007). Surprisingly this pathway is over-activated in the LCH cells of 100% of LCH patients (Badalian-Very *et al.*, 2010) and in the ECD cells and a small fraction (1-3%) of monocytes in ECD patients (Cangi *et al.*, 2014).
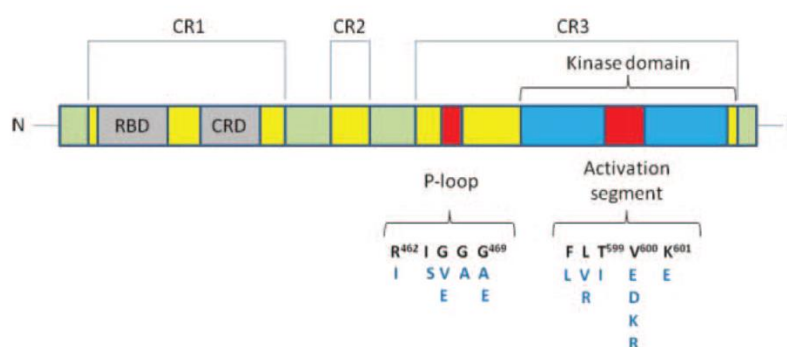
Figure 1.12. Schematic of BRAF Gene

Schematic representation of BRAF gene structure showing domains and some of the common BRAF mutations. CRD: Cystine-Rich Domain, RBD: RAS Binding Domain, CR. (Govender and Chetty, 2012)
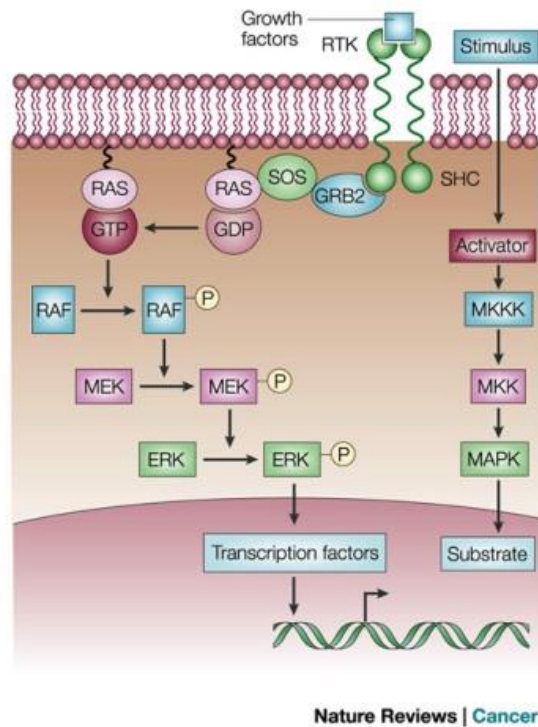
Figure 1.13. The MAPK (RAS-RAF-MEK-ERK) Pathway

A schematic of the MAPK Pathway(Chin, 2003).Briefly, when the GTPase RAS is activated by a growth factor binding to a cell surface receptor, it swaps it's GDP for a GTP which causes activation of the serine/threonine kinases of the RAF family. RAF then phosphorylates and activates the serine/threonine kinase MEK which in turn phosphorylates and activates the tyrosine/threonine kinase ERK. Finally, MEK can activate various transcription factors such as Myc, Elk1, Fos, Ets, Cyclin D for regulation of cell cycle and transcription and SPRY and DUSP for negative feedback.

1.5.6.1. *BRAF Mutations in Malignancies*

BRAF is one of the three members of the RAF family and is a member of the MAPK pathway. BRAF is known to have mutations in 7% of human cancers (Davies *et al.*, 2002) and can cause birth defects when inherited. There are more than 30 BRAF mutations associated with cancers (see Figure 1.12) but a thymine to adenine (T to A) substitution at nucleotide 1799, causing a valine (V) to glutamine (E) substitution at codon 600, and hence named $BRAF^{V600E}$on chromosome 7q32.7, constitutes 90% of BRAF mutations seen (Davies *et al.*, 2002).

This mutation is in the activation segment of the gene (Tan *et al.*, 2008) causing a constitutive activation of BRAF, leading to a RAS independent dysregulation of transcription, proliferation and feedback control. *BRAF^{V600E}* mimics the phosphorylation of the neighbouring T599 and S602 residues, which normally promotes BRAF activity (Michaloglou *et al.*, 2008), and give the gene almost 500 times the endogenous kinase activity of the wild-type allele (Curry *et al.*, 2012).

In cancers, *BRAF^{V600E}* is seen in 100% of Hairy Cell Leukaemia (HCL) patients (Tiacci *et al.*, 2011), ~80% of melanomas (Rubinstein *et al.*, 2010; Lovly *et al.*, 2012) and ~8% of colorectal cancers (Richman *et al.*, 2009; Tol *et al.*, 2009). However *BRAF^{V600E}* can also occur at high frequency in benign tumours such as melanocytic naevi, where 82% of tumours carry the mutation (Pollock *et al.*, 2003).

1.5.6.2. *BRAF Mutations in Histiocytosis*

Oncogene profiling has identified the same activating point mutation; *BRAF^{V600E}*, in approximately 50-60% of the LCH and ECD lesions of adults and children (Badalian-Very *et al.*, 2010; Haroche *et al.*, 2012b; Sahm *et al.*, 2012; Satoh *et al.*, 2012; Berres *et al.*, 2014). As previously discussed (section 1.5.6.1), the *BRAF^{V600E}* mutation causes the BRAF gene to be in a constitutively active state, causing a hyperactivation of the MAPK pathway.

In LCH, mutated BRAF appears to be confined to neoplastic cells, with a high but not completely uniform concordance with expression of the characteristic markers of LCH cells, including CD1a and Langerin (Badalian-Very *et al.*, 2010; Sahm *et al.*, 2012; Satoh *et al.*, 2012; Berres *et al.*, 2014).

Interestingly, in LCH and ECD patients who have *BRAF^{V600E}* positivity, the mutation is seen in all lesions, and in *BRAF^{V600E}* positive crossover patients with both LCH and ECD, the mutation is seen in both types of disease lesion (Hervier *et al.*, 2014).

There is no overall survival difference in either LCH or ECD patients associated with the $BRAF^{V600E}$ mutation (Rolland *et al.*, 2005; Haroche *et al.*, 2012b). However, $BRAF^{V600E}$positive LCH patients have a higher rate of relapse (Berres *et al.*, 2014) and $BRAF^{V600E}$ positive ECD patients have higher risk of comorbidities such as diabetes Insipidus (Haroche *et al.*, 2012) compared to $BRAF^{WT}$ patients with the same diseases.

To date, there is no evidence of $BRAF^{V600E}$ in other histiocytic disorders such as RDD (total 32 cases analysed), JXG (total 19 cases analysed) (Badalian-Very *et al.*, 2010; Sahm *et al.*, 2012; Satoh *et al.*, 2012; Berres *et al.*, 2014) or HLH. The absence of $BRAF^{V600E}$ in RDD and JXG does not exclude a connection between these diseases and LCH/ECD, as some patients have been found with further mixed histiocytosis diseases.

As 100% of HCL patients carry the $BRAF^{V600E}$ mutation in their peripheral blood, samples from these patients will be used as controls throughout this thesis. A general comparison of LCH, ECD and HCL is given in Figure 1.14.

1.5.6.3. *Other Mutations*

The presence of MAPK pathway over-activation in 100% of LCH and ECD patients, but the lower presence of $BRAF^{V600E}$mutations, implies that there are multiple mechanisms of activation.

Two recent studies using whole exome/targeted genome sequencing have identified high frequencies of MAP2K1 mutations in LCH patients which are mutually exclusive to $BRAF^{V600E}$mutations (Brown *et al.*, 2014; Chakraborty *et al.*, 2014). In both studies, no significance was seen between BRAF or MAP2K1 mutations and clinical characteristics; however, in the study by Chakraborty *et al*, functional studies showed that the efficacy of various MAPK pathway inhibitors did depend on which gene was mutated.

Using targeted sequencing, other mutations have been seen in the BRAF gene of $BRAF^{V600E}$ wild-type LCH patients; including a somatic insertion $BRAF^{V600DLAT}$, a germ line $BRAF^{T599A}$(Satoh *et al.*, 2012)and $BRAF^{V600D}$(Kansal *et al.*, 2013), each in one patient.

The RAF family of proteins includes ARAF, BRAF and CRAF. BRAF has the highest basal level of kinase activity, 15-20 times that of the other RAFs, and therefore requires less mutational activation to become fully promoting (Govender and Chetty, 2012). ARAF has the lowest basal kinase activity, although two LCH patients have been described with compound mutations in ARAF, which are able to promote pathway activation at a much higher rate than wild-type ARAF (Chakraborty *et al.*, 2014; Nelson *et al.*, 2014). The final RAF protein is CRAF, which unlike the other RAF proteins, has not been implicated in any known cancers or histiocytic disorders.
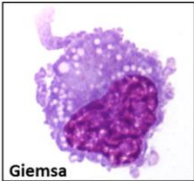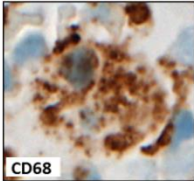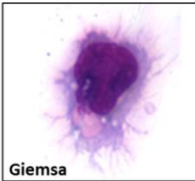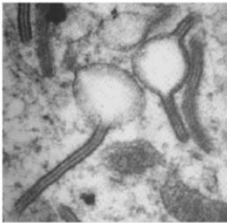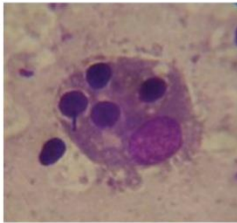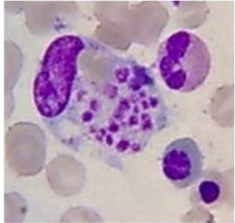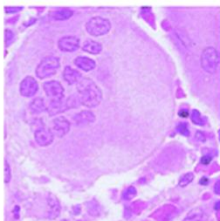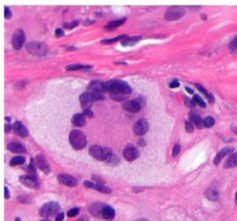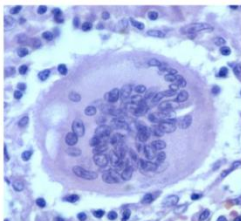
Finally, one single case of $BRAF^{WT}$ LCH has been seen with a mutation in the ERBB3 gene (Chakraborty *et al.*, 2014), which encodes a membrane bound epidermal growth factor receptor, which can activate the MAPK pathway (Citri *et al.*, 2003).

Due to the scarcity of samples, whole exome sequencing has not been performed on samples from ECD patients, though recent targeted sequencing studies have revealed that ECD patients can express mutations in the NRAS (3/25 patients) and PIK3CA (3/25 patients) genes (Emile *et al.*, 2014). In this study, the NRAS mutation was seen specifically in the CD14+ myeloid cell types by Sanger sequencing and not in the CD3+ T cells.

There are other possible mechanisms causing MAPK pathway hyper-activation, some of which have recently been explored. Although not explored in detail, no recurrent abnormalities in karyotype have been seen (Chu *et al.*, 2012), neither has BRAF locus duplication (Badalian-Very *et al.*, 2010). Other possible mechanisms could include overexpression of receptor tyrosine kinase on the disease cells, or

activation ligand overproduction by the disease cells or others (Badalian-Very *et al.*, 2012), indeed an unexplained overexpression of the protein osteopontin has been observed(Allen *et al.*, 2010).

A.

Disease	Langerhans Cell Histiocytosis	Erdheim Chester Disease	Hairy Cell Leukaemia
$BRAF^{V600E}$	57% $BRAF^{V600E}$ Positive (Badalian-Very et al, 2010)	54% $BRAF^{V600E}$ Positive (Haroche et al, 2012)	100% $BRAF^{V600E}$ Positive (Tiacci et al, 2011)
Cell Image	Giemsa	CD68	Giemsa
Cell Type	Langerhans Cell-Like	Foamy Macrophages	B Cell-Like
Markers	Langerin+CD1a+ S100+ Birbeck Granules	CD68+CD1a- Langerin-S100- No Birbeck Granules	CD103+CD11c+
Target Organs	Bone, Skin, BM	Long Bones, Skin, Cardiovascular	BM, Blood, Spleen

B.

i. ii. iii.

iv. v. vi.

Figure 1.14. General Comparison of LCH, ECD and HCL

A. The main differences seen between LCH, ECD and HCL

   LCH = Langerhans Cell Histiocytosis

   ECD = Erdheim Chester Disease

   HCL = Hairy Cell Leukaemia

HCL is used as a control in this thesis due to 100% of the patients being positive for the $BRAF^{V600E}$ mutation in the peripheral blood. LCH and HCL cells stained with May-Grünwald-Giemsa (pH 6.8). ECD cell stained with CD68 antibody.

B. i) Birbeck granules in LCH (Birbeck et al., 1961); ii) Emperipolesis in RDD(Vujhini et al., 2012); iii) Haemophagocytosis in HLH (Koul, 2010); iv) Osteoclast giant cell in LCH (Pernick, 2014); v) Touton giant cell in ECD (Nephron, 2011); vi) Foreign body giant cell in ECD (Shankar, 2012).

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1.6. Histiocytosis as Inflammatory or Malignant Disorders

There has been a longstanding question of whether LCH and ECD are reactive inflammatory disorders or malignancies, as they do not quite fit into either category. The WHO Classification of tumours of hematologic and lymphoid tissue, was last updated (4th edition) in 2008 (Swerdlow *et al.*, 2008). In this edition, LCH, ECD and JXG are listed as haematological malignancies. However, as of November 2014, patient information from organisations such as the Histiocytosis Association of America and the Histiocytosis Research Trust (UK) are careful not to explicitly label these diseases as leukaemic, in fact the Cancer Research UK - LCH-III clinical trial website explicitly states that "LCH is not cancer," just that "it can behave in a similar way to cancer and is usually treated by cancer specialists" (Windebank, 2012).

LCH and ECD patients have vastly varying clinical characteristics, ranging from spontaneously regressing disease, to systemic organ failure and death. Not all LCH/ECD patients respond well to regular treatments. These patients, particularly those with multi-system disease, are treated with chemotherapeutic regimens, but still have a poor prognosis with high rates of relapse and reactivation (Gadner *et al.*, 2008). Therefore, there is still a lot of controversy as to whether these diseases are inflammatory or malignant.

1.6.1. *Histiocytosis as an Aberrant Immune Response*

There is speculation that the disorders could be caused by a dysfunctional immune response to infection. This is due to the manifestations of disease in most patients, which are not characteristic of malignancies. Patients can often respond to biopsy alone or minimal treatment, or spontaneously resolve without any treatment at all. Also, it has been seen that the cellular makeup of the lesions only carries a small proportion of LCH cells (median 8%), the remainder comprising of other inflammatory cells (Berres *et al.*, 2014).

There is some potential evidence that viral infections such as human herpesvirus (HHV) and Epstein–Barr virus (EBV) may be involved in an aberrant immune response in RDD and HLH (Luppi *et al.*, 1998; Mehraein *et al.*, 2006; Bohne *et al.*, 2013). In msLCH, there are some indications of the involvement of Merkel cell polyomavirus in pathogenesis (Murakami *et al.*, 2014; Murakami *et al.*, 2015b), although these cohorts were small and the results have yet to be confirmed.

1.6.2. *Histiocytosis as a Malignancy*

In their seminal reviews, Douglas Hanahan and Robert Weinberg laid down 8 central hallmarks of cancer (Hanahan and Weinberg, 2000). Each of these hallmarks (as well as aspects of DNA instability, cell clonality and oncogenic driver mutations) in the context of histiocytosis are given in the following sections.

1.6.2.1. *Self-Sufficiency in Growth Signals*

Cancer cells are able to multiply without the requirement of external signals such as growth factors. Generally, LCH cells do not survive long in *in vitro* culture, only two LCH cell lines have been purported to be established; DOR-1 and PRU-1 (Gogusev *et al.*, 2005; Murakami *et al.*, 2015a), though neither of these cell lines showed the characteristic LCH cell markers: CD1a, Langerin or Birbeck granules. This suggests that LCH cells do require some external stimulus for growth.

1.6.2.2. *Insensitivity to Anti-Growth Signals*

Cancer cells are characteristically resistant to anti-proliferative signals from their neighbouring cells. In melanoma, it has been established that exogenous SPRY2, a protein which is a negative feedback regulator of the MEK/ERK pathway, is capable of inhibiting the pathway in $BRAF^{WT}$ melanocytes and melanoma cells, but not in cells carrying the $BRAF^{V600E}$ mutation (Tsavachidou *et al.*, 2004).

1.6.2.3. *Evading Apoptosis*

The ability to avoid apoptosis is a major factor in the survival of cancer cells. In immunohistochemistry studies, LCH lesions have been seen to have very few TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labelling), reactive cells (Petersen *et al.*, 2003), and exhibit only low levels of nuclear apoptotic changes (Marchal *et al.*, 2004), indicating levels of apoptosis are low. Furthermore, in LCH cells, levels of the anti-apoptotic factors Bcl-2 are high and pro-apoptotic factor caspase-3 are low (Marchal *et al.*, 2004; Amir and Weintraub, 2008). Interestingly, these differences were not observed in LCs in nearby non-lesional material, and expression differences were more apparent in patients with high risk disease. These results have been validated recently in gene expression studies showing that anti-apoptotic genes such as BCL2L1 and CFLAR are overexpressed in LCH cells compared to normal LCs (Allen *et al.*, 2010). Finally, the study by Petersen *et al*, 2003, showed that a small number of LCH cells co-expressed Fas and Fas-L, and suggested that this could lead to an 'autocrine apoptotic shortcut' possibly leading to spontaneous regression of LCH in some patients.

1.6.2.4. *Limitless Replicative Potential*

The Hayflick limit is the number of replications a cell can have before senescence, where no further cell division occurs (Hayflick and Moorhead, 1961), controlled by telomere shortening during replication. Most cancer cells are able to escape this limit and therefore have limitless replicative potential. In LCH, the aberrant cells appear to have very low rates of mitosis (Berres *et al.*, 2014), though similar low rates of mutation are seen in hairy cell leukaemia, a cancer where 100% of patients carry the *BRAF^{V600E}* mutation (Tiacci *et al.*, 2006; Tiacci *et al.*, 2011).

One result of the activated MAPK pathway in LCH is an upregulation of telomerase activity (Goueli and Janknecht, 2004; Dwyer *et al.*, 2007), thereby preventing telomere shortening, and thus

likely increasing the replicative potential of the cells carrying this mutation. However, LCH cells have been seen to have significant telomere shortening (da Costa *et al.*, 2007; Bechan *et al.*, 2008), which is characteristic of several cancers (Arceci, 2014) indicating that LCH cells must employ a mechanism to avoid shortened-telomere associated senescence and apoptosis.

Alternatively, *BRAF^{V600E}* has been suggested to promote cell senescence in benign naevi, and that a transformation to malignant melanoma occurs when a second growth and survival pathway (e.g. PI3K-Akt pathway) is over-activated by a decrease in tumour suppressors proteins (e.g. phosphatase and tensin homolog, PTEN) (Michaloglou *et al.*, 2005).

1.6.2.5. *Sustained Angiogenesis*

In most solid cancers, the cancer cells are able to promote angiogenesis, ensuring the supply of oxygen and nutrients to the tumour. Levels of the pro-angiogenic factor; vascular endothelial growth factor (VEGF) are increased in the serum of paediatric msLCH patients (Pavlakovic *et al.*, 2001). Furthermore, immunohistochemistry studies have shown that VEGF expression is present in msLCH lesions (5/5 lesions), some SS-LCH lesions (2/5 lesions) and more specifically on LCH cells, and that blood vessel density is significantly increased within lesions compared to normal margins (Dina *et al.*, 2005). In mouse models, it has been shown that BRAF deficient tumour cells are able to proliferate but are unable to promote angiogenesis at a high enough level for tumour expansion, leading to hollow tumour masses (Sobczak *et al.*, 2008). This indicates that an over-activation of the BRAF gene could very well lead to an increase in angiogenesis.

1.6.2.6. *Tissue Invasion and Metastasis*

Although some tumours can be benign, a characteristic of cancerous cells is their ability to metastasise; to invade adjacent tissue

or migrate to distant tissues and cause new lesions. Metalloproteases play a role in tumour migration through the rearrangement of the extracellular matrix. Some metalloproteases have been shown to have increased expression in pulmonary LCH (Hayashi *et al.*, 1997), and an increased expression of metalloprotease and adhesion factors, coinciding with a reduced expression of metalloprotease inhibitors has been seen in paediatric LCH (Allen *et al.*, 2010).

Heparanase, an enzyme required for cell penetration through the endothelial cell layer, is expressed in LCH (21/25 patients), although there was no association with disease activity (Dvir *et al.*, 2014). A review by Maurer *et al*, 2011; highlights several other mechanisms by which the *BRAF^V600E* mutation may potentially promote metastasis, such as inducing the production of TGFb and interleukin-8 and increasing the contractility of cells, all of which increase the ability of cells to migrate into the vasculature.

Interestingly, in LCH and ECD patients who have *BRAF^V600E* positivity, the mutation is seen in all lesions, and in *BRAF^V600E* positive crossover patients with both LCH and ECD, the mutation is seen in both types of disease lesion (Hervier *et al.*, 2014). Although this does not prove that co-existing lesions were initiated from metastasising cells from one original lesion, it seems unlikely that the multiple sites will develop the same mutation at the same time.

1.6.2.7. *Abnormal Metabolic Pathways*

The Warburg hypothesis of cancer growth is that due to a decreased respiratory function of mitochondria, cancer cells do not generate energy from the normal oxidative breakdown of pyruvate during glycolysis, and therefore must create energy by abnormal non-oxidative breakdown of glucose. Diabetes insipidus is frequently observed in LCH (Grois *et al.*, 2006), at around 6% of patients at diagnosis and carrying a 20% risk of developing later in the course of the disease. Anterior pituitary dysfunction is also commonly seen in

adult LCH patients (Kaltsas *et al.*, 2000). This indicates that abnormal carbohydrate and lipid metabolism pathways may be involved in LCH.

1.6.2.8. *Evading the Immune System*

A key aspect of cancer cells is their ability to evade the immune system, such as by the downregulation of differentiation antigens, resistance to cytotoxic T cell mediated death or by repressing the response of normal immune cells.

LCH patients have increased numbers of T regulatory cells (Tregs) in their peripheral blood, and a high proportion of the T cells in LCH lesions are Tregs (Senechal *et al.*, 2007). This high degree of Tregs in lesions is anticipated on the grounds that LCH cells express RANK, a potent stimulator of Treg proliferation (Senechal *et al.*, 2007). Tregs are able to suppress the action of T cells, likely preventing an immune response against the aberrant LCH cells. Tregs of paediatric LCH patients do in fact inhibit the immune system, as these patients have an impaired delayed-type hypersensitivity response (Senechal *et al.*, 2007).

In experiments with the $BRAF^{V600E}$+ melanoma cell line A375, inhibition of $BRAF^{V600E}$ causes a significant reduction in levels of the immunosuppressive cytokines IL-6, IL-10 and VEGF (Sumimoto *et al.*, 2006). Increased levels of IL-10 are seen in LCH lesions (Senechal *et al.*, 2007), indicating a cancer cell induced immune evasion mechanism.

1.6.2.9. *Unstable DNA*

Due to the ability of cancer cells to escape senescence, the cells can proliferate more times than they are normally able. The normal limit of cell proliferation prevents cells with damaged chromosomes from proliferating, whereas immortal cancer cells generally pick up more chromosome damage over time.

Early studies showed cytogenetic abnormalities in small (5-7 patient) LCH cohorts (Betts *et al.*, 1998; Murakami *et al.*, 2002), whereas a more recent and extensive study, using an array of different cytogenetics analysis techniques revealed no gross chromosomal abnormalities (e.g. aberrations in DNA ploidy, karyotype, copy number variations or single nucleotide polymorphisms) (da Costa *et al.*, 2009). Also, LCH and ECD patient lesions have extremely low rates of somatic mutations (median 1 mutation per sample) (Nelson *et al.*, 2014), which contrasts with high amounts of somatic mutations seen in most leukaemias, including HCL (Tiacci *et al.*, 2011).

1.6.2.10. *Clonality*

Although tumour cells can be heterogeneous, an original monoclonal or polyclonal population is an essential feature of cancer cells. From X chromosome inactivation studies in female patients, LCH and ECD are now known to be 'clonal' proliferations of cells, (Willman *et al.*, 1994; Yu *et al.*, 1994; Vencio *et al.*, 2007). These data are supported by the high enrichment of *BRAF^V600E*/*MAP2K1* mutations in sorted lesional LCH cells (Chakraborty *et al.*, 2014). However not all clonal populations are cancer, such as those seen in benign naevi.

1.6.2.11. *BRAF^V600E as an Oncogenic Driver Mutation*

A driver mutation is one which gives a selective advantage to a clone cell in terms of survival and/or proliferation in its microenvironment and thus leads to the initiation and progression of malignancies. The high rate of *BRAF^V600E* mutation in LCH and ECD (Badalian-Very *et al.*, 2010; Haroche *et al.*, 2012b), the fact that in the majority of cases, no other somatic mutations are present (Nelson *et al.*, 2014), and its presentation as a single allele, suggests that this is a dominant oncogenic driver of LCH and ECD.

In mouse models, expression of *BRAF^V600E* during embryonic development is lethal, whereas expression in somatic tissues leads to a histiocytic neoplasia (Mercer *et al.*, 2005) or adenomas (Dankort *et*

al., 2007). In a paper by Berres *et al* in 2014, the authors developed two more specific mouse models, expressing *BRAF^{V600E}* in DCs at differing states of maturation, to determine for certain whether *BRAF^{V600E}* is a driver mutation. Both models induced an LCH-like disease, indicating for certain that *BRAF^{V600E}* is a driver mutation in mice. Also it was seen that the *BRAF^{V600E}* mutation was consistent in coexistent tumours and serial relapse samples from human patients, indicating that *BRAF^{V600E}* is also a key early event in human disease. Interestingly, when *BRAF^{V600E}* was expressed under the CD11c promoter (less mature DCs), the mice developed a rapid and aggressive msLCH-like disease, whereas mice with *BRAF^{V600E}* expressed under the langerin promoter (more mature DCs) exhibited less severe disease.

1.6.3. *Histiocytosis as an Inflammatory Myeloid Neoplasm*

LCH has aspects of most of the tenets of cancer listed above, albeit the division of cells is much lower than other cancers, and the cells are difficult to grow in culture. Interestingly though, hairy cell leukaemia (HCL), which has BRAF mutations in 100% of cases (Tiacci *et al.*, 2011), also has a very low rate of proliferation (Tiacci *et al.*, 2006). The main arguments for a dysregulated immunological origin are that LCH has an obvious inflammatory and granulomatous pathology and in some cases is able to spontaneously regress. However the presence of a defined driver mutation which is known to be able to induce multiple oncogenic factors, would lead to the conclusion that LCH is a cancer. These separate conclusions may be united by the fact that LCH cells are able to promote an explicit inflammatory response, which has led some researchers to deduce that both a malignant and an inflammatory component may be essential for LCH pathogenesis, and therefore have determined to term the disease an 'Inflammatory Myeloid Neoplasm' (Berres *et al.*, 2014).

This is not yet a universally accepted notion and due to the highly variable manifestations of LCH, I believe that it is still possible that the disease could exist in malignant, inflammatory and combined forms. In

fact, the case may be that an original oncogenic driver mutation needs to be coupled with a secondary event, before pathogenesis occurs; such as a second hit mutation, or an inflammation promoting infection.

1.6.4. *The Origin of LCH Cells*

The cell of origin of LCH is unknown, though the history of LCH research points to the Langerhans cell. In the 1940s and 50s, the three disease entities eosinophilic granuloma, Hand-Schüller-Christian disease and Letterer-Siwe disease (see section 1.5.3, page 41) were united under the title Histiocytosis X, due to their concordant histiocytic cell morphology (Farber, 1941; Mallory, 1942; Lichtenstein, 1953). Later it was discovered that both Langerhans cells and the disease cells in Histiocytosis X contained Birbeck granules (Birbeck *et al.*, 1961; Basset and Turiaf, 1965), leading to the re-classification of the disease as Langerhans Cell Histiocytosis (Nezelof *et al.*, 1973). This definition is further enforced by the discovery of the markers Langerin and CD1a on both LC and LCH cells (Harrist *et al.*, 1983; Rousseau-Merck *et al.*, 1983; Valladeau *et al.*, 2000) see Figure 1.15.
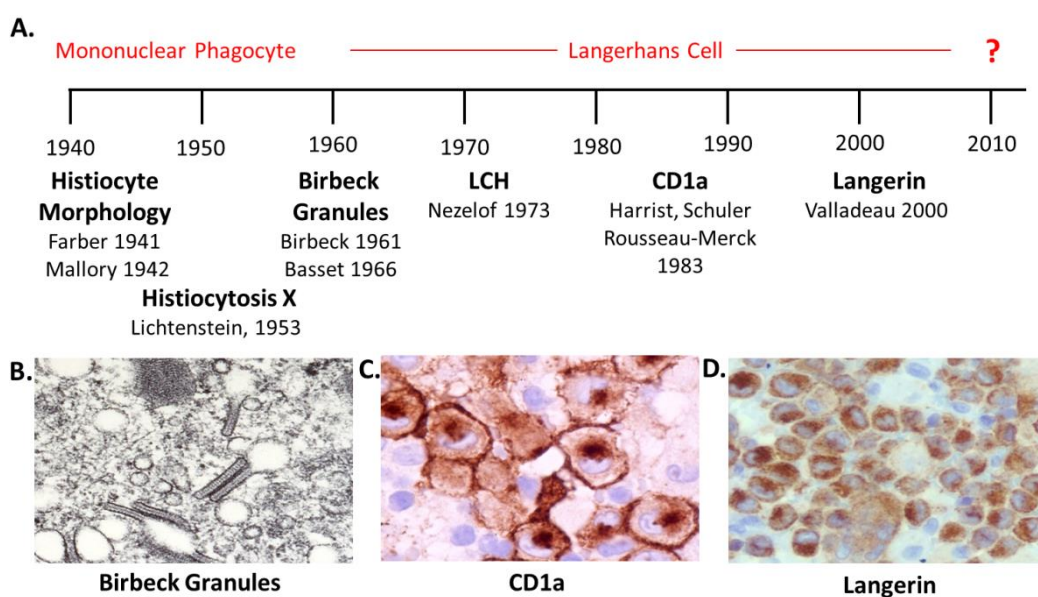
Figure 1.15. Brief History of Discoveries in LCH

- A. *Timeline of major discoveries. Red text indicates potential cells of origin.*
- B. *Electron microscopy of Birbeck granules in LCH cells.*
- C. *CD1a immunohistochemistry staining of CD1a on LCH cells.*
- D. *Langerin immunohistochemistry staining of CD1a on LCH cells.*

Photos B-D courtesy of Marian Malone (Malone, 2014)

Recent studies in dendritic cell biology and preliminary cell-specific gene expression studies cast doubt on the paradigm of the epidermal Langerhans cell as the cell of origin in LCH. These observations include:

i)   Immunohistochemistry and gene expression studies show substantial differences between LCH cells and native LCs. The LCH cells appear more like BM derived immature myeloid DCs than the LCs (Geissmann *et al.*, 2001; Pinkus *et al.*, 2002; Annels *et al.*, 2003; Fleming *et al.*, 2003; Rust *et al.*, 2006; Allen *et al.*, 2010; Hutter *et al.*, 2012).

ii)   Recent murine data have shown the existence of LC-like Langerin+ cells within most non-lymphoid tissues (Ginhoux *et al.*, 2007; Poulin *et al.*, 2007), and our own recent work has demonstrated a langerin+ CD1a+ DC subset in the skin which is phenotypically and functionally distinct from LCs(Bigley *et al.*, 2014).

iii)   LCH can occur in organs usually devoid of LCs such as bone, viscera, brain and lungs.

These observations are more consistent with a model that LCH cells arise by differentiation of a precursor that acquires some but not all the characteristics of epidermal LCs and has the capacity to migrate to multiple sites that do not normally contain LCs. It is therefore possible that, similarly to normal tissue DCs, LCH cells derive from circulating precursors rather than tissue-resident LCs.

It is known that human CD34+ progenitor cells can differentiate into cells expressing langerin and CD1a, very similar to LCs (Caux *et al.*, 1992; Strobl *et al.*, 1997; Ratzinger *et al.*, 2004; Klechevsky *et al.*, 2008). Monocytes can also acquire langerin and CD1a (Geissmann *et al.*, 2001; Hutter *et al.*, 2012). Circulating myeloid DCs are increased in LCH (Rolland *et al.*, 2005) but have not been shown to express markers typical of LCH cells. In addition, several studies have consistently found that blood myeloid DCs may express low levels of Langerin in healthy

individuals (MacDonald *et al.*, 2002; Haniffa *et al.*, 2012; Harman *et al.*, 2013).

These *in vitro* observations suggest that CD34+ progenitors and monocytes might have the capacity to form LCH cells(Weitzman and Jaffe, 2005). A review of studies to date, looking at the LC-like differentiation potential of human CD34+ progenitors and monocytes, is given in Table 1.5, page 64.

The origin of the aberrant cells in LCH and ECD remains obscure but the presence of mutated BRAF is a possible means of identifying putative precursor cells in the peripheral blood or bone marrow. Previous analysis of blood monocytes and bone marrow did not detect mutated BRAF by next generation sequencing or immunohistochemistry, respectively (Sahm *et al.*, 2012; Satoh *et al.*, 2012). A recent study, using allele-specific PCR, has reported *BRAF^{V600E}* in the CD14+ monocytes and CD14-CD11c+ fraction of peripheral blood and CD34+ bone marrow populations, of children with MS LCH (Berres *et al.*, 2014). The CD14-CD11c+ fraction of cells in human peripheral blood includes CD16+ monocytes, CD1c+ mDCs, CD141+ mDCs and a residual CD11c+16-1c-141- population, leaving the exact identity of the cells containing the *BRAF^{V600E}* mutation in this fraction open to further investigation. A clonal disorder of myelopoiesis was also suggested by the detection of *BRAF^{V600E}* in CD34+ bone marrow cells and their *in vitro* differentiated progeny.

Table 1.5. LC-Like Cell Induction Studies

Reference	Inducers	Non-Inducers	Cells	LC Markers	Selection	Method	Media
Caux et al, 1992	GM-CSF with TNF-α		CB CD34+ Progenitors	CD1a, BGs	Positive	Minimacs columns, CD34 mAb	RPMI + 10% FCS
Strobl et al, 1996	TGFβ (With TNF-α, GM-CSF and SCF)		CB CD34+ Progenitors	CD1a	Positive	MACS CD34 Progenitor Cell Isolation Kit	X-VIVO 15 + 10% Human Plasma
Caux et al, 1996	GM-CSF & TNF-α (with SCF)		CB CD34+ Progenitors	CD1a, Lag, E-Cad, BGs	Positive	Minimacs columns, CD34 mAb	RPMI + 10% FCS
Strunk et al, 1996	GM-CSF & TNF-α (+/- IL-4)		PB CD34+ Progenitors	CD1a, BGs	Positive	CD34 mAb coated beads	IMDM + 10% FCS
Strobl et al, 1997	FLT3L & TGFβ (With FLT3L, TGF-B, TNF-a, GM-CSF and SCF)		CB CD34+ Progenitors	CD1a, Lag	Positive	MACS CD34 Progenitor Cell Isolation Kit	X-VIVO 15, Serum Free
Geissmann et al, 1998	TGFβ (with GM-CSF & IL-4)		PB Monocytes	CD1a, E-Cad, BGs, Lag, CLA	Negative	MACS monocyte isolation kit I	Complete Media
Caux et al, 1999	TNF-α (with SCF & GM-CSF)		CB CD34+ Progenitors	CD1a, E-Cad, Lag, BGs	Positive	Minimacs columns, CD34 mAb	RPMI + 10% FCS
Ito et al, 1999	GM-CSF with IL-4 and TGFβ		PB CD11c+1c+ DCs (mistakenly thought to be CD11c+1a+)	Langerin, E-Cad, Lag, BGs	Positive	Flow Cytometry Sorted Cells DR+3-7-14-16-19-CD11c+1c+	50% Monocyte-Conditioned Medium
Mohamadzadeh et al, 2001	IL-15 (with GM-CSF)	IL-4 with GM-CSF	PB Monocytes	E-Cad, Langerin, CCR6, no BGs	Negative	CD1a-3-19-56-GlyA- Dynabead Depletion	RPMI + 10% FCS
Guironnet et al, 2002	GM-CSF with TGFβ	GM-CSF with IL-4 +/- TGFβ	PB Monocytes	Langerin, E-Cad, BGs	Negative	MACS monocyte isolation kit I	RPMI + 10% FCS
Mollah et al, 2002	M-CSF and TGFβ without GM-CSF (with FLT3L, SCF and TNF-a)		CB CD34+ Progenitors	CD1a, BGs, Langerin, E-Cad, Factor XIIIa	Positive	MACS CD34 Progenitor Cell Isolation Kit	X-VIVO 15, Serum Free
Chomarat et al, 2003	TNF-a (with GM-CSF and IL-4 (+/- IL-1a, IL-1b or TNF-α)	IL-1	PB Monocytes	CD1a, DC-Sign, Langerin	Negative	mAbs and Dynabeads	RPMI + 10% FCS
Arrighi et al, 2003	TNF-α, IL-1B, LPS (pre-culture with GM-CSF and IL-4 (+/- TGF-B).	CD40L	CB CD34+ Progenitors	Langerin, Lag, E-Cad	Positive	CD34 mAb coated dynabeads.	IMDM + 10% FCS
Mollah et al, 2003	IL-3 & TGFβ without GM-CSF		CB CD34+ Progenitors	CD1a, Langerin, E-Cad no BGs	Positive	CD34 Progenitor Cell Isolation Kit	X-VIVO 15, Serum Free
Barbaroux et al, 2006	GM-CSF, TGF-B (with TNF-α, SCF, pre-culture with M-CSF)	TNF-a, IL-4	CB CD34+ Progenitors	Langerin, CD1a, CCR6, BGs	Positive	MACS CD34 Progenitor Cell Isolation Kit	RPMI + 10% FCS
Hoshino et al, 2006 Shibasaki et al, 2007	Notch Ligand Delta-1 with TGFβ & GM-CSF	Notch Ligand Delta-1 with TGF-B & IL-3	PB CD14+ Monocytes	CD1a, Langerin, CLA, CCR6, E-Cad, BGs	Positive	CD14 mAb coated beads.	RPMI + 10% FCS
Musso et al, 2008	Activin A (TGFβ family member) (With GM-CSF and IL-4, without TGFβ)		PB CD14+ Monocytes	Langerin, BGs, E-Cad, CLA, CCR6	Positive	CD14 mAb coated beads.	RPMI + 10% FCS
Eljaafari et al, 2009	INF-γ (GM-CSF and IL-4.)		PB CD14+ Monocytes	Langerin (but also CD123)	Positive	MACS CD14 positive selection kit	RPMI + 10% AB Serum
Van de Ven et al, 2012	ABCG2 (BCRP) (with TGFβ, GM-CSF, TNF-α, FLT3L & SCF)		MUTZ3 AML Cell Line CD34+	Langerin, CD1a	Positive	Cell Line	MEM + 20% FCS
Yasmin et al, 2013	B-Catenin, +/- Vitamin D (With FLT3L, TGFβ, TNF-α, GM-CSF and SCF)		CB CD34+ Progenitors	CD1a, Langerin, E-Cad	Positive	Direct CD34 Progenitor Cell Isolation Kit	X-VIVO 15, Serum Free
Yasmin et al, 2013	BMP7, BMP4 (With FLT3L, TGFβ, TNF-α, GM-CSF and SCF)	BMP2, BMP6	CB CD34+ Progenitors	CD1a, Langerin, E-Cad	Positive	Direct CD34 Progenitor Cell Isolation Kit	X-VIVO 15, Serum Free

Acronyms given on following page.

ABCG2 = ATP-binding cassette sub-family G member;

AB Serum = Human Serum from male AB+ Plasma;

BG = Birbeck Granule;

BMP = Bone Morphogenetic Protein;

CB = Cord Blood;

CCR6 = Chemokine Receptor Type 6;

CD40L = CD40 Ligand;

CLA = Cutaneous Lymphocyte Antigen;

DC-SIGN = Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin;

E-Cad = E-Cadherin (CD324);

FCS = Foetal Calf Serum;

FLT3L = Fms-like Tyrosine kinase 3 Ligand;

GM-CSF = Granulocyte/Macrophage-Colony Stimulating Factor;

IMDM = Iscove's Modified Dulbecco's Medium;

IFN-γ = Interferon Gamma;

IL = Interleukin;

Lag = Langerhans-associated granule (Kashihara et al., 1986);

LPS = Lipopolysaccharide;

mAb = Monoclonal Antibody;

MACS = Magnetic-Activated Cell Separation;

M-CSF = Macrophage Colony Stimulating Factor;

MEM = Minimal Essential Media;

PB = Peripheral Blood;

RPMI = Roswell Park Memorial Institute medium;

SCF = Stem Cell Factor;

TNF-α = Tumour Necrosis Factor Alpha;

TGFβ = Transforming Growth Factor Beta;

X-VIVO = A Chemically Defined, Serum-free Hematopoietic Cell Medium.

1.6.5. *The Origin of ECD Cells*

The question of the origin of disease cells in ECD is harder to approach. The only known marker for the disease is an aberrant expression of CD68 in the target organs. The functions of CD68 are not entirely clear, though it is thought to have a role in the clearance of debris from cells, which would bear an association with the high degree of phagocytosis seen in ECD. Unfortunately, CD68 is a fairly ubiquitous marker, seen in virtually all peripheral blood cell subsets (Kunz-Schughart *et al.*, 2003; Kunisch *et al.*, 2004), so it would be impossible to determine the relationship between ECD cells and circulating cells using this marker alone. Therefore this thesis will concentrate on using the *BRAF*^{V600E} mutation to attempt to track the precursors of LCH and ECD, and the markers Langerin and CD1a to find which circulating cells have the potential to become LCH-cell-like.

Chapter 2. General Materials and Methods

This chapter covers the general methodology used throughout this thesis.

2.1. Sample Collection

Informed consent was sought for all human samples used in this project in accordance with a favourable ethical opinion from Newcastle & North Tyneside 1 Research Ethics Committee (Appendix 8).

2.2. Media and Buffers

2.2.1. *Flow Buffer*

Flow buffer was made up of Dulbecco's Phosphate Buffered Saline (D-PBS) + 2% Heat Inactivated Foetal Calf Serum (HI-FCS) (both from PAA; www.paa.com) + 0.4% EDTA (Sigma-Aldrich; www.sigmaaldrich.com).

2.2.2. *Sort Buffer*

Sort buffer was made up of Dulbecco's Phosphate Buffered Saline (D-PBS) + 0.5% Heat Inactivated Foetal Calf Serum (HI-FCS) (both from PAA; www.paa.com) + 0.4% EDTA (Sigma-Aldrich; www.sigmaaldrich.com).

2.2.3. *RF-10*

RF-10 was made up of RPMI 1640 Media + 10% Heat Inactivated Foetal Calf Serum (HI-FCS) + 1% Penicillin-Streptomycin + 2% L-Glutamine (all from PAA; www.paa.com).

2.2.4. *Freezing Solution*

Freezing Solution was made up of 90% Heat Inactivated Foetal Calf Serum (HI-FCS) (PAA; www.paa.com) + 10% DMSO (NBS Biologicals; www.nbsbio.co.uk).

2.2.5. *Red Blood Cell (RBC) Lysis Buffer*

A 10x concentration stock of RBC lysis buffer was made up of 41.4g NH4Cl (Sigma-Aldrich; www.sigmaaldrich.com), 5.0g KHCO3 and 0.18g EDTA (both from VWR; www.uk.vwr.com) dissolved in 400ml sterile water. This stock was pH adjusted to 7.35 by addition of 1M NaOH (Fisher Scientific; www.fisher.co.uk) and topped up to 500ml with sterile water. The buffer was diluted to 1x concentration with sterile water before use.

2.3. General Cell Processing

2.3.1. *Isolation of Peripheral Blood and Bone Marrow Aspirate Cells*

Mononuclear cells were extracted from fresh peripheral blood (PB) and bone marrow (BM) aspirates following standard protocols. Briefly, samples of PB or BM were diluted1:2 or 1:4 respectively in Dulbecco's Phosphate-Buffered Saline (D-PBS) and then layered on top of a volume of Lymphoprep^{TM} density-gradient media (Axis-Shield Diagnostics Ltd; www.axis-shield.com) equivalent to half the total volume of diluted sample. The mononuclear cells were then separated from the rest of the sample by room temperature density centrifugation (800g, 15 minutes). The interface between the lymphoprep and the supernatant was aspirated (See Figure 3.1) with a Pasteur pipette and washed twice in D-PBS (500g, 5 minutes followed by 200g for 7 minutes to remove platelets). 1-2mls of RBC lysis buffer was added to the cell pellet and allowed to incubate in the dark for 5 minutes before a final wash in D-PBS (500g, 5 minutes).
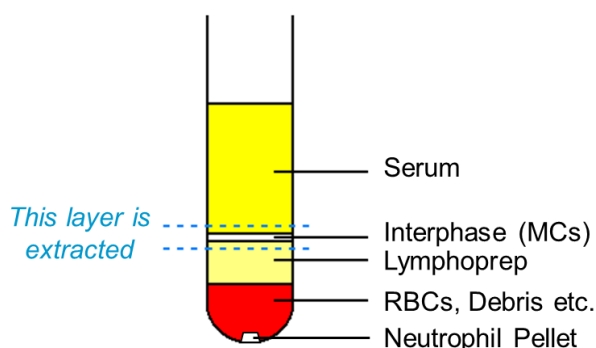
Figure 2.1. Diagram of Lymphoprep Separated Sample

MC = Mononuclear Cells; RBC = Red Blood Cells

2.3.2. *Isolation of Neutrophils*

Neutrophils are isolated from the pellet formed after PB or BM samples are lymphoprepped (Figure 2.1). Briefly; after the mononuclear cells were aspirated from the samples, the remaining lymphoprep was discarded leaving RBCs and a cellular pellet which is 66-88% composed of neutrophils (see section 4.3; pg. 122). RBC lysis buffer was added equivalent to the same volume as the RBCs. The sample was vortexed and left at room temperature in the dark for 15 minutes before being topped up with D-PBS and centrifuged (500g, 5 minutes). Supernatant was discarded, leaving a cellular pellet which could be counted. If RBCs were still present, the RBC lysis, incubation and washing steps were repeated.

2.3.3. *Isolation of Cells from Tissue*

LCH lesions and tissues were digested to single cell suspensions as previously described (Haniffa *et al.*, 2012).Briefly; the tissue was placed into a 24 well plate with 1ml RF-10 media and cut down to ~5mm2 sections. 1/100 collagenase (1.6mg/ml) was added and the plate was incubated at 37°C, 5% CO_2 for eight hours. Digested cells were then diluted with room temperature PBS and centrifuged at 500g for 5 minutes.

2.3.4. *Isolation of Cells from Hip Femoral Head Bone Marrow*

Control BM was collected either from normal donor material excess to clinical BM transplantation or from hip femoral heads after hip replacement surgery. From femoral heads, BM was carefully extracted from the surgical opening using bone clippers while wearing chain mail safety gloves. This semi-solid BM was placed into a 100µl nylon cell strainer in a 50ml falcon tube and washed with PBS until white. The washed cell solution was then layered onto lymphoprep as previously described in section 2.3.1.

2.3.5. *Cell Counting*

Cells were counted at 1:1 with trypan blue exclusion dye (Invitrogen; www.invitrogen.com) on an Improved Neubauer Haemocytometer (Weber Scientific International; www.weberscientific.com).

2.3.6. *Cryopreservation and Thawing of Cells*

Cells were frozen at a maximum concentration of 0.5×10^7 Cells/ml of freezing solution in Nunc cryovials (Sigma-Aldrich; http://www.sigmaaldrich.com/). Vials were wrapped in bubble-wrap and frozen in a -80°C freezer before being transferred to a -140°C freezer within 24 hours.

If defrosting of samples was required, vials were removed from the freezer and defrosted in a 37°C water bath before quickly transferring to a 15ml tube of warm RF-10 in a dropwise manner. This tube was then centrifuged for 5 minutes at 500g and the cells were resupended in 1ml of the appropriate media (e.g. sort buffer/RF-10) before counting (see 2.3.5) to ascertain post-thawing count and viability.

2.3.7. *Cytospin Slides and May-Grünwald-Giemsa Staining*

Cytospin slides were prepared from sorted cells in 200µl RF-10 using a Shandon Cytospin 4, with Shandon filter cards and Shandon Cytospin coated slides (Thermo Scientific; www.thermoscientific.com) spinning at 600rpm for 10 minutes. Slides were left to dry for 30 minutes and then fixed in 100% methanol for 2 minutes. Next, the slides were stained in May-Grünwald stain (pH 6.8) for 2 minutes, rinsed with water and stained in Giemsa stain (pH 6.8) for 2 minutes. Photographs were taken on a Zeiss Axioimager Z2 microscope with AxioVision 4.8 software at 100 times magnification. Scales as indicated.

2.4. General Flow Cytometry

2.4.1. *Mononuclear Cell Flow Cytometry Phenotyping*

According to standard protocols for flow cytometric analysis, cells were diluted/concentrated to $1x10^6$(BM & DC panels) or $0.2x10^6$(Lymph panel) in 50µl flow buffer. 3µl anti-mouse IgG (Sigma-Aldrich; www.sigmaaldrich.com) was added to each sample tube and allowed to incubate for 10 minutes to help prevent non-specific binding. The relevant antibodies were then added to the sample tubes (See Table 3.1) and allowed to incubate in the dark at 4°C for 30 minutes. Cells were then washed in flow buffer (500g, 5 minutes) and finally re-suspended in 250µl flow buffer. LSRII and Canto cytometers (Becton Dickinson; www.bd.com/uk) were used to perform the flow cytometry and the data was analysed on FlowJo software version 7.6.5 (Treestar; www.treestar.com).

2.4.2. *Flow Cytometry Cell Sorting*

Fluorescence activated cell sorting was performed using a BD FACS Aria (custom 100 mW 488 sapphire, 60mW 355 UV, 40 mW 640 red, 50 mW 407 violet, 70 µm nozzle and 20 psi) or BD FACS Aria-Fusion (standard50 mW 488 sapphire, 15mW 355 UV, 100 mW 640 red, 85 mW 405 violet, 50mw 561 yellow-green, 70 µm nozzle and 20 psi).Cells were sorted using the flow cytometry panels described in section 2.6, pg. 76.

Sorted cells were collected into 1.5ml Eppendorf tubes containing 200µl RF-10.After sorting, collection Eppendorfs were topped up with RF-10 and kept on ice until processing. For cell culture, Eppendorfs were spun at 500g for five minutes and the supernatant was removed. 1ml of the relevant cell culture media was added and the Eppendorfs were spun again at 500g for five minutes. Finally, the supernatant was replaced with the required volume for culture. For DNA extraction, centrifuge spins were increased to maximum speed, and supernatants were replaced with water.

2.4.3. Stains and Antibodies

4,6-diamidino-2-phenylindole (DAPI; Partec; www.partec.com) was used to exclude dead cells. Details of the antibodies used are displayed in Table 2.1. Unless otherwise stated, the term 'lineage markers' or Lin includes the antibodies for CD3, CD19, CD20 and CD56.

2.4.4. TruCOUNT

An absolute count of cells in each sample was achieved using TruCOUNT™ tubes (Becton Dickinson; www.bd.com/uk) and following the manufacturers' protocols. Briefly, 5µl of each antibody (CD3 APC; CD14 PE; CD45 APCCy7 and CD34 FITC; See Table 3.1) was added to the mesh of the TruCOUNT™ tube followed by50µl whole blood (added by reverse pipetting). The tube was vortexed and allowed to incubate in the dark at 4°C for 20 minutes before a further vortex. 150µl of RBC lysis buffer (see section 2.2.5) was added and the tube was allowed to incubate in the dark at room temperature for 15 minutes before a further vortex. An LSRII cytometer (Becton Dickinson; www.bd.com/uk) was used to perform the flow cytometry and the data was analysed on FlowJo software, version 7.6.5 (Treestar; www.treestar.com). The absolute count of each cell population was discerned using Equation 2.1.

$$\frac{\text{\# of events in region containing cell}}{\text{\# of events in absolute count bead region}} \quad X \quad \frac{\text{\# of beads per test}}{\text{test volume}} = \frac{\text{absolute count}}{\text{of cell}}$$

Equation 2.1. Absolute Cell Population Counting Equation

Antibody	Fluorochromes	Company	Clone	Isotype
CD1a	A700	BioLegend	HI149	Mouse IgG$_1$,κ
CD1a	BV421	BioLegend	HI149	Mouse IgG1, κ
CD1c	APC	Miltenyi Biotec	AD5-8E7	Mouse, IgG$_{2a}$, κ
CD1c	PeCy7	BioLegend	L161	Mouse IgG$_1$,κ
CD3	APC	BD Biosciences	UCHT1	Mouse IgG$_1$,κ
CD3	FITC	BD Biosciences	SK7	Mouse IgG$_1$,κ
CD3	PERCPCy5.5	BD Biosciences	SK7	Mouse IgG$_1$,κ
CD3	V500	BD Biosciences	UCHT1	Mouse IgG1, κ
CD4	PE	BD Biosciences	SK3	Mouse IgG$_1$,κ
CD7	FITC	BD Biosciences	4H9	Mouse, IgG$_{2a}$, κ
CD8	APCCy7	BD Biosciences	SK1	Mouse IgG$_1$,κ
CD10	PE-TR	Beckman Coulter	ALB1	Mouse IgG$_1$
CD11b	APC	BioLegend	ICRF44	Mouse IgG$_1$,κ
CD11c	A700	BD Biosciences	B-ly6	Mouse IgG1, κ
CD11c	V450	BD Biosciences	B-Ly6	Mouse IgG$_1$,κ
CD14	APCCy7	BD Biosciences	MФP9	Mouse, IgG$_{2b}$, κ
CD14	BV650	BioLegend	M5E2	Mouse IgG2a, κ
CD14	ECD	Beckman Coulter	RMO52	Mouse IgG$_1$,κ
CD14	FITC	BD Biosciences	M5E2	Mouse, IgG$_{2a}$, κ
CD14	PE	BD Biosciences	M5E2	Mouse, IgG$_{2a}$, κ
CD14	PE-Cy7	BD Biosciences	M5E2	Mouse, IgG$_{2a}$, κ
CD14	Q655	Invitrogen	Tük4	Mouse, IgG$_{2a}$
CD16	APC-H7	BD Biosciences	3G8	Mouse IgG$_1$,κ
CD16	FITC	BD Biosciences	NKP15 (Leu-11a)	Mouse IgG$_1$,κ
CD16	PE	BD Biosciences	3G8	Mouse IgG$_1$,κ
CD16	PeCy7	BD Biosciences	B73.1	Mouse IgG$_1$,κ
CD16	PE-TR	Invitrogen	3G8	Mouse IgG$_1$,κ
CD19	FITC	BD Biosciences	4G7	Mouse IgG$_1$,κ
CD19	PE	BD Biosciences	HIB19	Mouse IgG$_1$,κ
CD19	PeCY7	BD Biosciences	SJ25C1	Mouse IgG$_1$,κ
CD20	FITC	BD Biosciences	L27	Mouse IgG$_1$,κ
CD25	PECy7	BD Biosciences	2A3	Mouse IgG$_1$,κ
CD34	APC	BD Biosciences	581	Mouse IgG$_1$,κ
CD34	APCCy7	BioLegend	581	Mouse IgG$_1$,κ
CD34	FITC	BD Biosciences	8G12	Mouse IgG$_1$,κ
CD34	PE	BD Biosciences	8G12	Mouse IgG$_1$,κ
CD38	PECy7	BD Biosciences	HB7	Mouse IgG$_1$,κ
CD45	APCCy7	BD Biosciences	2D1	Mouse IgG$_1$,κ
CD45	PE-TR	Invitrogen	HI30	Mouse IgG$_1$
CD45	V450	BD Biosciences	2D1	Mouse IgG$_1$,κ
CD45	V500	BD Biosciences	HI30	Mouse IgG$_1$,κ
CD45RA	BV510	BioLegend	HI100	Mouse, IgG$_{2b}$, κ
CD45RA	V500	BD Biosciences	HI100	Mouse, IgG$_{2b}$, κ
CD56	APC	BD Biosciences	B159	Mouse IgG$_1$,κ
CD56	FITC	BD Biosciences	NCAM16.2	Mouse, IgG$_{2b}$, κ
CD83	FITC	BD Biosciences	HB15e	Mouse IgG2b, κ
CD90	PERCPCy5.5	BioLegend	5E10	Mouse IgG$_1$,κ
CD110	APC	BD Biosciences	1.6.1	Mouse, IgG$_{2b}$, κ

Antibody	Fluorochromes	Company	Clone	Isotype
CD114	APC	BioLegend	LMM741	Mouse IgG$_1$,κ
CD123	BV421	BioLegend	6H6	Mouse IgG$_1$,κ
CD123	PE	BD Biosciences	9F5	Mouse IgG$_1$,κ
CD123	PERCPCy5.5	BD Biosciences	7G3	Mouse, IgG$_{2a}$, κ
CD135 (FLT3)	PE	BD Biosciences	4G8	Mouse IgG$_1$,κ
CD141	APC	Miltenyi Biotec	AD5-14H12	Mouse IgG$_1$
CD172a (SIRP-α)	PE	BioLegend	SE5A5	Mouse IgG$_1$,κ
CD207 (Langerin)	PE	Beckman Coulter	DCGM4	Mouse IgG$_1$,κ
CD235a (Glycophorin A)	PE	BD Biosciences	GA-R2 (HIR2)	Mouse, IgG$_{2b}$, κ
CD324 (E-Cadherin)	APC	BioLegend	67A4	Mouse IgG1, κ
CD326 (EpCAM)	APC	BD Biosciences	EBA-1	Mouse IgG1, κ
HLA-DR	A700	BD Biosciences	L243 (G46-6)	Mouse, IgG$_{2a}$, κ
HLA-DR	V450	BD Biosciences	L243 (G46-6)	Mouse, IgG$_{2a}$, κ
HLA-DR	V500	BD Biosciences	L243 (G46-6)	Mouse, IgG$_{2a}$, κ

Table 2.1. Antibodies Used Throughout This Thesis

2.5. Flow Cytometry Controls

2.5.1. *Fully Stained Controls*

A series of fully stained normal controls were run for all panels, to create standard ranges of cell numbers (see section 2.7, pg.81). One fully stained normal control was run with each cell sort experiment for comparison.

2.5.2. *Unstained Controls*

During the setup for each panel, a tube of unstained cells was run to rule out any background or cellular autofluorescence.

2.5.3. *Single Stain Controls*

During the setup for each panel, a series of single stained tubes of 'normal control' cells were run as positive controls.

2.5.4. *CompBead Controls*

A series of unstained and single stained positive and negative Compensation Bead (Becton Dickinson; www.bd.com/uk) tubes were run for the setup of each panel to give distinct positive and negative stained populations to set compensation values and to make adjustments for spectral overlap.

2.5.5. *Fluorescence Minus One (FMO) & Isotype Controls*

A combined 'fluorescence minus one' and isotype system of controls was used to confirm true positive staining and to aid in defining gating boundaries. Isotype controls were used at the same concentration as the corresponding antigen specific antibodies. This is especially important as some cells such as macrophages have a high autofluorescence, which can result in false positives.

2.6. Multiparameter Flow Cytometry Panels

In order to study the development of DCs in haematological malignancies and histiocytic disorders, it was necessary to develop methods to determine quantities of these cells in the peripheral blood (absolute counts) and bone marrow (relative counts) in the steady state. These methods were built upon previous work of the Human Dendritic Cell (HuDC) Lab and developed in this thesis.

2.6.1. *Antigen Presenting Cells*

A fourteen parameter panel was adapted to enable analysis of the antigen presenting cell (APC) compartment, comprising monocytes and dendritic cells (DC) in PB and other tissues. The panel can identify 8 different populations (See Table 2.2). The gating strategy for the panel is given in Figure 2.2.

Cell Type	Markers
CD14+ Monocytes	CD45+DR+Lin-CD14+CD16-
CD14+CD16+ Monocytes	CD45+DR+Lin-CD14+CD16+
CD16+ Monocytes	CD45+DR+Lin-CD14-CD16+
CD123+Plasmacytoid DC	CD45+DR+Lin-CD14-CD16-CD123+CD34-
CD34+ Circulating Progenitors	CD45+DR+Lin-CD14-CD16-CD123-CD34+
CD141+ Myeloid DC	CD45+DR+Lin-CD14-CD16-CD123-CD34-CD141+
CD11c+ Myeloid DC	CD45+DR+Lin-CD14-CD16-CD123-CD34-CD11c+
CD1c+ Myeloid DC	CD45+DR+Lin-CD14-CD16-CD123-CD34-CD11c+CD1c+

Table 2.2. Cell Populations Identified by the APC Panel
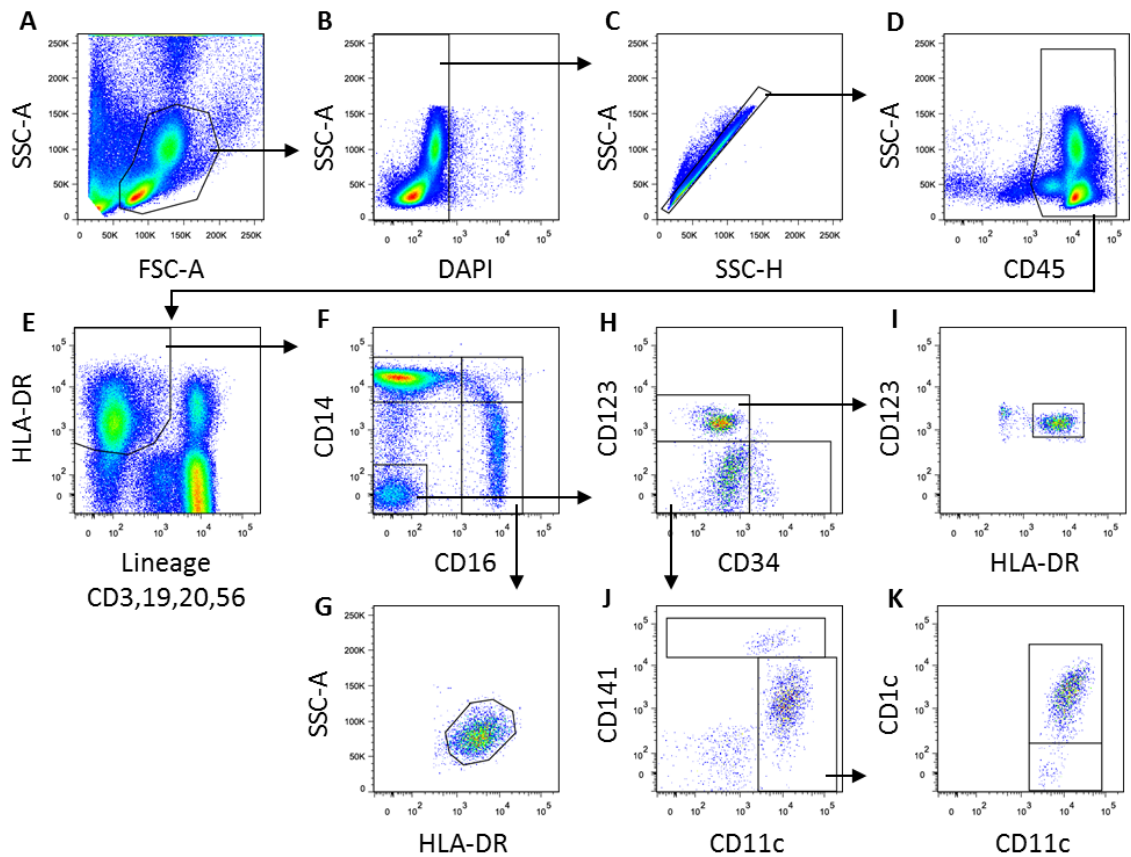
Figure 2.2. Antigen Presenting Cell Panel Gating Strategy

(A) The cell population was gated on FSC-A and SSC-A, excluding debris. (B) Live cells were gated by excluding DAPI positive cells. (C) Singlets were gated on SSC-H and SSC-A, excluding doublet cells. (D) Leukocytes were isolated by expression of CD45. (E) Monocytes and Antigen Presenting Cells (APCs) were isolated by expression of HLA-DR and lack of Lineage markers (CD3, CD19, CD20, and CD56). (F) Monocytes were separated by expression of CD14 and/or CD16. (G) CD16+ monocytes were isolated by their expression of HLA-DR to exclude any CD16+ NK cell contamination. (H) pDCs were separated by expression of CD123 and CD34+ circulating progenitor cells were isolated by CD34 expression. (I) pDCs were isolated by their expression of HLA-DR to exclude any CD123+ Basophil contamination. (J) CD141+ and CD11c+ mDCs were separated from the CD123-CD34- group. (K) The CD11c+ mDC group was further divided by expression of CD1c into true CD1c+ mDCs and a residual CD1c- fraction.

2.6.2. Lymphocytes

An eight parameter panel was set up to analyse the lymphoid compartments in PB and other tissues. The panel can identify 7 different populations (See Table 2.3). The gating strategy for the panel is given in Figure 2.3.

Cell Type	Markers
T Cells	CD3+
CD4+ T Cells	CD3+CD4+
CD8+ T Cells	CD3+CD8+
CD56+ T Cells	CD3+CD56+
T Regulatory Cells (Tregs)*	CD3+CD19-CD8-CD4+CD25+
B Cells	HLA-DR+CD19+
Natural Killer Cells	CD3-CD56+

Table 2.3. Cell Populations Identified by the Lymphocyte Panel

* This fraction may also contain CD4+CD25+ activated T cells.
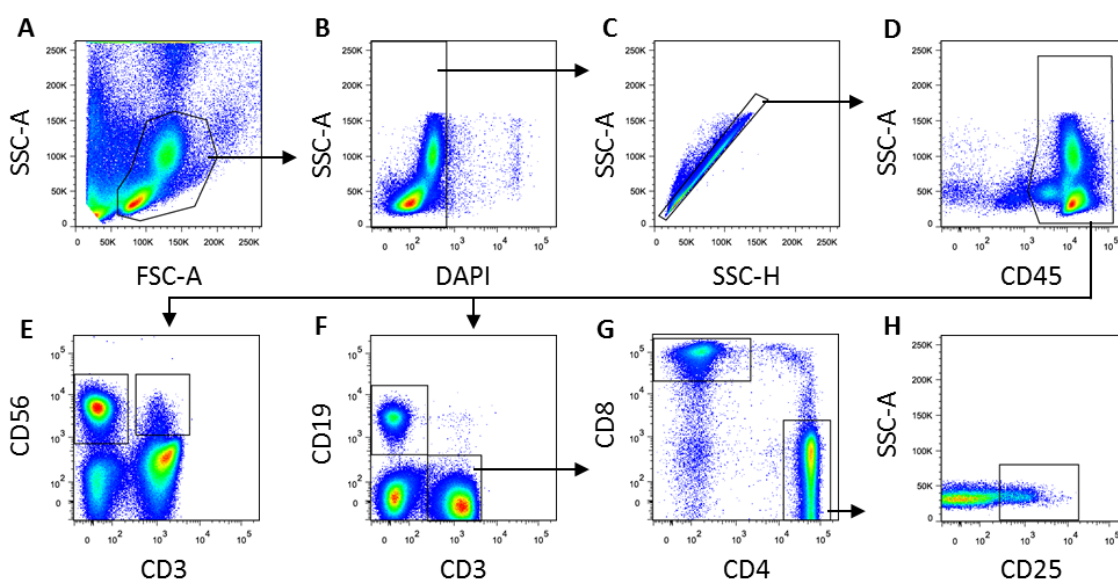
Figure 2.3. Lymphocyte Panel Gating Strategy on Fresh PBMC

(A) The cell population was gated on FSC-A and SSC-A, excluding debris. (B) Live cells were gated by excluding DAPI positive cells. (C) Singlets were gated on SSC-H and SSC-A, excluding doublet cells. (D) Leukocytes were isolated by expression of CD45. (E) CD3-CD56+ NK cells and the small CD3+CD56+ T cell fraction isolated. (F) B cells identified by expression of CD19 and T cells by expression of CD3. (G) T cells split by CD4 and CD8. (H) Tregs isolated from CD4+ T cells by expression of CD25.

2.6.3. *Progenitor Cells*

A thirteen parameter panel was set up to analyse the CD34+ progenitor compartment in BM, PB and other tissues. The panel can identify 7 different progenitor cell populations (See Table 2.4), according to recent descriptions (Manz *et al.*, 2002; Doulatov *et al.*, 2010; Goardon *et al.*, 2011), as well as an uncharacterised CD34+38+10-45RA+123^{high} cell fraction. The gating strategy for the panel is given in Figure 2.4.

Cell Type	Markers
Haematopoietic Stem Cells (HSCs)	CD34+CD38-CD90+CD45RA-
Multi-Lymphoid Progenitors (MLP)	CD34+CD38-CD90-CD45RA+
Multi Potent Progenitors (MPP)	CD34+CD38-CD90-CD45RA-
B cell and NK Progenitors (BNK)	CD34+CD38+CD10+
Common Myeloid Progenitors (CMP)	CD34+CD38+CD10-CD123+CD45RA-
Megakaryocyte/Erythroid Progenitors (MEP)	CD34+CD38+CD10-CD123-CD45RA-
Granulocyte/Macrophage Progenitors (GMP)	CD34+CD38+CD10-CD123+CD45RA+
CD123^{High} Fraction (CD123^{High})	CD34+CD38+CD10-CD123^{high}CD45RA+

Table 2.4. Cell Populations Identified by the CD34+ Panel

None of the following markers proved to be more efficient than CD123 (Interleukin-3 receptor) at splitting the CMP and MEP populations: CD11b (Integrin alpha M), CD16 (a low affinity Fc receptor) region receptor), CD110 (Thrombopoietin receptor), CD114 (granulocyte colony-stimulating factor receptor), CD135 (Fms-like tyrosine kinase 3), CD172a (Signal regulatory protein α), CD235a (Glycophorin A) (data not shown).
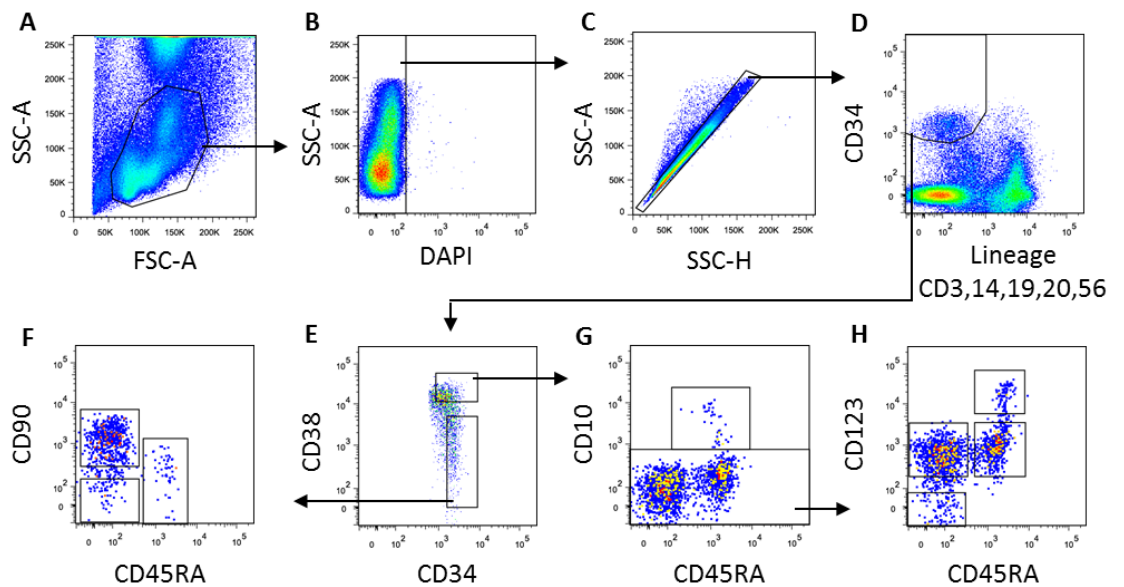
Figure 2.4. CD34+ Progenitor Panel Gating Strategy on Fresh BM

(A) The cell population was gated on FSC-A and SSC-A, excluding debris. (B) Live cells were gated by excluding DAPI positive cells. (C) Singlets were gated on SSC-H and SSC-A, excluding doublet cells. (D) Progenitor cells where isolated by their expression of CD34. (E) Progenitor cells were split by their expression of CD38. These gates were arbitrarily but consistently set at 25% for CD38+ cells and 17% for CD38- cells. (F) Primitive CD38- Progenitors where split by their expression of CD90 and CD45RA into CD90-CD45RA+ MLPs, CD90+CD45RA- HSCs and CD90-CD45RA- MPPs. (G) From the more mature CD38+ cells, BNKs were isolated by their expression of CD10. (H) CD38-CD10- progenitors where split by expression of CD123 and CD45RA into CD123+CD45RA- CMPs, CD123+CD45RA+ GMPs, CD123-CD45RA- MEPs and an unclassified CD123^{high}CD45RA+ cell fraction.

2.7. Normal Ranges

2.7.1. Peripheral Blood Mononuclear Cell Profile

In order to create normal ranges for the mononuclear cell types present in peripheral blood, samples from 22 healthy adult volunteers (age range 25 – 55 years) were analysed using the preceding APC and Lymphocyte multi-parameter flow cytometry panels (see sections 2.6.1 and 2.6.2). A whole blood TruCOUNT^{TM} tube (see section 2.4.4) was run alongside each PBMC sample including the antibodies CD14 and CD3 which allowed for the determination of the absolute counts of the mononuclear cell and lymphoid compartments respectively (Figure 2.5).
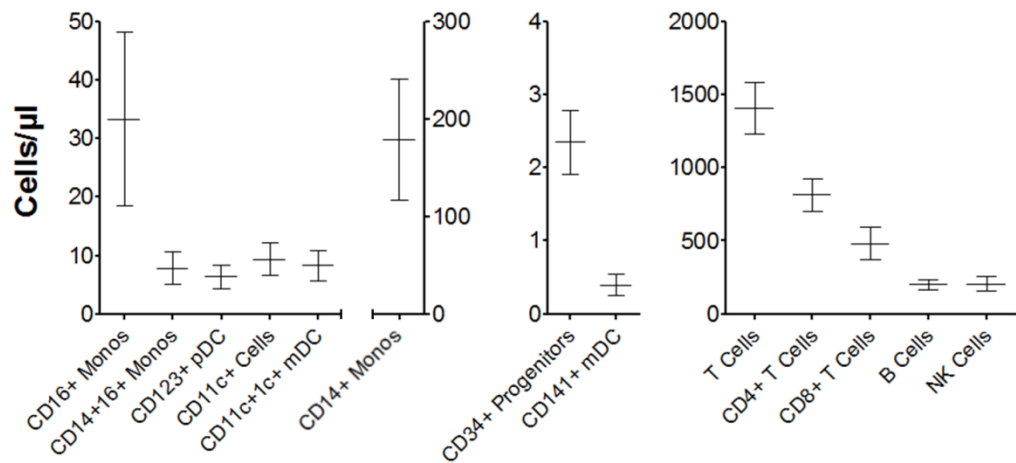
Figure 2.5. Absolute Quantified Normal Ranges of PBMCs

Standard ranges of mononuclear and lymphocyte cell subsets from 22 healthy adult volunteers. Absolute quantifications based on TruCOUNT^{TM} tube analysis. Error bars are means with 95% confidence interval (CI). All axes are as cells/μl. Monos = Monocytes; pDC = plasmacytoid DC; mDC = myeloid DC; NK = Natural Killer Cell.

2.7.2. *Progenitor Cell Profile (BM and PBMC)*

It is more difficult to create standard ranges of the progenitor cell subsets in steady-state bone marrow, as this is rarely extracted from healthy volunteers. Therefore normal BM was sourced from two different sources (see section 2.3.4) and analysed using the progenitor cell panel (see section 2.6.3).

Firstly, donor material was sourced from the Stem Cell Transplantation unit at the Royal Victoria Infirmary, but as peripheral blood stem cells (PBSCs) are now more regularly used for allogeneic transplantation than bone marrow, this source was limited to 6 samples (age range 18-33). The remaining samples (n=14) were taken from hip femoral head bone marrow post hip replacement surgery (age range 38-92), see Figure 2.6. A further set of peripheral blood samples (n=10) was taken from healthy adult volunteers (age range 24 – 48) to form a comparison of the progenitor cells in the circulating peripheral blood, see Figure 2.6. Samples were not G-CSF mobilised.

There were statistical differences between BM and PBMC for all progenitor cell subsets, apart from MPP. HSCs were seen to be variable in BM, but most frequently represented the largest population (21%), whereas in PBMC, very few HSCs were present (3%). MLP were enriched in the PBMC (38%), but low in proportion in the BM (8%), whereas B/NK were enriched in BM (18%) and reduced in PBMC (2%). In the myeloid pathway; CMP, GMP and MEP were similar in the BM (9-15%), although in the PBMC, CMPs were predominant (28%). CD123^{high} cells were low in both cell sources but slightly higher in BM than PBMC (4% and 1% respectively).
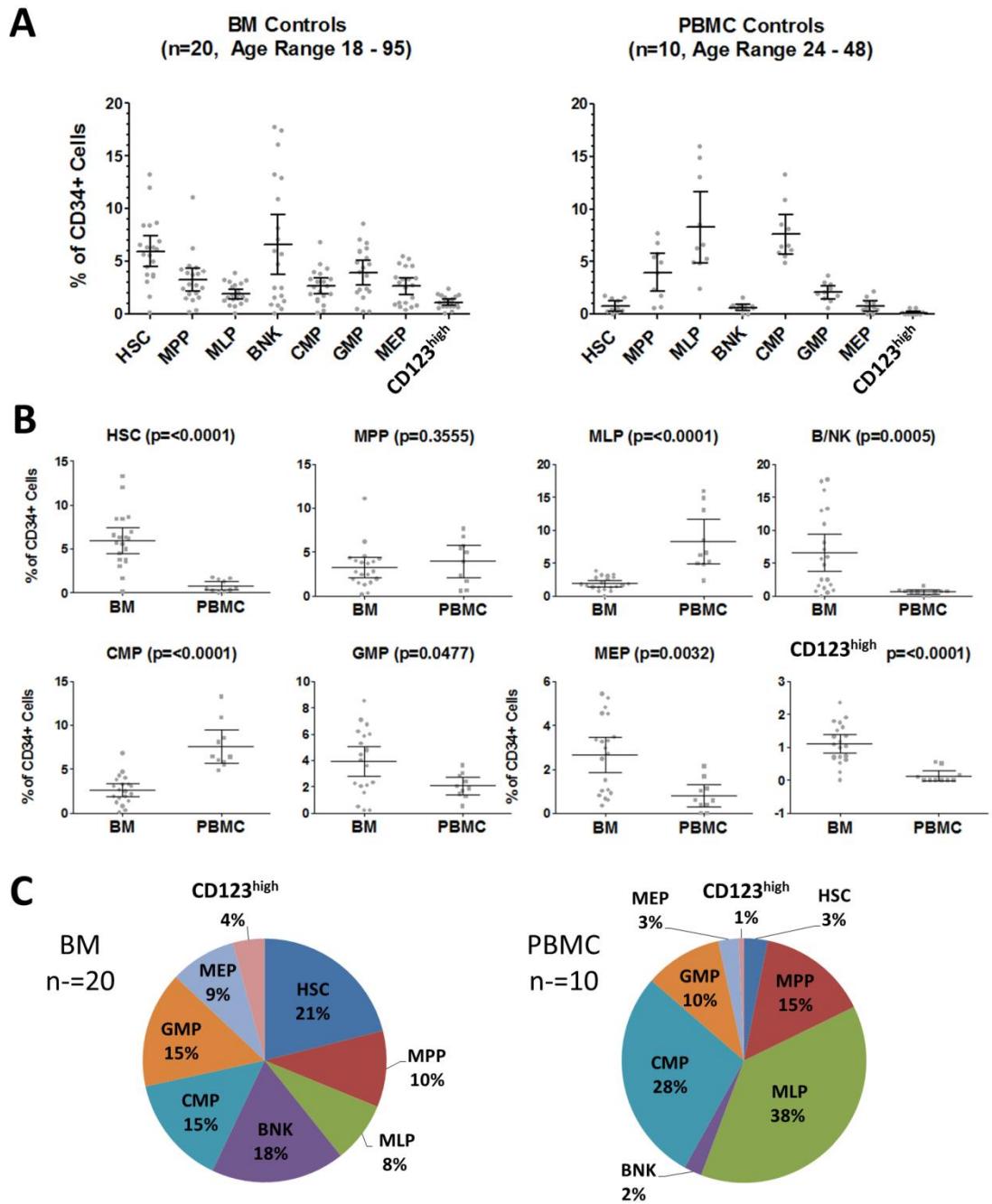
Figure 2.6. Normal BM Cell Type Ranges

HSC = Haematopoietic Stem Cell; MPP = Multi-Potent Progenitor; MLP = Multi-Lymphoid Progenitor; B/NK = B/NK Cell Progenitor; GMP = Granulocyte/Macrophage Progenitor; CMP = Common Myeloid Progenitor; MEP = Megakaryocyte/Erythroid Progenitor; CD123high = CD123highCD45RA+ undefined progenitor population. Error bars indicate mean with 95% CI.

A) *Standard ranges of BM (n=20) and PBMC (n=10) progenitor cell subsets.*

B) *Comparison of the % of CD34+ cells of each progenitor cell type. Statistics are two-tailed Mann-Whitney T tests.*

C) *Mean proportional percentage of progenitor subsets in the CD34+ gate.*

2.8. General Molecular Biology

2.8.1. *DNA Extraction*

DNA was extracted using one of two Qiagen kits (www.qiagen.com). For less than $1x10^6$ cells, a QIAamp DNA Micro Kit was used; for cell numbers between $1x10^6$ and $1x10^7$, a QIAamp DNA Mini Kit was used, including the optional addition of 4µl RNAse A. Company protocols were followed, with the modification of increasing all centrifugations to full speed for five minutes and repeating the final elution step.

2.8.2. *NanoDrop DNA Quantification*

DNA was quantified using a NanoDrop model ND1000 (www.nanodrop.com) as per standard protocol.

Chapter 3. Mononuclear Cell Development in Myeloid Malignancies

3.1. Introduction

As discussed in the introduction section (section 1.4.5, page 35), data on risk markers for AML prognosis and outcome at the time of diagnosis are lacking in the literature, therefore this chapter concerns the exploration of possible risk markers at diagnosis. This analysis involves comparative flow cytometry and ELISA assays of acute myeloid leukaemia (AML) and the pre-leukaemic conditions sporadic myelodysplastic syndrome (MDS) and dendritic cell, monocyte and B and NK cell (DCML) deficiency.

3.1.1. *Chapter Aims*

1) To use state of the art multiparameter flow cytometry and enzyme-linked immuno assays (ELISAs) to evaluate the differences between DCML deficiency, MDS and AML patients and their related immunodeficiencies.

2) To use the tools developed in this thesis to test the hypothesis that acute leukaemia is associated with an 'extended phenotype' of cellular immunodeficiency.

3) To analyse whether acute leukaemia is variably associated with immunodeficiency due to depletion of mononuclear cells, in addition to global bone marrow failure, and that this cellular immunodeficiency is a risk factor for infection during the initial treatment of leukaemia.

4) To use the tools developed in this thesis to explore new leukaemia biology including the correlation of the extended phenotype of leukaemia with standard and novel genetic alterations in leukaemia and new marker panels for residual disease.

3.1.2. *Acute Leukaemia*

Acute leukaemia (AL) is a life-threatening accumulation of abnormal haematopoietic cells, which causes bone marrow failure and leads to

death in ~50% of patients. Therefore it is crucial to find out more about the basic biology of leukaemia, in order to develop a wider range of effective treatments and ways to diagnose and monitor the disease. Further details of acute leukaemia are given in introduction section 1.4.2, pg. 26.

3.1.1. *Classifying Leukaemic Cells by Immunophenotype*

Multi-parameter flow cytometry facilitates complex analysis of peripheral blood and stem cell compartments (Autissier *et al.*, 2010; Jardine *et al.*, 2013). Recently developed flow cytometry protocols created in the Human Dendritic Cell Lab (HuDC) and developed in this thesis are capable of resolving 23 distinct components from the peripheral blood mononuclear cell and CD34+ progenitor cell compartments (see section 2.6.3, pg. 79). Using these flow cytometry methods, It may be possible to reproduce the AML progenitor phenotypes introduced by Goardon *et al*, 2011 (see section1.4.3.1 pg. 32), but also to group AML by the phenotypic pattern of mature markers seen on AML blast cells.

3.1.2. *The Extended Phenotype of Leukaemia*

Loss or aberrant expansion of specific cell populations during the evolution of leukaemia may lead to immunodeficiency. At presentation, the failure of erythropoiesis, granulopoiesis and thrombocytopoiesis are paramount but the extent to which mononuclear cell differentiation has failed has not been examined. Recent studies show clear relationships between leukaemic blasts and normal progenitor compartments (Cox *et al.*, 2004; Kong *et al.*, 2008; le Viseur *et al.*, 2008; Goardon *et al.*, 2011) and it is likely that different extended phenotypes will be associated with each biological type of acute leukaemia. Understanding how defects in myelopoiesis affect circulating DC populations, has the potential to provide: 1) new insight into the nature of immunodeficiency and the attendant clinical problems of bone marrow failure; 2) new information about the basic biology of acute myeloid leukaemia; 3) new insights into the homeostasis of antigen presenting cells. Further details of this hypothesis are given in the introduction section 1.4.6, pg. 35. Multi-parameter flow cytometry using TruCOUNT tubes is a testable model for

this project, as it gives absolute quantification of all of the mononuclear cell types present in peripheral blood.

3.1.3. *Dendritic Cell, Monocyte and B and NK Lymphoid Deficiency*

The theory of an extended phenotype of leukaemia is supported by the recently described Dendritic Cell, Myeloid and B and NK Lymphoid (DCML) deficiency, otherwise referred to as MonoMac (Vinh *et al.*, 2010; Calvo *et al.*, 2011).DCML deficiency patients have a common patient presentation of atypical mycobacterial and viral infections with an underlying specific deficiency of DCs, monocytes, B and NK cells, and an overall deficiency of the MLP progenitor cell (Bigley and Collin, 2011; Calvo *et al.*, 2011). DCML deficiency is caused by mutations in the GATA2 gene (Dickinson *et al.*, 2011; Hsu *et al.*, 2011) and up to 50% of patients develop MDS (Calvo *et al.*, 2011; Hsu *et al.*, 2011; Bodor *et al.*, 2012; Holme *et al.*, 2012; Hsu *et al.*, 2013) and may progress further to leukaemia (Vinh *et al.*, 2010; Bigley and Collin, 2011).

Although the cellular losses seen in DCML deficiency could imply that it is in fact a form of MDS, the specific immunodeficiencies appear to be an 'accessory phenotype' rather than playing a significant role. In fact, many DCML deficiency patients present with clinical problems prior to their meeting the standard criteria for MDS, indicating that the cell-intrinsic phenotype of the disease directly effects haematopoiesis. Therefore, it is not certain how closely DCML can be generalised to sporadic MDS and AML.

Mononuclear cytopenia, in particular the loss of DCs, has not been systematically investigated in symptomatic GATA2 mutation patients. Furthermore, it is not known how distinct the phenotype of DCML deficiency is from cytopenias occurring in acquired MDS with wild-type GATA2. A more precise understanding of the evolution of cellular deficiency and progression of DCML deficiency, in comparison to other forms of BMF may further assist the recognition and clinical management of these disorders. These data may also be very useful in the prognosis of

leukaemia and leukaemia biology itself, due to possible progression of DCML deficiency to MDS to AML.

3.1.4. *Fms-like Tyrosine Kinase Ligand*

Fms-like Tyrosine Kinase 3 (FLT-3) is a cytokine receptor involved in normal haematopoiesis. FLT3 Ligand (FLT3L) may be increased in the refractory anaemia form of MDS (Zwierzina *et al.*, 1999), and it has more recently been found to be massively elevated in cases of DCML deficiency (Bigley *et al.*, 2011). No studies to date have examined serum levels of FLT3L in AML, though AML cells express high levels of the FLT3 receptor (Birg *et al.*, 1992), and *in vitro* studies have reported that FLT3 ligand can promote AML cell proliferation and survival (Drexler *et al.*, 1999; Meyer and Drexler, 1999). It is therefore possible that variable levels of serum FLT3 ligand may be used as a simple differentiating biomarker of the diseases.

3.1.5. *Chapter Hypotheses*

1) Acute leukaemia is variably associated with immunodeficiency due to failure of development of mononuclear cells, independently of the effect of global bone marrow failure.

2) Distinct patterns of cytopenia are observed with GATA2 mutation, sporadic MDS and acute leukaemia.

3) The 'extended phenotype' of acute leukaemia is related to the immunophenotype of blast cells.

4) The 'extended phenotype' of leukaemia correlates with the genotype of acute leukaemia.

3.2. Materials and Methods for Chapter Three

3.2.1. *Sample Collection*

Blood surplus to diagnostic requirement was collected from GATA2 mutation and MDS patients. Samples of blood and BM which remained after diagnostic requirements were collected from AML patients. Samples from all patients were linked with clinical data, including the age and gender of the patient, the date of presentation, and the treatment protocol used. Laboratory data obtained at the time of diagnosis, including the immunophenotype and karyotype of the leukaemic cells, was also collected from AML patients. A summary of all patients recruited for this study is given in Figure 3.1. Detailed patient characteristics are given in Table 5.1. All patient and control samples were acquired with ethical approval from the Newcastle and North Tyneside Research Ethics Committee (Appendix 8). Informed consent was obtained in accordance with the Declaration of Helsinki.

3.2.1.1. *DCML Deficiency (GATA2 Mutation) Patients*

Patients with DCML deficiency carrying GATA2 mutations were referred from a wide range of clinicians suspecting a GATA2-related disorder. There were no specific inclusion criteria and all patients found to have GATA2 mutation by direct sequencing are reported (n=18). Sequencing was performed in a separate study (Dickinson *et al.*, 2014).

3.2.1.2. *Myelodysplastic Syndrome Patients*

Patients with acquired myelodysplasia (MDS; WHO classification: refractory cytopenia and multilineage dysplasia) were recruited from the FRH ambulatory clinic. Inclusion criteria was for those patients who displayed a tri-lineage MDS as defined by BM morphology and histology and dysplastic features in the cells of all three haematopoietic lineages (myeloid, erythroid and megakaryocytic).All MDS patients were symptomatic and some required transfusion support but none had received high dose

cytoreductive therapy prior to testing. These patients were seen to have no GATA2 mutations in a separate study (Dickinson *et al.*, 2014).

3.2.1.3. *Acute Myeloid Leukaemia Patients*

Samples of peripheral blood and bone marrow were gathered from acute myeloid leukaemia patients presenting to Haematology at Northern Centre for Cancer Care (NCCC), Freeman Hospital (FRH) (Figure 5.1). There were no specific recruitment criteria, other than the availability of material surplus to diagnostic requirements and the ability to consent. A total of 43 AML patients were recruited into the study at time of diagnosis. AML was diagnosed by blast percentage of 20% or higher by qualified diagnostic laboratory staff. 38 patients had a diagnosis of *de novo* AML, consisting of specific diagnosis of AML (30), AMML (2), APML (4) and BPDCN (2). 5 patients were transformations of AML from MDS. PBMC samples were analysed from 38 patients and BM from 15 patients (10 patients with both PBMC and BM). The PBMC samples from 2 patients were found to have insufficient cell numbers for analysis.

3.2.1.4. *Control Peripheral Blood and Bone Marrow*

Normal peripheral blood samples were taken with informed consent from voluntary donors. Bone marrow was taken either from donations to the bone marrow transplantation service (informed consent for research taken by the donor registries) or from patients undergoing hip replacement surgery (informed consent at site).

3.2.2. *FLT3L Serum Biomarker ELISA Screening*

Serum ELISA was performed with Quantikine Human FLT3/Flk-2 Ligand Immunoassay, according to manufacturer's instructions (R&D Systems; www.rndsystems.com). All samples were performed in duplicate.

3.2.3. *Bioinformatics Data Clustering*

Unsupervised hierarchical clustering was performed using a Euclidean distance matrix and Ward method agglomeration. These analyses were performed using base packages of the 'R' programming language (ver. 3.1.1 "Sock it to Me").

3.2.4. *Statistical analysis*

ANOVA with Bonferroni Multiple Comparisons Test were used to compare groups in most analyses. Cell count data were obviously skewed in DCML deficiency patients and so non-parametric tests were preferred (Mann-Whitney or Kruskal-Wallis with Dunn's Multiple Comparison Test).

3.3. Results

A summary of patient recruitment is given in Figure 3.1, detailed patient characteristics are given in Table 3.1.A (DCML deficiency) B (MDS) and C (AML).
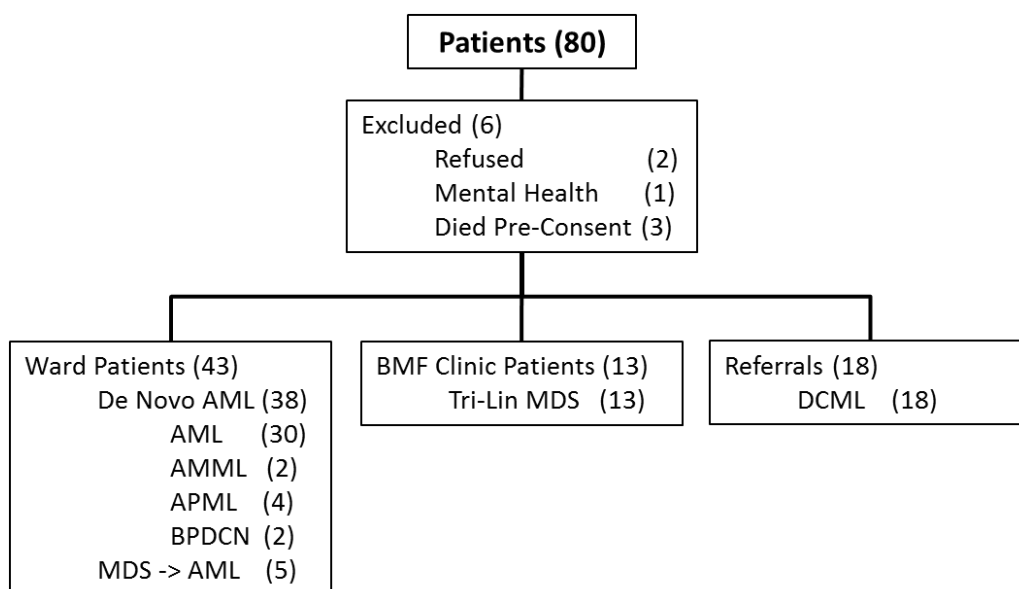
Figure 3.1. Haematological Disorder Patient Recruitment

AML = Acute Myeloid Leukaemia; AMML = Acute Myelomonocytic Leukaemia; APML = Acute Promyelocytic Leukaemia; BPDCN = Blastic Plasmacytoid Dendritic Cell Neoplasm; MDS = Myelodysplastic Syndrome; Tri-Lin = Tri-lineage (Red blood cells, neutrophils and platelets); DCML = Dendritic Cell, Monocyte, B and NK Cell Deficiency.

A

A Number	Age at Diagnosis	Gender	Hb (g/dL)	Plt (x10^9/L)	Neut (x10^9/L)	FLT3L (pg/ml)	GATA-2 Mutation
A1946	12	M	13.0	271	3.05	8750	G200fs
A2052	22	F	10.6	431	3.42	2500	R398W
A2162	18	M	14.1	163	3.29	6874	D340-381
A2352	18	M	14.3	570	2.4	4752	G199fs
A2354	17	M	14.3	123	0.39	1933	G199fs
A2355	13	F	11.8	183	1.4	6935	G199fs
A2375	40	M	17.2	210	6.89	67	R398W
A2472	31	F	14	116	4.2	8345	T354M
A2473	29	F	13.4	221	4.2	5328	T354M
A2478	8	M	12.6	157	0.602	8452	C85fs
A2483	22	F	12.5	116	4.18	4686	A372T
A2516	36	M	11.8	100	0.3	7925	R398Q
A2576	17	M	-	-	-	2311	T354M
A2627	22	F	10.6	123	2.09	2715	D340-381
A2628	19	F	15.2	184	1.65	7896	P245fs
A2744	30	M	10.4	19	6.26	294	G200fs/390delK
A2824	60	?	11.5	375	6.47	2662	G200fs
A2896	4	F	8.2	33	0.9	1267/851	R361C

B

A Number	Age at Diagnosis	Gender	PBMC WBC (x10^9/L)	Hb (g/dL)	Plt (x10^9/L)	Neut (x10^9/L)	FLT3L (pg/ml)	Karyotype
A2650	75	M	1.88	12.6	107	0.25	21.36	Unknown
A2774	67	M	1.52	8.8	10	0.59	162.835	Monosomy 11
A2775	54	M	2.51	9.3	78	1.40	613.379	Trisomy 8
A2803	59	M	3.10	11.2	94	1.74	56.598	Unknown
A2808	61	F	4.08	12.9	81	1.13	17.294	Normal
A2818	79	F	2.41	10.4	59	1.33	278.928	Unknown
A2928	58	F	1.56	11.4	32	0.55	133.178	Monosomy 7
A2986	42	F	3.14	8.3	36	0.93	<min	Unknown
A6967	71	M	1.13	7.6	78	0.35	216	Normal
A6968	64	F	2.35	9.5	17	0.68	-	Normal
A6969	68	M	1.97	9	65	0.4	139	Complex
A6970	49	M	2.41	13.6	-	0.67	174	Normal
A6991	83	M	2.69	9.7	26	1.39	123.965	Trisomy 8

Table 3.1. Patient Clinical Characteristics

A) DCML Deficiency Patients; B) MDS Patients; C) AML Patients (Next Page)

A review of the common clinical features of the cohort is presented.

Hb = Haemoglobin; Plt = Platelets; Neut = Neutrophils; FLT3L = Fms-like tyrosine kinase 3; GATA2 = The gene which codes for GATA binding protein 2, found to be mutated in almost all patients with the DCML deficiency phenotype (37/43 to data, unpublished data). PBMC = Peripheral Blood Mononuclear Cells; BM = Bone Marrow; WBC = Whole Blood Count; w/RGA = with Recurrent Genetic Abnormalities; wMDRC = with Myelodysplasia Related Changes; NOS = Not Otherwise Specified.

FLT3L was measured in Serum/Plasma Samples taken at time of diagnosis.

Details of the classifications of leukaemia are given in section 1.4.2.1, pg. 28.

FAB = French American British; WHO = World Health Organisation; Cytogenetic Risk Status based on Grimwade et al, 2010.

A Number	Main Diagnosis	Stage	Age at Diagnosis	Gender	PBMC WBC (x10⁹/L)	BM Blasts (%)	Blast % CD34	FLT3L (pg/ml)	Cytogenetics	FAB Type	WHO Type	Cytogenetic Risk Status	PBMC Blast Phenotype	BM Blast Phenotype	Cluster Group
A2577	BPDCN	De Novo	72	M	4.87	20	-	-	Normal	-	BPDCN	Intermediate	-	GMP/MLP	5
A2650	AML	Progression	76	M	1.50	5	63	-	Normal	-	wMDRC	Intermediate	-	-	-
A2691	AML	De Novo	53	M	7.67	57	1	-	Normal	M1	NOS	Intermediate	GMP/MLP	GMP/MLP	4
A2695	AML	De Novo	45	M	1.89	20	90	-	Normal	-	NOS	Intermediate	GMP/MLP	-	2
A2698	AML	De Novo	68	M	273.00	99	0.1	-	Normal, FLT3 ITD, NPM1	M0	wRGA	Intermediate	CMP/MPP	-	-
A2790	AML	De Novo	71	M	2.38	82	3	<min	Normal	-	NOS	Intermediate	-	-	4
A2792	AML	De Novo	47	F	9.72	Occasional	-	-	Normal, FLT3 ITD, NPM1	-	wRGA	Intermediate	GMP/MLP	GMP/MLP	-
A2865	AML	Progression	74	M	0.50	20	59	40.214	Trisomy 8, NPM1	M1	wMDRC	Intermediate	GMP/MLP	-	-
A2880	AML	De Novo	55	M	1.32	35	7	62.106	Normal, FLT3 ITD, NPM1	M0	wRGA	Intermediate	CMP/MPP	-	-
A2913	AML	De Novo	56	F	17.22	Present	0.1	<min	Isochromosome 21, Inv3, NPM1	-	wRGA	Adverse	-	CMP/MPP	3
A2917	AML	De Novo	52	F	-	High	0.1	<min	Normal, NPM1	-	wRGA	Intermediate	CMP/MPP	CMP/MPP	3
A2918	AML	De Novo	64	F	2.69	78	52	<min	Deletion 5Q	M4	wMDRC	Adverse	CMP/MPP	CMP/MPP	-
A2926	AML	De Novo	48	F	41.60	61	-	<min	Normal, FLT3 ITD, NPM1	-	wRGA	Intermediate	GMP/MLP	-	4
A2935	AML	De Novo	70	M	1.24	-	-	<min	Normal	-	NOS	Intermediate	-	-	-
A2939	APML	De Novo	23	F	0.85	90	3	<min	Translocation 15:17	M3	wRGA	Favourable	CMP/MPP	-	3
A2966	AML	De Novo	33	F	7.00	99	67	<min	Translocation 8:21, Deletion 9	M2	wRGA	Favourable	GMP/MLP	-	-
A2977	AMML	De Novo	37	F	3.10	No BM	-	<min	Inversion 16	M4	wRGA	Favourable	GMP/MLP	-	1
A2990	AML	De Novo	28	M	40.33	85	80	25.224	Translocation 8:21	M2	wRGA	Favourable	GMP/MLP	GMP/MLP	1
A2991	AML	De Novo	69	M	1.05	50	50	<min	Complex 1	-	wMDRC	Adverse	-	-	3
A6492	AML	De Novo	64	F	306.35	80	91	<min	Trisomy 8	M0	NOS	Intermediate	Hybrid	Hybrid	2
A6916	AML	De Novo	54	M	2.00	15	2	<min	Normal	M0	NOS	Intermediate	-	-	3
A6917	AML	De Novo	71	M	1.00	Present	0.1	<min	Trisomy 8, FLT3 ITD, NPM1	M0	NOS	Intermediate	-	-	-
A6931	AML	De Novo	63	F	7.95	33	0.1	119.634	Normal	M3	NOS	Intermediate	GMP/MLP	GMP/MLP	3
A6951	APML	De Novo	43	F	21.86	Some	0.1	85.169	Translocation 15:17, NPM1	M3	wRGA	Favourable	CMP/MPP	-	3
A6956	AML	Progression	69	F	5.37	20	32	<min	Complex 2, FLT3 ITD, NPM1	-	wMDRC	Adverse	GMP/MLP	-	4
A6961	AML	De Novo	67	F	-	60	14	<min	Normal	-	NOS	Intermediate	GMP/MLP	-	4
A6989	APML	De Novo	48	M	2.40	Present	0.1	60.831	Normal	M3	NOS	Intermediate	-	-	-
A6994	AML	De Novo	70	F	34.80	Present	0.1	60.656	Normal	M0	NOS	Intermediate	-	-	3
A7021	AML	De Novo	57	F	4.20	30	12	<min	Normal	M0	NOS	Intermediate	-	GMP/MLP	2
A7022	AML	De Novo	51	F	120.60	99	-	<min	Normal	-	NOS	Intermediate	-	-	3
A7027	AML	De Novo	72	M	6.52	70	0.1	17.643	Normal	-	NOS	Intermediate	-	CMP/MPP	3
A7043	AML	De Novo	20	M	9.53	74	0.1	<min	Normal	-	NOS	Intermediate	-	-	-
A7055	AML	De Novo	38	M	46.50	80	52	<min	Complex 3	M0	wMDRC	Adverse	GMP/MLP	-	1
A7072	AML	De Novo	48	F	3.26	70	53	63.039	Normal, FLT3 ITD	-	NOS	Intermediate	GMP/MLP	-	2
A7080	AML	De Novo	51	F	15.35	50	36	<min	Normal	M5	NOS	Intermediate	GMP/MLP	-	2
A7092	AML	Progression	70	M	0.99	80	17	<min	Translocation 2:3	M5	wMDRC	Intermediate	GMP/MLP	-	4
A7113	AML	Progression	66	M	14.64	25	21	<min	Complex 4	M4	wMDRC	Adverse	GMP/MLP	-	1
A7129	AMML	De Novo	52	M	2.36	Many	60	12.193	Complex 5	-	wMDRC	Adverse	GMP/MLP	GMP/MLP	2
A7140	AML	De Novo	41	F	12.82	35	80	<min	Deletion 9	-	wMDRC	Favourable	GMP/MLP	-	-
A7148	BPDCN	De Novo	76	M	-	Numerous	0.1	17.564	Normal	-	BPDCN	Intermediate	-	-	-
A7208	APML	De Novo	49	M	4.41	76	2	n/a	Translocation 15:17	M3	wRGA	Favourable	-	-	-
A7219	AML	De Novo	77	M	164.66	90	-	n/a	Trisomy 21	-	NOS	Intermediate	-	-	-
A7221	AML	De Novo	59	M	-	High	68	8.602	Trisomy 15	M1	NOS	Intermediate	GMP/MLP	GMP/MLP	2

Complex
1) 47,XY,t(1;12)(q25;q24),del(4)(q31), del(5)(q1?3),add(15)(p11.1),-18,-22,-22,+4mar [7] / 46,XY [3]
2) 46,XX,del(5)(q22q35), del(12)(p1?)[11] flt3- npm1-

Karyotype
3) 45,XY,der(3)add(3)(p1?3) add(3)(q27),7,add(12)(1?2),add(12)(p13),-13,+mar [6] FISH: MECOM REARRANGED [99/100]

Cytogenetics
4) 45~47,XY,inv(3)(q21q26),-7,+8[cp6] , FISH: MECOM NOT rearranged [100]
5) 45~46,XY,dic(2;?)(q37;?),add(3)(p2?),add(3)(q2?),-5,-12,-12,del(17)(p1?),-22, -22,+5~7mar[5]

C

3.3.1. General Clinical Features

Age of presentation and gender were collected from all patients. DCML deficiency patients were diagnosed at a significantly younger age than MDS and AML patients (Figure 3.2). No statistical significance was seen for patient gender.
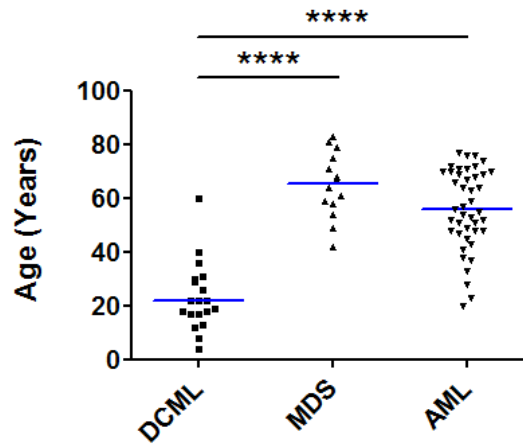
Figure 3.2. Patient Age of Diagnosis

*Age of diagnosis of patients with dendritic cell, monocyte, B and NK lymphoid deficiency (DCML), myelodysplastic syndrome (MDS) and acute myeloid leukaemia (AML). Blue lines are medians. Statistics were Kruskal-Wallis One Way ANOVA. **** = <0.0001*

3.3.2. *Diagnostic Laboratory Results*

Data from the diagnostic lab for haemoglobin (Hb), platelets (Plts) and neutrophils were collected from DCML deficiency (18/18), tri-lineage MDS (13/13) and AML (42/43) patients.

There were significant reductions in all three factors in MDS and AML patients compared to DCML deficiency patients. None of the factors were significantly different between MDS and AML.
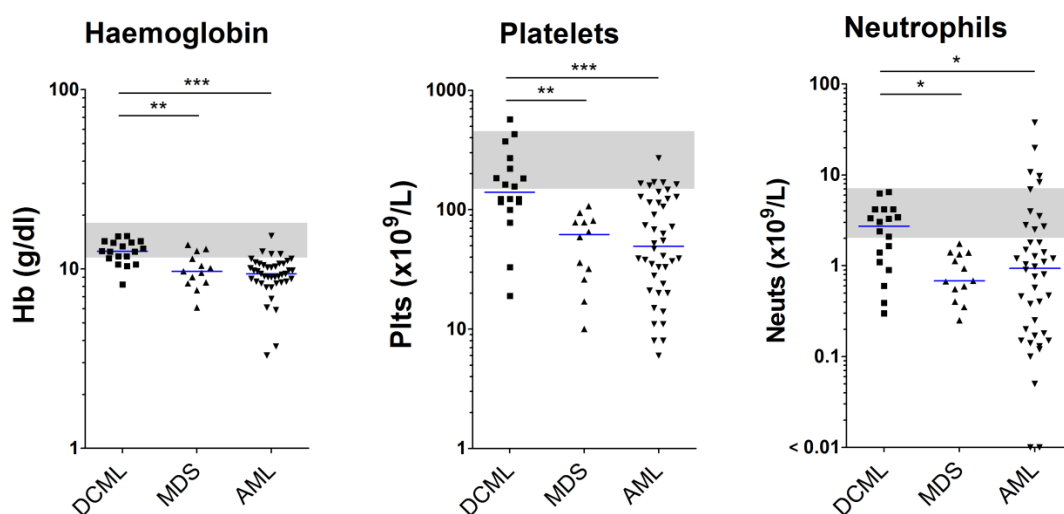
Figure 3.3. Automated Diagnostic Blood Counts

Comparison of automated diagnostic blood counts of patients with DCML deficiency (n=18), MDS (n=13) and AML (n=42). Grey line is the standard range. Blue lines are medians. Hb = Haemoglobin; Plts = Platelets; Neuts = Neutrophils.

Statistics with Kruskal-Wallis with Dunn's Multiple Comparison Test. * = <0.05; ** = <0.01; *** = <0.001.

3.3.3. *Mononuclear Cell Profiles*

In order to profile the extent of mononuclear immunodeficiency, PBMC samples from DCML deficiency (18/18), tri-lineage MDS (13/13) and AML (36/43) patients and 20 normal controls were run on the APC and Lymph panels (see section2.6, pg. 76) and TruCOUNT tubes were used to extrapolate absolute counts.

As expected, DC, monocyte, B and NK counts were significantly reduced in all symptomatic carriers of *GATA2* mutation, whereas T cells, although appearing reduced in some patients, were maintained. DCML deficiency was significantly different from MDS in levels of CD16+ Monocytes, CD141+ mDCs and NK Cells (all $p = <0.05$), and from AML in all cell subtypes other than T cells (all $p = <0.001$). The only subtype significantly different between MDS and AML patients was circulating non-classical CD16+ monocytes, which appeared expanded in AML ($p = <0.05$). There were trends for lower mDCs and B cells in MDS, but these did not achieve significance after adjustment for multiple comparisons. NK cells were difficult to isolate from blast cells by flow cytometric analysis, so care must be taken when interpreting NK cell count data.
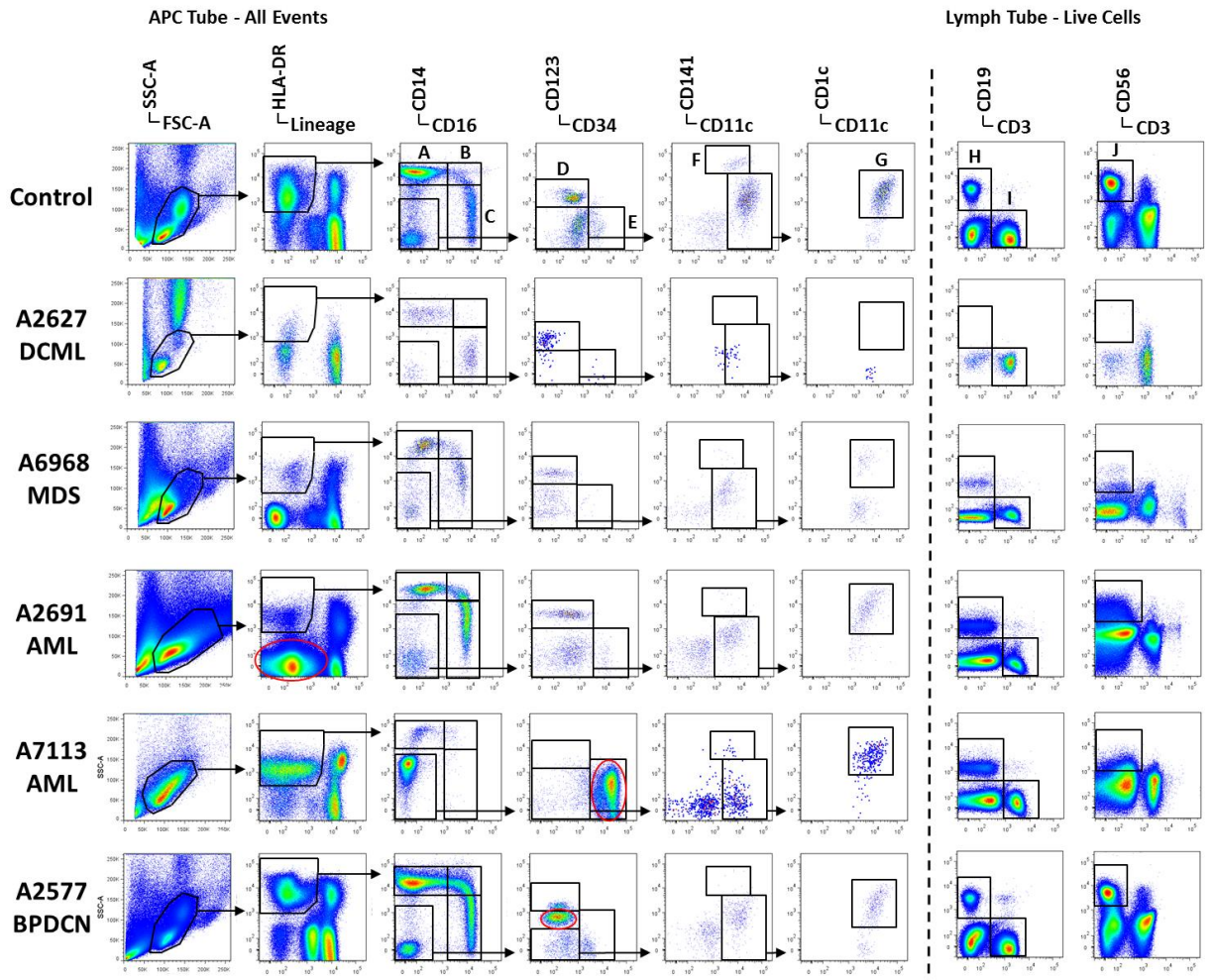
Figure 3.4. DCML Deficiency, MDS and AML Gating Examples

Examples of gating strategies for Dendritic Cell, Monocyte and B and NK Lymphoid (DCML) Deficiency, Myelodysplastic Syndrome(MDS) and Acute Myeloid Leukaemia(AML) patients. Gating strategies were kept as consistent as feasibly possible. As seen, AML phenotyping profiles can vary massively. Most likely blastic populations are ringed in red in the three AML examples. BPDCN = Blastic Plasmacytoid Dendritic Cell Neoplasm, a subset of AML.

Tube One (APC Panel)- Cells gated on FSC-A and SSC-A and gate for live (DAPI negative) singlets (not shown); A = CD14+ monocytes; B = CD14+16+ monocytes; C = CD16+ monocytes; D = CD123+ pDCs; E = CD34+ circulating progenitor cells; F = CD141+ mDCs; G = CD1c+ mDCs.

Tube Two (Lymph Panel) -Cells gated on FSC-A and SSC-A and gate for live (DAPI negative) singlets (not shown); H = CD19+ B Cells; I = CD3+ T Cells; J = CD56+ NK Cells. BPDCN patient NK cell data not used, due to CD56 positivity on BPDCN cells.

A

CD14+ Monocytes

CD16+ Monocytes

CD123+ pDCs

CD11c+1c+ mDCs

CD141+ mDCs

T Cells

B Cells

NK Cells
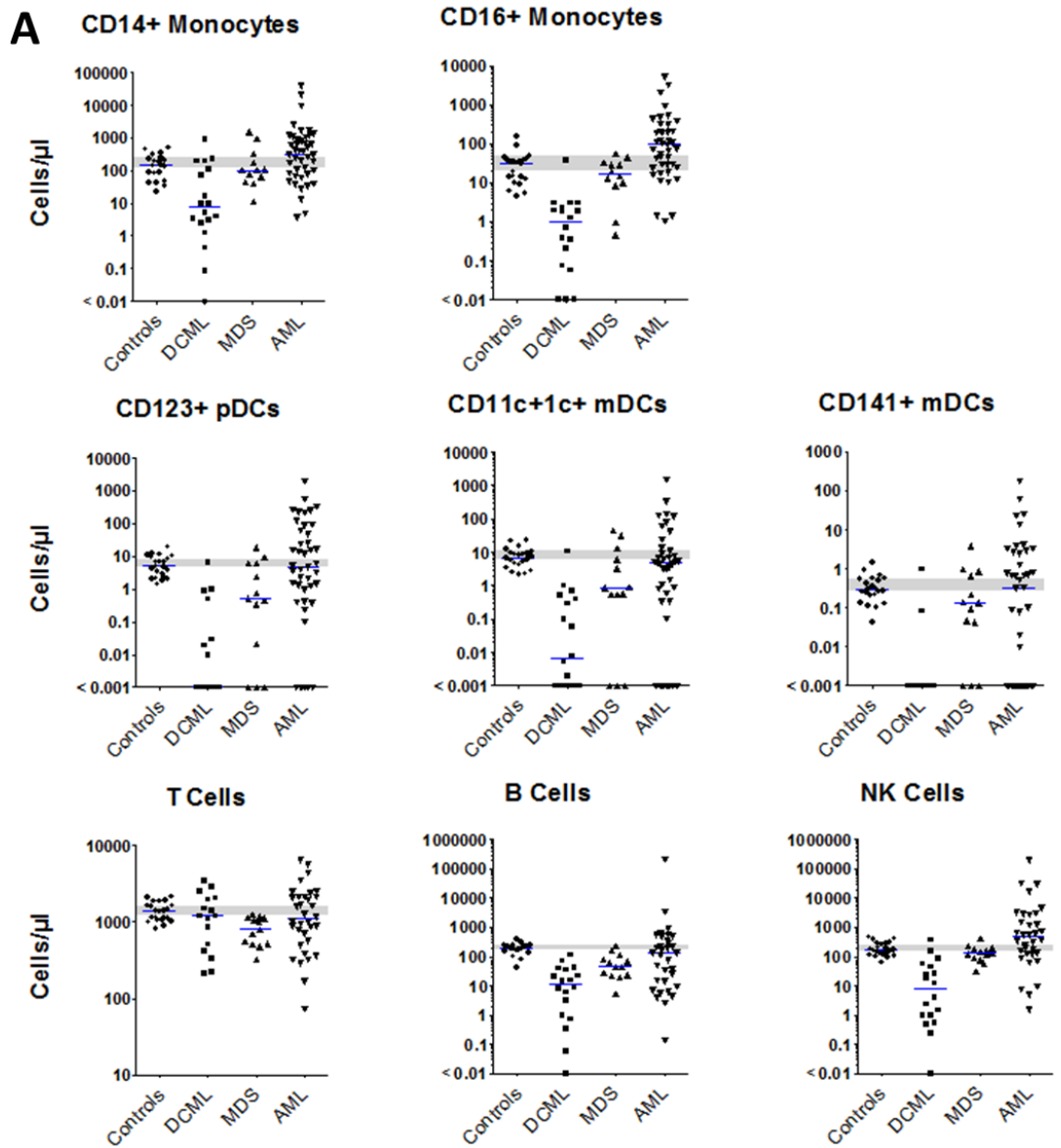
B

	Controls vs DCML	Controls vs MDS	Controls vs AML	DCML vs MDS	DCML vs AML	MDS vs AML
CD14+ Monocytes	*	ns	ns	ns	***	ns
CD16+ Monocytes	***	ns	ns	ns	***	*
CD123+ pDCs	***	ns	ns	ns	***	ns
CD1c+ mDCs	***	ns	ns	ns	***	ns
CD141+ mDCs	***	ns	ns	*	***	ns
T Cells	ns	ns	ns	ns	ns	ns
B Cells	***	*	ns	ns	***	ns
NK Cells	***	ns	ns	ns	***	ns

Figure 3.5. DCML Deficiency, MDS and AML Mononuclear Profiles

A) Comparison of automated diagnostic blood counts of patients with DCML Deficiency (n=18), MDS (n=13) and AML (n=42). Grey line is the standard range. Blue lines are medians.

B) Statistical analysis of the data in figure A. Statistics with Kruskal-Wallis with Dunn's Multiple Comparison Test. * = <0.05; ** = <0.01; *** = <0.001.

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3.3.4. *Fms-like Tyrosine Kinase 3 Ligand (FLT3L)*

In order to see whether FLT3L levels varied in the patients, serum from symptomatic DCML deficiency (n=18), tri-lineage MDS (n=12) and AML (n=36) patients were analysed for levels of FMS-Like Tyrosine Kinase 3 Ligand (FLT3L) using an Enzyme-Linked Immunosorbent Assay (ELISA) (Figure 3.6.A). Results indicated significantly elevated FLT3L for symptomatic *GATA2* mutation, and significantly decreased in AML (p=<0.0001 for both vs. normal and each other). In fact 24/36 AML patients did not have any detectable FLT3L above the limit of the assay (7pg/ml), and 3/8 of the remaining patients were below the normal range of 48.30 - 173.8pg/ml. The highest level of FLT3L in an AML patient was 119.634pg/ml. Both DCML deficiency and AML were also significantly different to MDS (p=<0.0001 for both), where there was a wide spread of FLT3L levels, some high; others low and one patient had undetectable levels, though this was not seen to be significantly different to normal controls.

As might be expected, there was a statistically significant correlation coefficient between % BM blasts and diagnostic laboratory WBC in AML patients (p=0.0152; Figure 3.6.B), therefore levels of FLT3L were compared to both of these factors. Neither % BM blasts nor WBC had a statistically significant correlation coefficient in comparison with FLT3L levels (Figure 3.6.C&D). Detectable or not detectable FLT3L in AML patients did not correlate with cytogenetics, FAB or WHO types, immunophenotypic types or blast percentage CD34 positivity, however CD14+ and CD16+ monocytes were increased in patients with undetectable FLT3L (p=<0.05, data not shown).
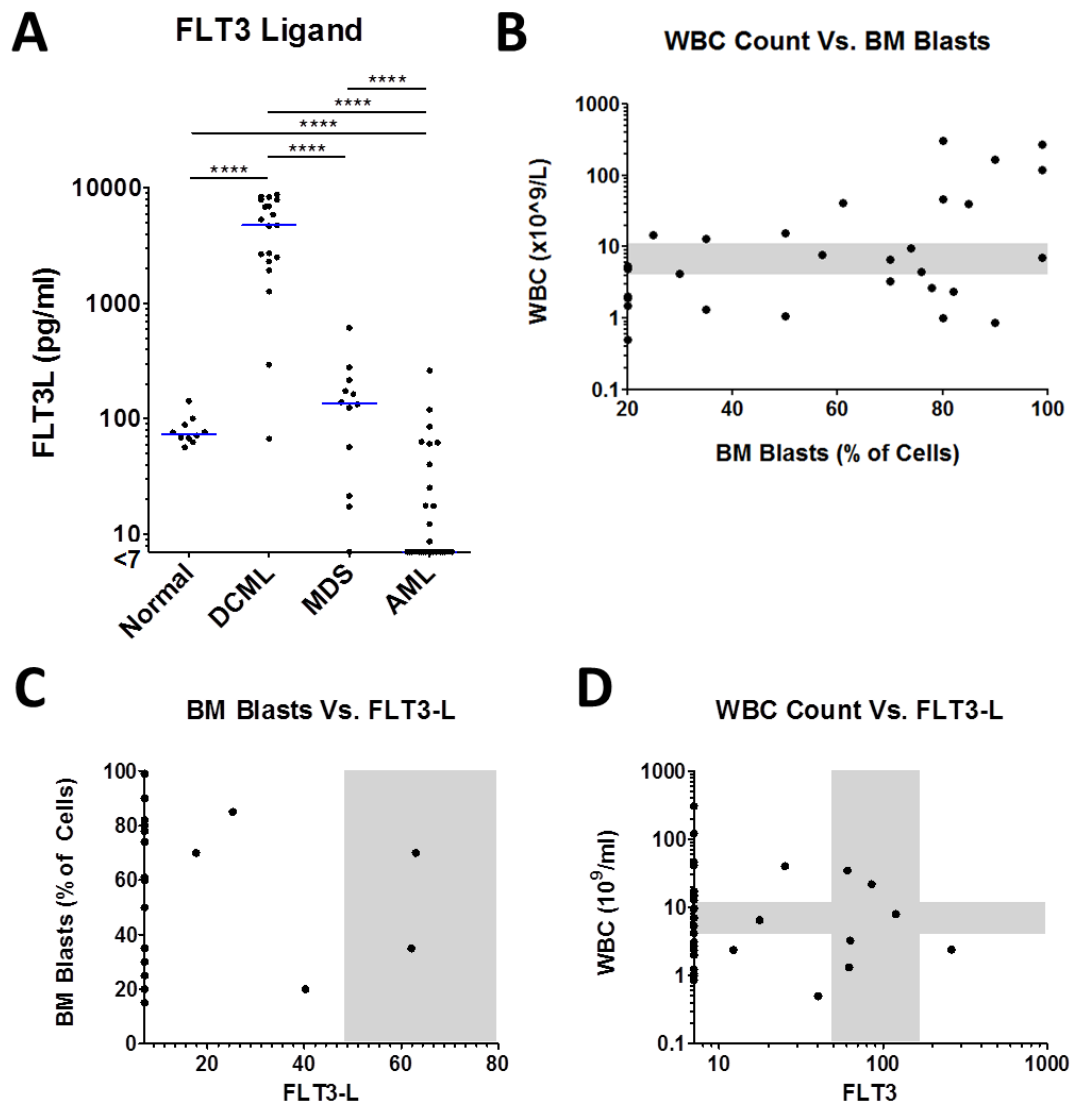
Figure 3.6. FLT3-Ligand Analysis

A) Comparison of serum levels of Fms-like tyrosine kinase 3 ligand (FLT3L) in patients with DCML deficiency (n=18), tri-lineage MDS (n=12), AML (n=36) and normal controls (n=10). Error bars indicate medians. For the purposes of statistics, readings below the ELISA readable sensitivity were analysed as 7pg/ml. Statistics were individual T-tests. **** = <0.0001. 24/36 AML patients had FLT3L <7pg/ml.

B) Comparison of diagnostic laboratory WBC readings versus BM blast count percentages. The diagnosis for AML is above 20% BM blasts.

C) Comparison of FLT3L serum levels versus BM Blast count percentage. Grey bars indicate normal ranges.

D) Comparison of FLT3L serum levels versus diagnostic laboratory WBC. Grey bars indicate normal ranges.

3.3.5. *AML Blast Cell Analysis*

In order to see if the results from the Goardon *et al* paper were reproducible, BM from 15 AML patients was analysed using the progenitor cell panel described in section2.6.3, pg.79(see Figure 3.7). In both CD34+ and CD34- AML patients, gating originated from the CD34+ cells in the bone marrow. In CD34- patients, blast cells were also gated for progenitor cell markers to confirm the phenotype (see Figure 3.7). Phenotypes matched between CD34+ cells and blast cells in all CD34- AML patients analysed. Two patients were excluded due to low cell numbers making analysis impossible. In the remaining thirteen patients; three immunophenotypic groups could be seen. 8/13 patients matched the GMP/MLP immunophenotype, 4/13 patients matched CMP/MPP immunophenotype. One final patient (A6492) represented a 'Hybrid' type, where the highest expansions were seen in the CMPs and MLPs, with lesser expansions in the GMPs, MPPs and HSCs (see Figure 3.7).

In pursuance of classification of AML patients where BM was unavailable, the flow cytometric progenitor cell panel was performed upon the PBMCs of 30 of the AML patients. Both BM and PBMC was thus analysed from 9 AML patients. For all 10 patients, the phenotypic type of leukaemia matched between BM and PBMC (see Table 3.1). Thus, PBMC progenitor immunophenotypes were used to further this analysis. Four patients were excluded due to low cell numbers making analysis impossible. From the PBMC results; 19/26 patients were GMP/MLP type, 6/26 patients were CMP/MPP type. The BPDCN patient with progenitor immunophenotyping data (A2577) had a GMP/MLP phenotype.

Comparative graphs of the different progenitor fractions in the two main immunophenotypes are given in Figure 3.8. HSCs, and MEPs were proportionally and statistically increased in CMP/MPP patients. Interestingly, all but one CMP/MPP patients were completely deficient in CD123^{high} cells.
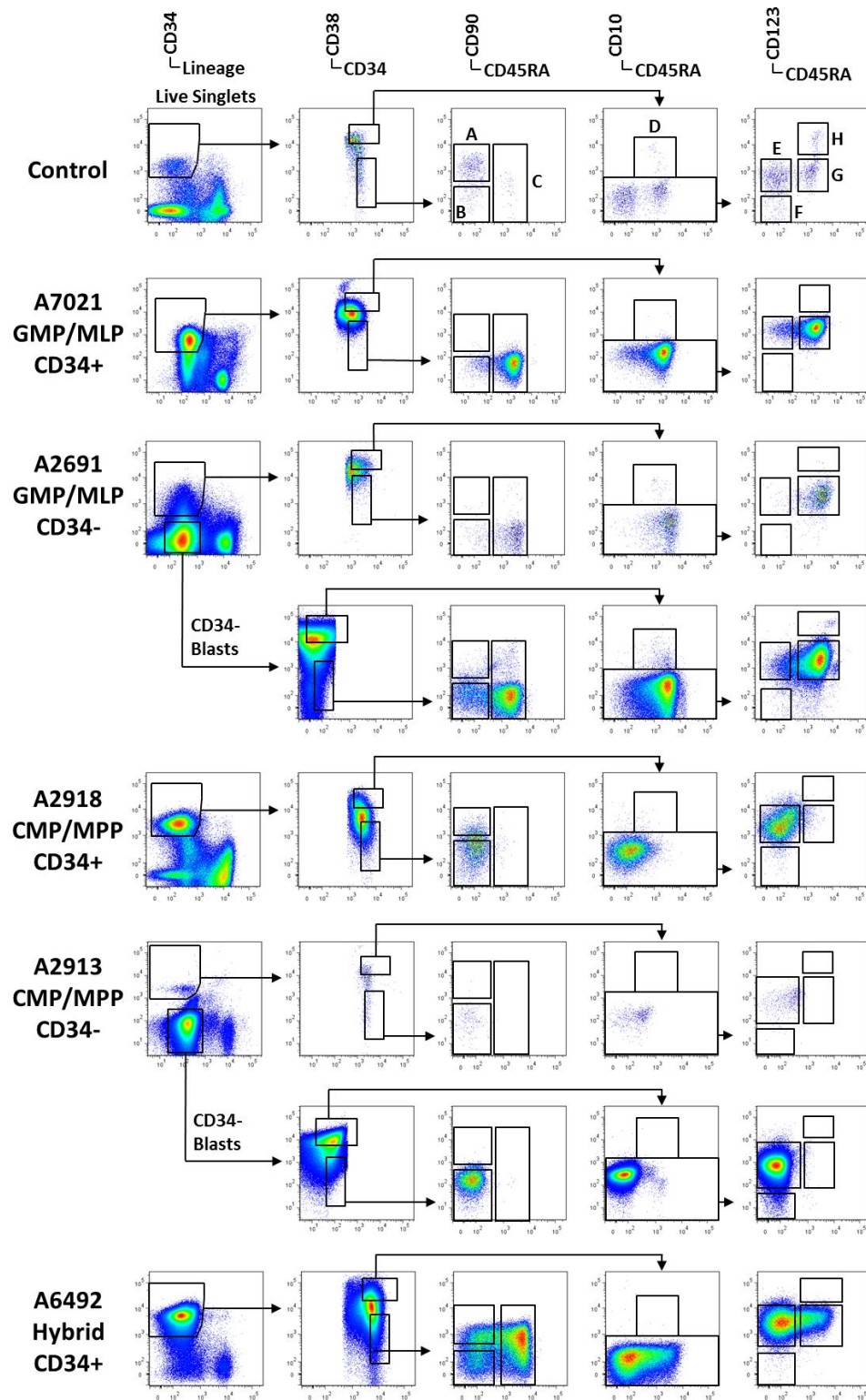
Figure 3.7. AML Progenitor Gating Examples

AML BM progenitor gating examples. In cases where blasts were CD34-, the blasts were gated using the same strategy as the CD34+ cells, in order to confirm the phenotype. A = Haematopoietic Stem Cells; B = Multi-Potent Progenitors; C = Multi-Lymphoid Progenitors; D = B/NK Cell Progenitors; E = Common Myeloid Progenitors; F = Megakaryocyte/Erythroid Progenitors; G = Granulocyte/Macrophage Progenitors; H = CD123^{high} unclassified fraction.
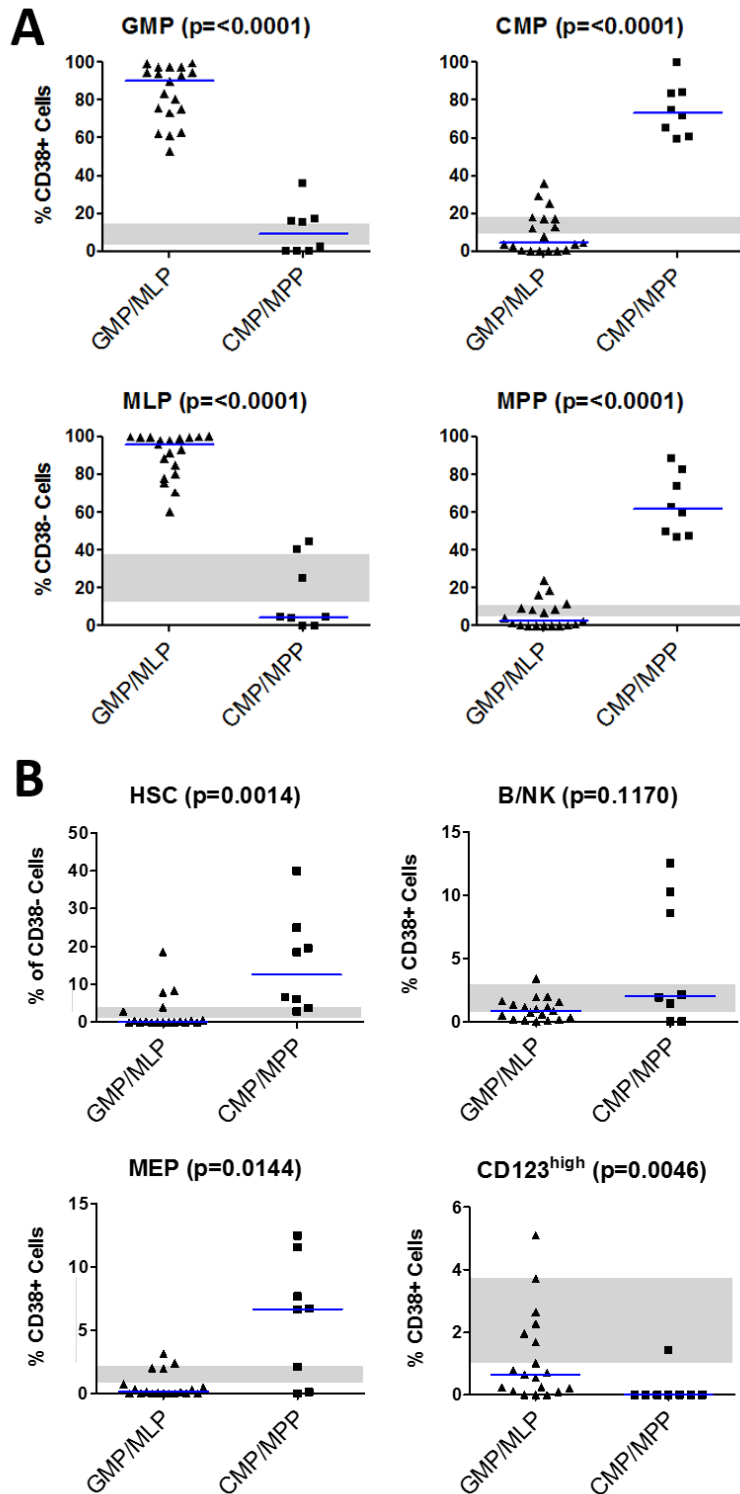
Figure 3.8. AML Patient PBMC CD34+ Cell Phenotyping Results

A. *Percentage composition of CD34+38+ cells by GMP and CMP and of CD38- cells by MLP and MPP as used in the detection of phenotypic blast type.*

B. *Percentage composition of CD34+38- cells by HSCs and CD38+ cells by B/NKs, MEPs and CD123^high cells.*

Blue error bars indicate medians. Grey bars are standard ranges derived from 10 normal PBMC samples. Statistics are Mann Whitney t tests.

3.3.6. *Mononuclear Subset Comparison by Phenotypic Blast Type*

In order to see whether the different phenotypic types of AML had varying effects on the normal mononuclear cell content of the peripheral blood, the previously derived data on circulating mononuclear cell subsets were re-analysed from 30 AML patients where phenotypic typing results were available (Figure 3.9).

Results varied for all cell types. The GMP/MLP group had a significantly higher median level of CD14+ monocytes and CD1c+ mDCs than the CMP/MPP group. The CMP/MPP group had a significant decrease in B cells, whereas the median of the GMP/MLP was within the normal range. The GMP/MLP group appeared to have higher levels of CD16+ monocytes, and in the CMP/MPP group DCs appeared reduced, however these results were not statistically significant.
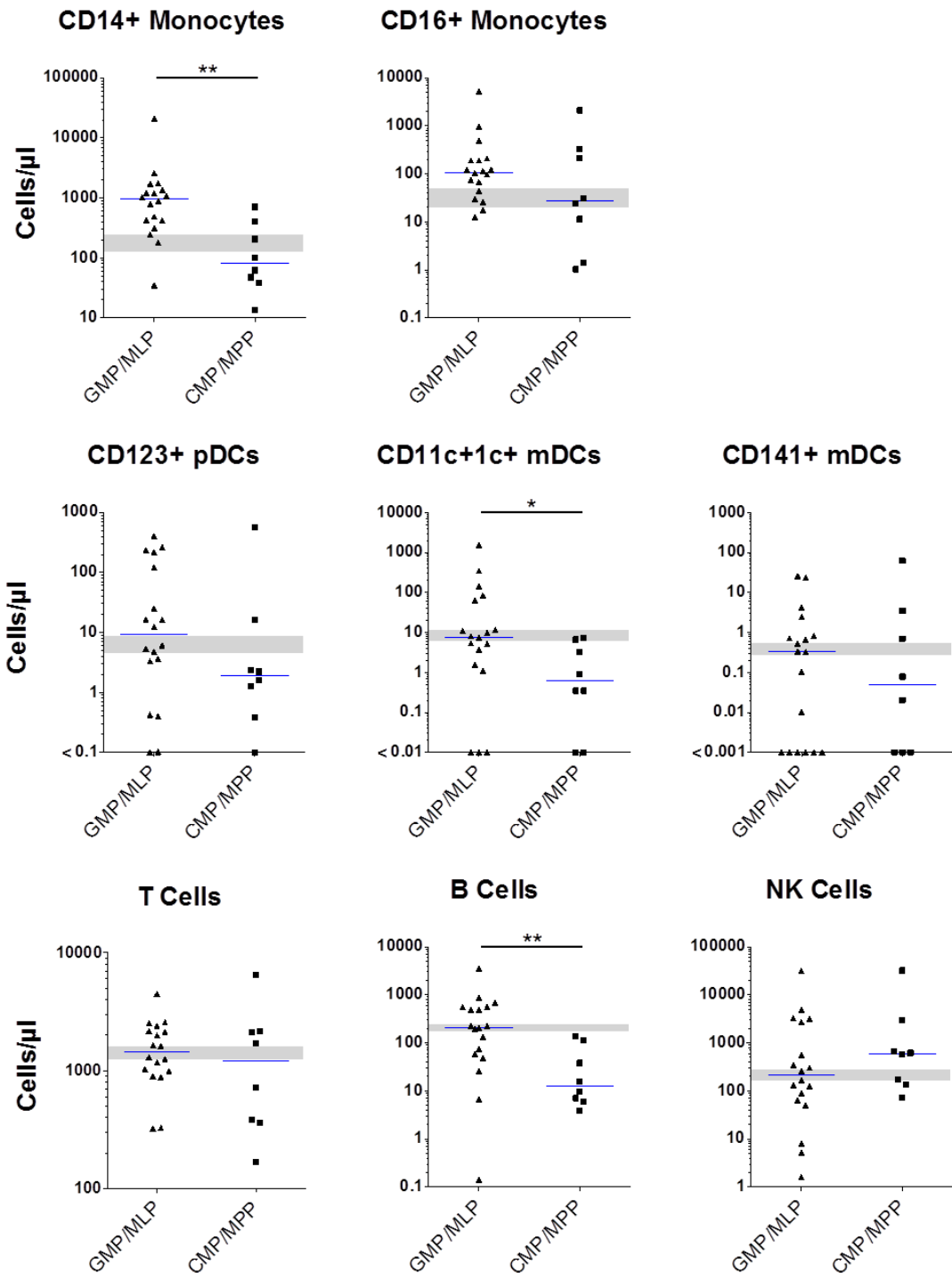
Figure 3.9. 'Goardon' Type Mononuclear Cell Analysis

Comparison of controls (n=21, grey bars, indicating 95% CI) and patients with GMP/MLP type blasts (n=20) and CMP/MPP type blasts (n=8) by TruCOUNT analysis. Analysis was performed as previously described. pDC: plasmacytoid Dendritic Cell; mDC myeloid Dendritic Cell. Error bars show medians. Statistical analysis was Mann Whitney t tests.* p=<0.05; ** p=<0.01.

3.3.7. *AML Blast DC Marker Clustering Analysis*

To examine the expression of DC related markers on the blastic cells of AML, and to see if these expressions could be used to group AML subtypes, blast cells were isolated. The majority of blast cells appeared to fall into two groups visually. These were Lineage-DR- blasts or Lineage-DR+CD14-16-34+ blasts. No blast cells were seen which were positive for CD16, CD141 or CD1c.

After the blast cells were isolated, the Mean Fluorescence Intensity (MFI) of each marker on the cells could be collected (n=29). This included MFIs for HLA-DR, Lineage (CD3,19,20,56), CD14, CD16, CD123, CD34, CD141, CD11c, CD141 and CD1c. These data were then clustered using the statistical software 'R'.

Clustering analysis showed that the AML patients could be split into 5 groups by DC marker MFIs (Figure 3.10.A). Cluster 1 and 2 patients look very similar visually, though CD34 expression was higher and CD14 expression lower in cluster 1.The third cluster of patients had a 'null' phenotype, negative for all DC panel markers. Cluster 4 patients had Lineage-DR^{low}CD14-16-123^{low}11c-141-1c- blasts, which could be either CD34 positive or negative. Finally cluster 5 was specific for a single BPDCN patient, where the blasts were Lineage-DR+CD16-123^{low}34-11c-141-1c-. Flow cytometric examples and representations of each clustering type are given in Figure 3.10.B.
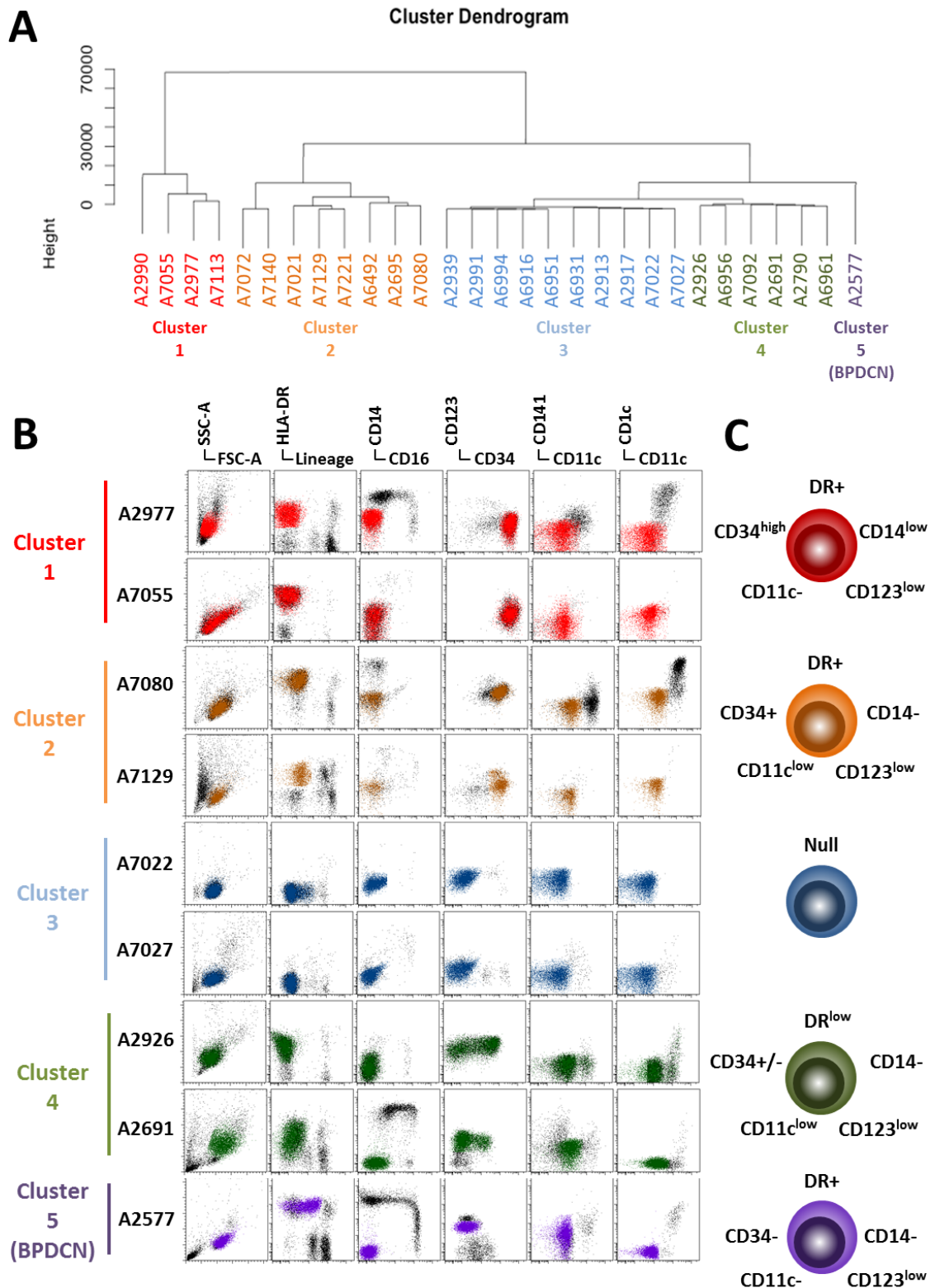
Figure 3.10. AML DC Marker MFI Clustering Analysis

A) Cluster dendrogram of AML patient (n=29) based on mean fluorescence intensity (MFI) of DC related flow cytometry markers.

B) Flow cytometry examples of each clustered AML type. Red dots are blast cells, black dots are normally differentiated cells.

C) Illustrative examples of the markers identifying each clustered AML type.

3.3.8. *AML Comparison Summary Tables*

Where available, data from the previous analyses were cross-compared in order to see if the progenitor and mature marker immunophenotypes were related, and to correlate with cytogenetics (Table 3.2).

Patients with the GMP/MLP progenitor immunophenotype had a wide range of mature immunophenotypes and cytogenetics. Only one GMP/MLP patient had the 'null' phenotype of cluster 3. Nine patients had normal cytogenetics and 4 patients had complex abnormalities. The BPDCN patient had a normal karyotype, though due to the nature of BPDCN, this patient had an adverse phenotype. There were no blast phenotyping data available from the final patient with complex abnormalities.

All five CMP/MPP patients were seen to have the mature phenotype of cluster 3, where all mature cell markers are negative. Three of these patients had a normal karyotype, and two had 15;17 translocations. Of the three CMP/MPP not shown, two had a normal karyotype and one carried a Deletion 5Q mutation. No 15;17 translocations or 5Q deletions were seen in any of the GMP/MLP type leukaemias.

Similarly, only AML patients with blasts of mature phenotype cluster 3 appeared to have an association with WHO or FAB type, being exclusive to AML with recurrent genetic abnormalities or AML not otherwise specified in the WHO classification system and to the M0 and M3 FAB type AMLs (see Table 3.2).

Cytogenetic Risk Group	Cytogenetic Abnormality	GMP/MLP (CD45RA+)	CMP/MPP (CD45RA+)
Favourable	Translocation 15;17		③ ③
	Translocation 8;21	①	
	Inversion 16	①	
	Deletion 9	②	
Intermediate	Normal	② ② ② ② ③ ④ ④ ④	③ ③ ③
	Translocaton 2;3	④	
	Trisomy 15	②	
Adverse	Complex*	①C3 ①C4 ②C5 ④C2	
	Normal (BPDCN)	⑤	

Table 3.2. Comparison of phenotypic and clustered blast types.

Summary of cytogenetics, phenotypic blast type and cluster blast type (where known). Cytogenetic risk groups based on Grimwade et al, 2010.

① =Cluster 1; ② = Cluster 2; ③ = Cluster 3; ④ = Cluster 4; ⑤ = Cluster 5. BPDCN = Blastic Plasmacytoid Dendritic Cell Neoplasm

* Complex cytogenetics given below; indicated by superscript numbers:
Complex 1: *47,XY,t(1;12)(q25;q24),del(4)(q31),del(5)(q1?3),add(15)(p11.1),-18,-22,-22,+4mar [7] /46,XY [3] (not shown in table)*
Complex 2: *46,XX,del(5)(q22q35), del(12)(p1?)[11] flt3- npm1-*
Complex 3: *45,XY,der(3)add(3)(p1?3) add(3)(q27),7,add(12)(1?2),add(12)(p13),-13,+mar [6] FISH: MECOM REARRANGED [99/100]*
Complex 4: *45~47,XY,inv(3)(q21q26),-7,+8[cp6], FISH:MECOM NOT rearranged [100]*
Complex 5: *45~46,XY,dic(2;?)(q37;?),add(3)(p2?),add(3)(q2?),5,-12,-12,del(17)(p1?),-22, -22,+5~7mar[5]*

A

WHO Type	Cytogenetic Abnormality	GMP/MLP (CD45RA+)	CMP/MPP (CD45RA+)
AML with Recurrent Genetic Abnormalities	Translocation 15;17		③ ③
	Translocation 8;21	①	
	Inversion 16	①	
	NPM1+/-FTL3ITD	② ④	③
AML with Myelodysplasia Related Changes	Deletion 9	②	
	Translocaton2;3	④	
	Complex	① C3 ① C4 ② C5 ④ C2	
AML Not Otherwise Specified	Trisomy 15	②	
	Normal	② ② ② ③ ④ ④	③
BPDCN	Normal	⑤	

B

FAB Type	GMP/MLP (CD45RA+)	CMP/MPP (CD45RA+)
M0	① C3 ② ③	③
M1	② ④	
M2	①	
M3		③ ③
M4	① ② C5	
M5	② ④	

Figure 3.11. Comparison of Blast Types and WHO/FAB Classifications

Summary of WHO/FAB AML types, cytogenetics, phenotypic blast type and cluster blast type (where known).

① =Cluster 1; ② = Cluster 2; ③ = Cluster 3; ④ = Cluster 4; ⑤ = Cluster 5.

WHO = World Health Organisation AML Classification System

FAB = French-American-British AML Classification

For details of classification systems, see section 1.4.2.1, page 28.

BPDCN = Blastic Plasmacytoid Dendritic Cell Neoplasm

* Complex cytogenetics given on previous page

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3.4. Discussions for Chapter Three

This chapter describes the comparative analysis of DCML deficiency, MDS and AML patients in terms of; major diagnostic parameters, cell profiling of normal mononuclear cell fractions and levels of FLT3L in serum. Also more detailed analysis was performed on the blast cells of AML patients in terms of phenotypic markers (related to progenitor cells and mature cells) and cytogenetics.

3.4.1. *Comparative Phenotyping of DCML Deficiency and MDS*

Although *GATA2* mutation is a constitutive genetic risk for developing MDS (Hahn *et al.*, 2011; Hsu *et al.*, 2011; Holme *et al.*, 2012; Kazenwadel *et al.*, 2012; Pasquet *et al.*, 2013), patients with *GATA2* mutation may be distinguished from those with acquired MDS and AML on several grounds. *GATA2* mutation is associated with a much younger age of presentation, better preserved haemoglobin, neutrophils and platelets and much more severe defects of DCs, monocytes, and lymphoid cells than patients with MDS and AML. The observation that control MDS patients did not have *GATA2* mutations is consistent with a recent large cohort study showing an incidence of mutation in only 4 of 603 MDS patients (Papaemmanuil *et al.*, 2013).

Overall, it was seen that GATA2 mutation causes a specific mononuclear cell loss prior to the development of leukaemia. This is distinct from sporadic MDS, where there is a less severe and more generalised tri-lineage failure of haematopoiesis. Now that I know that the profile of cell loss differs between GATA2 mutation and MDS, it would be interesting to explore if the cells in both diseases are lost due to different mechanisms.

3.4.2. *Immune Dysfunction in Acute Leukaemia*

Leukaemia is a known cause of bone marrow failure (BMF); however the specific order of failure has not previously been explored. The hypothesis proposed in this thesis was that leukaemia has an 'extended

phenotype' of cellular immunodeficiency relating to the progenitor compartment from which it arises.

In a small cohort of patients (n=30), it was seen that variable patterns of normal cell loss can be seen in *de novo* AML, however the majority of patients had an unexpectedly intact level of immune cell differentiation even at high levels of BM blasts and/or circulating WBCs. This finding is surprising as it has been assumed that the normal progeny of a stem cell compartment will contract when displaced by leukaemic clones, owing to impaired differentiation. The next stage of this line of research would be to define whether these circulating 'normal' mononuclear cells are indeed normal, or are in fact part of the AML clone.

Although large degrees of variation were seen, some patients with expanded subsets and others with specific deficiencies, the only subset significantly altered in MDS or AML was B cells in MDS patients which were reduced from normal. Interestingly, some AML patients had expansions of DC numbers, while others were completely deficient in all three DC subtypes. Also, patients with acquired MDS also had mild mononuclear cytopenias of pDCs, and both classical and non-classical monocytes.

The theory of an extended phenotype of acute leukaemia assumes a model of haematopoiesis based on the most recently published data with the caveat that certain features (e.g. the possible LMPP/MLP split in the murine haematopoietic system, see section 1.1.4, pg. 7), are disputed. These are just the closest approximations; it is likely that models of haematopoiesis will change significantly in the coming years.

3.4.3. *Serum FLT3-Ligand in Haematological Disorders*

FLT3L levels were significantly increased in symptomatic DCML deficiency patients seen in this study, whereas DCML deficiency patients who progress to MDS have normal levels of FLT3L (Dickinson *et al*., 2014), similar to the levels seen in the control tri-lineage MDS patients. These results suggest a biphasic relationship, that FLT3L becomes

progressively elevated in cases of DCML deficiency but then declines as MDS develops. Levels of serum FLT3L in sporadic MDS patients were not significantly different from normal. In a study by Zwierzina *et al*, there was a correlation seen between WHO MDS types and serum FLT3L levels (Zwierzina *et al.*, 1999). Unfortunately data on WHO/FAB types were unavailable for the MDS patients in this thesis.

In previous studies, high rates of mutation in the FLT3 gene, which encodes the receptor for FLT3L have been seen in AML (Yamamoto *et al.*, 2001). FLT3L is vital for the production of DCs in the steady-state (Shortman and Naik, 2007; Rahman and Aloman, 2013). Mice deficient in FLT3L have been seen to have reduced DC numbers (McKenna *et al.*, 2000), whereas administration of FLT3L to mice or healthy humans has been shown to substantially increase DC numbers (Maraskovsky *et al.*, 1996; Maraskovsky *et al.*, 2000). It is unclear which cell types produce the FLT3L required for DC maturation, though some suggestions are T cells (Saito *et al.*, 2013) or bone marrow endothelial cells (Solanilla *et al.*, 2000). In this thesis, AML patients were seen to have significantly decreased levels of FLT3L at diagnosis, quite often lower than the detectable range of the ELISA assay. Also, the blast cells of the AML patients in this study were seen to bear little or no mature myeloid cell markers. These two observations could suggest that the deficiency of FLT3L in the AML patients could lead to an inhibition of maturation of the blastic cells.

3.4.4. *AML Blast Cell Progenitor Phenotype*

The study by Goardon *et al*, involved phenotyping the blast cells of CD34+ (>5%) AML patients using progenitor cell markers (Goardon *et al.*, 2011). In that study, two immunophenotypic patterns of AML were observed; 87.8% of patients were GMP/MLP type and 13.8% of patients, were CMP/MPP type.

In this thesis, it was possible to reproduce the AML typing data seen in the Goardon *et al* paper, using both BM and PBMC samples. It

was seen in nine patients that the progenitor immunophenotypic pattern of blasts in the BM, was reproduced in the blasts of the PBMCs, therefore the pattern observed in either sample type can be used in this analysis.

In the paper by Goardon *et al*, patients were excluded when blasts were less than 5% positive for CD34. In this thesis I was able to include CD34- AML patients in the analysis by first analysing the progenitor profile of the CD34+ fractions of cells, and then comparing to the phenotypic profile of the actual blastic cells using the same gating strategy. In all eight CD34- AMLs analysed in this study, the phenotypic bias seen in the CD34+ progenitor cells was matched within the phenotypic profile of the blastic cells.

This analysis of both CD34+ and CD34- AML patients led to a disparity in the proportion of blast cell progenitor immunophenotypic patterns, with a slightly higher rate of CMP/MPP patients (63% GMP/MLP; 24% CMP/MPP). In this thesis it was seen that nearly all GMP/MLP patients had a CD34 expression greater than 5% (14/15) whereas the majority CMP/MPP patients had a CD34 expression of less than 5% (6/8). This was in fact a statistically significant difference between the two AML types (p=0.0023).

In terms of extended phenotyping, it was seen that patients with the GMP/MLP phenotype had a better preserved mononuclear cell profile, including DC and lymph levels averaging on normal and expanded levels of monocytes.

Interestingly, a third blast cell progenitor immunophenotypic pattern was observed in one patient (A6492). In this patient there were clear expansions of the CMP and MLP progenitor groups, although there were also lesser expansions of GMPs, MPPs and HSCs, while B/NK and MEP progenitors were almost completely absent. This patient had an extremely high level of CD34 positivity (91%), and appeared to have normal levels of T and B cells, with slightly increased monocyte, pDC and CD141+ mDC fractions. NK cells looked to be greatly increased whereas CD1c+ mDCs

were completely absent (data not shown). It would be interesting to gather more data on this patient to observe possible differences from the two main patterns.

3.4.5. *AML Blast Cell Mature Immunophenotypic Type*

Secondary to the progenitor-marker immunophenotypic patterns discussed above, AML patients were also bioinformatically clustered according the MFIs of DC-related markers upon the blast cells, the 'mature immunophenotype'. This is a form of analysis not attempted before in AML, and may give important information about the relationship between AML blasts and DC lineages. FSC-A and SSC-A MFIs were added in an additional analysis, but these were found to confound the data, most likely due to the high levels of variability of these measurements (data not shown). In this analysis it was seen that the AML patients (n=29) could be clustered into 5 distinct groups.

Interestingly, it was seen that 5/5 of the CMP/MLP patients with clustering data available, fell into cluster 3, which had a 'null' phenotype, with no obvious expression of any DC related markers. The GMP/MLP patients fell into all five clusters, 4/18 in cluster 1; 6/18 in cluster 2; 1/18 in cluster 3, 5/18 in cluster 4. One BPDCN patient, who also displayed a GMP/MLP phenotype was included in the analysis and clustered separately from all other patients (cluster 5), which is characterised by HLA-DR positivity and CD34 negativity.

3.4.6. *Genetic Abnormalities and the Extended Phenotype of AML*

In the Goardon *et al* study, no correlations were found between morphology or cytogenetics and Blast Cell Immunophenotypic Patterns. However, in this thesis, I have seen that the CMP/MPP pattern correlates with both clustering analysis as a mature marker 'null' phenotype, as well as cytogenetics, notably 15:17 translocations and normal karyotype. These correlations were likely missed by the Goardon *et al* group due to the exclusion of CD34- patients, which overall account for ~32-55% of AML patients (Del Poeta *et al.*, 1994; Krasinskas *et al.*, 1998; Legrand *et*

al., 2000; Thalhammer-Scherrer *et al.*, 2002). As patients with normal cytogenetics were found in both immunophenotypic types, there is a possibility that these patients could be grouped and observed for any outcome differences, potentially splitting the intermediate risk group.

3.4.7. *Summary*

Using techniques established earlier in this thesis, I have successfully derived peripheral blood and bone marrow phenotypic profiles in DCML deficiency, MDS and AML. I have seen that both DCML deficiency and MDS have deficiencies in mononuclear cells, though the reduction is less severe in MDS, while in AML, mononuclear differentiation is remarkably well preserved.

Secondly, I have seen that serum levels of FLT3L are vastly increased in DCML deficiency patients, and almost completely abated in AML patients. FLT3is a gene with a high frequency of mutation in AML patients (Yamamoto *et al.*, 2001), so this is an interesting finding, and may be exploitable in a clinical setting.

Also, I have reported in AML that the phenotypic profiles of the blastic cells can be used to group different types by both the previously described progenitor cell phenotyping analysis or by clustering analysis of the DC-related markers. These groups, particularly the CMP/MPP, cluster 3 types showed correlations to normal and translocation 15;17 cytogenetic groups, which may be able to be exploited in the future.

The final aims of this chapter were to attempt to correlate the extended phenotype of AML patients with FAB/WHO types, rates of infection and extra-medullary disease. Unfortunately, due to the low numbers of patients recruited into the study, and the unavailability of data on FAB/WHO types, these analyses were not performed.

Chapter 4. *BRAF*^{V600E} Mutation Detection Assay Setup

4.1. Introduction

This chapter describes the development of methods to analyse the *BRAF*^{V600E} mutational allele levels of sorted cell types using real-time PCR (RT-PCR).

4.1.1. *Chapter Aims*

1) To develop polymerase chain reaction (PCR) protocols to detect *BRAF*^{V600E} in lesions and sorted peripheral blood and bone marrow fractions.

4.2. Methods of Detection of *BRAF*^{V600E}

As technology is expanding at an exponential rate, new and modified methods of mutation detection arise each year. The following is a brief summary of the current methods available for the detection of *BRAF*^{V600E}. For a full summary of the sequencing, mass spectrometry and melting curve analysis techniques, plus 454 Pyrosequencing and Cobas 4800 BRAF test, see (Curry *et al.*, 2012).

4.2.1. *First Generation Sequencing - Sanger Sequencing*

Sanger sequencing, pioneered in the 1970s (Sanger and Coulson, 1975; Smith *et al.*, 1986), involves DNA polymerase controlled selective incorporation of chain-terminating dideoxynucleotides during DNA replication. This technique has been largely supplanted by next-generation sequencing for large scale genome work, but is still often used for single gene confirmationary studies, including for *BRAF*^{V600E} in Histiocytic disorders (Go *et al.*, 2014).

4.2.2. *Sequenom (Mass Spectrometry)*

The company Sequenom provides a mass-spectrometry based method for diagnostic laboratory*BRAF*^{V600E} testing in lesions, and is typically used for Formalin Fixed Paraffin Embedded (FFPE) samples. The Sequenom protocol has a sensitivity of 10%, however, the diagnostic

laboratory warns of the possibility of false negatives under 30% (NewGene, 2014; Sherwood *et al.*, 2014).

4.2.3. *Melting Curve Analysis*

PCR product double stranded DNA has specific melting temperatures dependant on the sequence of bases in the strand. Melting curve analysis depends on these slight differences to tell between PCR products containing the series of bases specific to wild-type BRAF compared to mutated BRAF (Ansevin *et al.*, 1976; Ririe *et al.*, 1997). This method has a high sensitivity to detect mutations, however as it only shows a melting point difference compared to normal, it is not possible to tell whether the mutation detected is $BRAF^{V600E}$ or another mutation.

4.2.4. *Next Generation Sequencing - Pyrosequencing*

Pyrosequencing is a form of next-generation sequencing which differs from Sanger sequencing, in that instead of detecting chain-terminating dideoxynucleotides, it detects the release of pyrophosphates when certain nucleotides are incorporated (Ronaghi *et al.*, 1996; Ronaghi *et al.*, 1998). The benefit of pyrosequencing is that it has a much greater sensitivity than Sanger sequencing, it is able to detect the $BRAF^{V600E}$ mutation down to a percentage of 2%, rather than the limit of detection of 20% of Sanger sequencing (Tan *et al.*, 2008). Recently, pyrosequencing was used on whole blood (23 patients) and CD14+ monocytes (7 patients) of LCH patients, with no positivity in either group, with a limit of detection of 1-2% (Satoh *et al.*, 2012).

4.2.5. *VE1 Antibody*

Recently, a $BRAF^{V600E}$ specific antibody has been developed which can differentiate between wild-type and V600E mutated BRAF (Andrulis *et al.*, 2012), and has been used for detection of lesional $BRAF^{V600E}$ in LCH (Sahm *et al.*, 2012). Although this antibody is chiefly used for FFPE samples, it may be useful in this study for detection of the mutation on cytospins of cellular fractions which are in too small a quantity for allele-specific PCR. However several studies have noted that the VE1 antibody

has a low sensitivity (71%) and specificity (74%) and can have non-specific nuclear staining (Kuan *et al.*; Adackapara *et al.*, 2013). Indeed in personal experience I have seen that the VE1 antibody can have low-level non-specific staining of macrophages in healthy controls (data not shown). Therefore much care must be taken when interpreting these results, strong staining is likely to be true positive, but weak staining may be low or false positive.

4.2.6. *Allele Specific PCR*

Allele-specific PCR can be used as a direct detection method for Single Nucleotide Polymorphisms (SNPs), by enzymatic amplification of the specific DNA sequence to be studied by oligonucleotide primers. The primers are specific for the mutation or wild-type and produce different size products, so a difference can be observed when running the products out on polyacrylamide gel. Recently, allele specific Mutation Detection RT-PCR Assays have been produced by several companies which can detect both wild type and SNP from a sample, and give a result of quantity based on fluorescent probes, removing the need for gel analysis. These techniques can have specificity down to 0.1%, and were therefore chosen for this analysis. Recently the techniques have been used to show that $BRAF^{V600E}$ can be found in the peripheral blood of LCH patients, specifically in the CD14+ and CD11c+ cells (Berres *et al.*, 2014).

Allele specific PCR is the method of choice for this thesis due to its high levels of sensitivity and specificity, cost-effectiveness and lower DNA input requirements and current expertise in the lab. It was therefore necessary to optimise methods of cellular subset sorting, DNA extraction and quantification specific for this study, and the best allele specific PCR assay to use.

4.3. Cellular Composition of the 'Neutrophil Cell Pellet'

Monocyte, DC and lymphocyte subsets were sorted from lymphoprepped PBMCs by the methods described previously (see section 2.4.2, pg. 71). However, due to the higher density of neutrophils, they cannot be isolated from peripheral blood in the same way. Therefore it was important to determine the exact cellular composition of the 'neutrophil pellet' after lymphoprepping samples, to ensure that this could be defined as a pure neutrophil fraction in further experiments in this thesis.

As described in section 2.3.1 (pg. 68), neutrophils were instead isolated from the cell pellet formed at the bottom of the tube during lymphoprepping of samples (Figure 4.1.A). As this isolation is based only on density centrifugation, it was important to ascertain the true cellular content of the cell pellet. This was achieved by running 6 'neutrophil cell pellets' from fresh LCH patient samples on the DC profile panel discussed insection2.6.1, pg. 76.

Neutrophils were distinguished by their high SSC-A, medium FSC-A and positivity for CD11c (Figure 4.1.B). The majority (66-88%) of events were neutrophils, there was also 9-20% debris and <2% 'other cells' (Figure 4.1.C). From the 2% 'other cells', 40-85% (0.8-1.7% of total events) were T cells, and 11-44% (0.2-0.9% of total events) were HLA-DR-Lineage- cells (Figure 4.1.D). All other cell types constituted <4% of the 'other cell' fraction, <0.08% of the total.
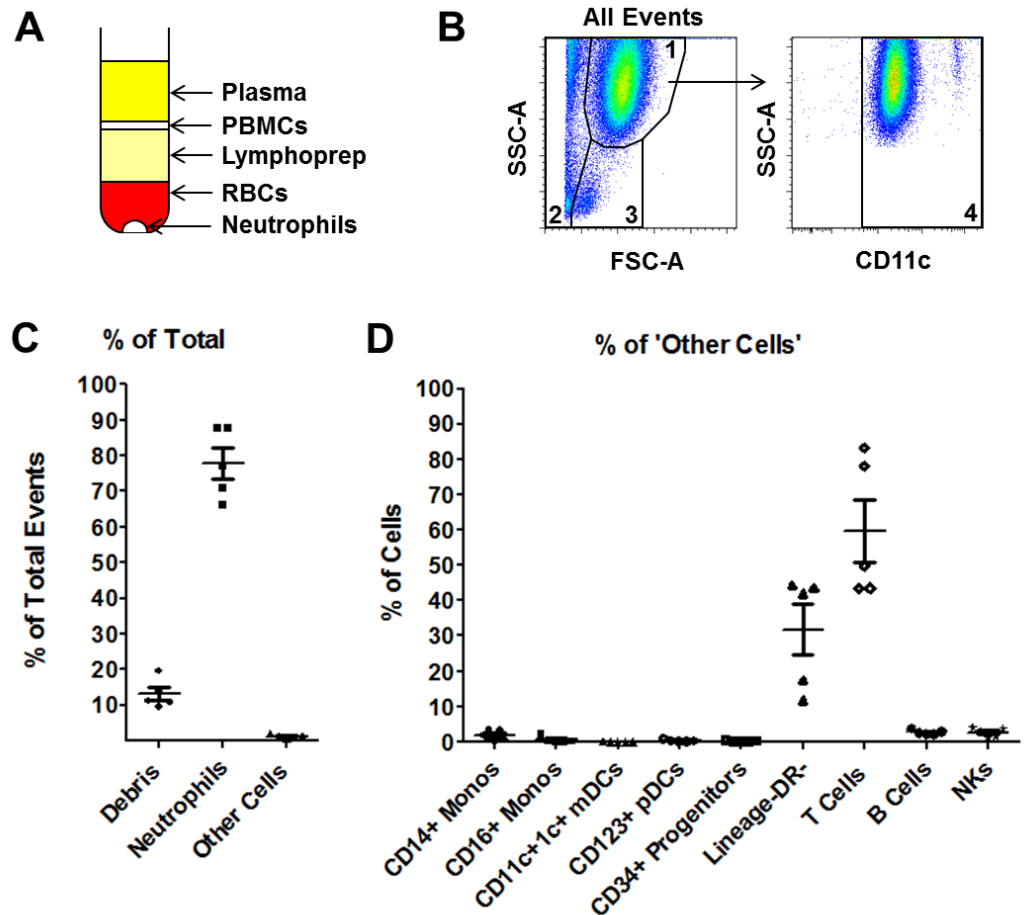
Figure 4.1. Cellular Composition of the 'Neutrophil Cell Pellet'

A. *Diagrammatic representation of a sample post lymphoprep. Showing density based separation of plasma, PBMCs, lymphoprep solution, RBCs and the 'neutrophil cell pellet'.*

B. *Flow cytometry schematic of neutrophil isolation from the 'neutrophil cell pellet'. Neutrophils are identified from all events by high SSC-A and medium FSC-A (1) and then by positive expression of CD11c (4). Contaminates include debris (2) and 'other' cells (3).*

C. *Percentage of total events which are debris, neutrophils or 'other' cells of the fresh 'neutrophil cell pellets' of six LCH patients. Showing the majority of events (66-88%) to be true neutrophils.*

D. *Composition of the 'other' cell gate of (3) by percentage of cell types (see section Figure 2.2, pg. 77for gating strategy) 40-85% (0.8-1.7% of total events) was T cells, and 11-44% (0.2-0.9% of total events) was HLA-DR-Lineage- cells.*

4.4. DNA Recovery Optimisation

The processes involved in sample preparation for the allele specific PCR assay are complex, including sample density centrifugation, cell staining and sorting, DNA extraction and finally PCR. There are several variable options to use during this processing, so the outcomes of certain variables were compared to optimise the results.

4.4.1. *Pre-Amplification*

One option to allow for a greater sensitivity of mutation detection would be to amplify the DNA pre-PCR. This would allow for small numbers of cells >1,000 per well to be analysed. For this study, pre-amplification was not employed, as it reduces the potential sensitivity of the assay. I.e. if less than 1,000 cells are used per well, it is impossible to say with certainty that the mutation can be detected down to 0.1%.

4.4.2. *NanoDrop Readings*

A total of 2-20ng DNA is recommended for use in the different allele specific PCR kits. In order to keep input DNA consistent, at the outset of testing a NanoDrop (www.nanodrop.com) was used to quantify DNA in the samples. However, the sensitivity of the NanoDrop analyser at the low concentrations of DNA expected from patient samples is unknown.

In order to test the sensitivity of the NanoDrop at low DNA concentrations three separate serial dilutions were created from normal PBMCs from three different donors, originally quantified by viCell analysis. These dilutions were at the following cell numbers: 100k, 50k, 10k, 1k, 100, 10, 1 and a further 10x dilution which would theoretically equate to 0.1 cells. DNA was extracted from these preparations using Qiagen DNA micro kits and the quantity of DNA was measured on the NanoDrop analyser. The average DNA content of mononuclear cells is 6pg(Baechtel, 1989), therefore an expected curve could be created of the maximum DNA quantity possible to extract from each set number of cells.

As can be seen in Figure 4.2.A, samples with a DNA concentration of less than ~10ng/µl, may have falsely high NanoDrop analyser concentration readings. Figure 4.2.B shows the NanoDrop analysed 260/280 and 260/230 ratios of DNA purity. As can be seen, there appears to be correlations between low DNA concentrations and less pure samples. This could be due to incorrect NanoDrop analysis at low DNA concentration, or there could be a link between low DNA concentration and higher levels of contaminates from the DNA extraction process.

As this study involves samples with small numbers of cells, and to make sure enough DNA was used in the PCR assay, it was decided to base the volume of DNA used on the original cell count prior to DNA extraction, instead of potentially inaccurate NanoDrop DNA concentration readings.
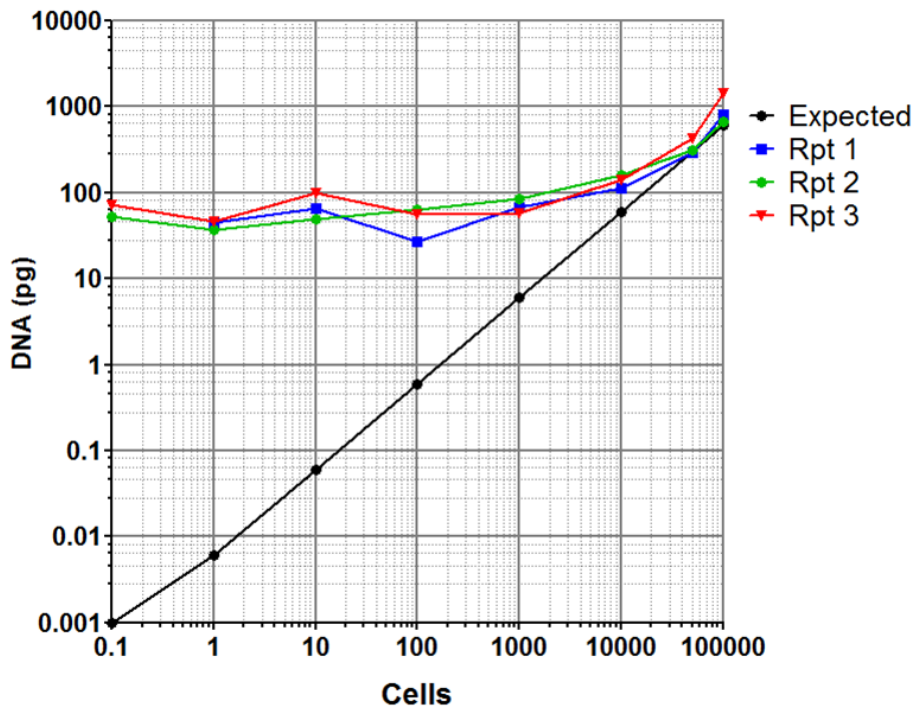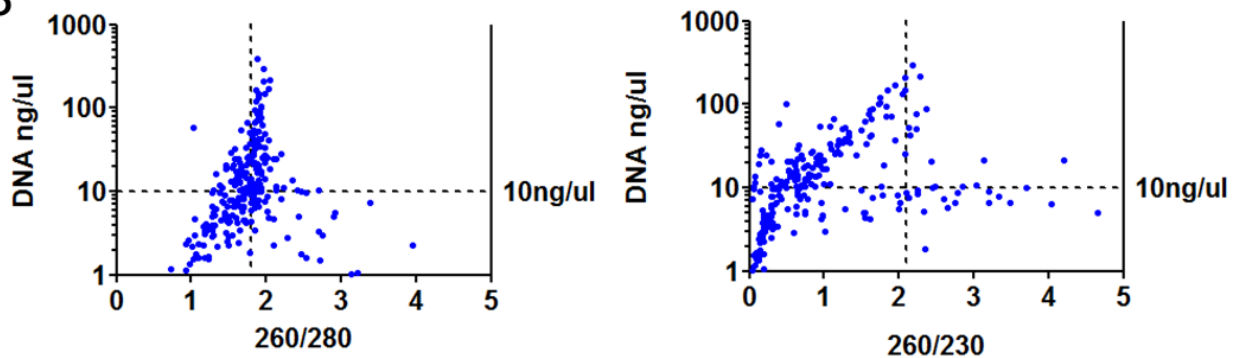
Figure 4.2. Known Cell Number NanoDrop Sensitivity Testing

A) NanoDrop readings (ng/µl) in samples of DNA extracted from set numbers of cells and eluted into 20µl. Black line indicates the expected maximum quantity of DNA extractable, based on 6pg DNA per cell. Coloured lines indicate 3 replicates of the sample preparation and DNA extraction.

B) NanoDrop 260/280 and 260/230 readings from samples with differing expected concentrations of DNA. DNA samples with 260/280 ratios of ~1.8 and 260:230 ratios of 2.0-2.2 are considered pure.

4.5. Available Mutation Detection RT-PCR Assays

There are currently three $BRAF^{V600E}$ specific mutation detection PCR assays available from; Qiagen (www.qiagen.com); Life Technologies (www.lifetechnologies.com) and GeneFirst (www.genefirst.com). The following section of this chapter covers a comparison of the available assays, in order to ensure that the most reliable and cost-efficient assay was used.

4.5.1. *Qiagen Assay*

The assay from Qiagen is a 'qBiomarker Somatic Mutation PCR Assay' which employs Amplification Refractory Mutation System (ARMS$^{®}$) primer-based allele discrimination and Hydrolysis Probe-based quantitative real-time PCR technology.

ARMS$^{®}$ technology, otherwise known as allele-specific PCR can be used to detect single base pair mutations. First described by Newton *et al*, (Newton *et al.*, 1989), the system works by splitting 5-10ng of DNA between two PCR wells; one containing a mutation primer specific for $BRAF^{V600E}$, the other containing a reference primer specific for a non-variable region of the BRAF gene (Figure 4.3). Taq polymerase is then able to discriminate between a match and a mismatch at the 3' prime end of the PCR primer.

Post RT-PCR, the threshold cycle (CT) values of each well are compared to get the delta CT (dCT). The reference primer specific well gives a quantification of the total number of the alleles in the well, and the mutation primer specific well gives the percentage of alleles which contain the $BRAF^{V600E}$ mutation. The CT value (CT^{Mut}) from the mutation specific assay is inversely correlated to the abundance of mutant DNA in the sample (Equation 4.1).

$$\text{Delta CT}^{Test} = Ct^{Mut} - Ct^{Ref}$$

Equation 4.1. Calculation of Delta CT (dCT) of the Test

The QIAGEN assay uses HotStart DNA Polymerase to prevent non-specific amplification during the set up process, before a 95°C, 10 minute activation step and a ROX passive reference dye to compensate for non-PCR related variations in fluorescence detection.

4.5.2. *Life Technologies Assay*

The assay from Life Technologies is a 'TaqMan® Mutation Detection Assay'. This works in the same way as the Qiagen, although it has two additional benefits; Competitive Allele-Specific TaqMan® PCR (castPCR), and optional Internal Positive Controls. CastPCR, developed by Life Technologies combines the mutation allele specific TaqMan® quantitative PCR (qPCR) with MGB oligonucleotide blockers which are specific for the wild-type allele, thus blocking any wild-type background amplification and allowing higher specificity and sensitivity than normal qPCRs (Figure 4.3). Life Technologies have performed spiking experiments which demonstrate that the assay sensitivity is below 0.1%.

The optional Internal Positive Control (IPC) can be duplexed with any TaqMan® Mutation Detection Assay, using an exogenous non-human DNA target and probe for a robust PCR-positive signal. This allows detection of low copies or target DNA while providing a distinction between a mutation target negative result and a PCR fail result.

4.5.3. *GeneFirst Assay*

In January 2011, a new molecular diagnostics company named GeneFirst was founded by Guoliang Fu from Oxford University. This company now offers a further $BRAF^{V600E}$ mutation detection assay using Multiplex Mutation Detection (MMD) PCR. The benefit of this type of PCR, is that only one well is required which contains a mutation primer (HEX/FAM signal), and an endogenous control (ROX signal), thus less DNA is required. This assay has a stated sensitivity of 1% (1.5-3ng DNA) or 0.1% (15-30ng DNA). Although this assay was not used in this study, comparative assessment of this assay versus the used Life Technologies assay may be beneficial to future work.
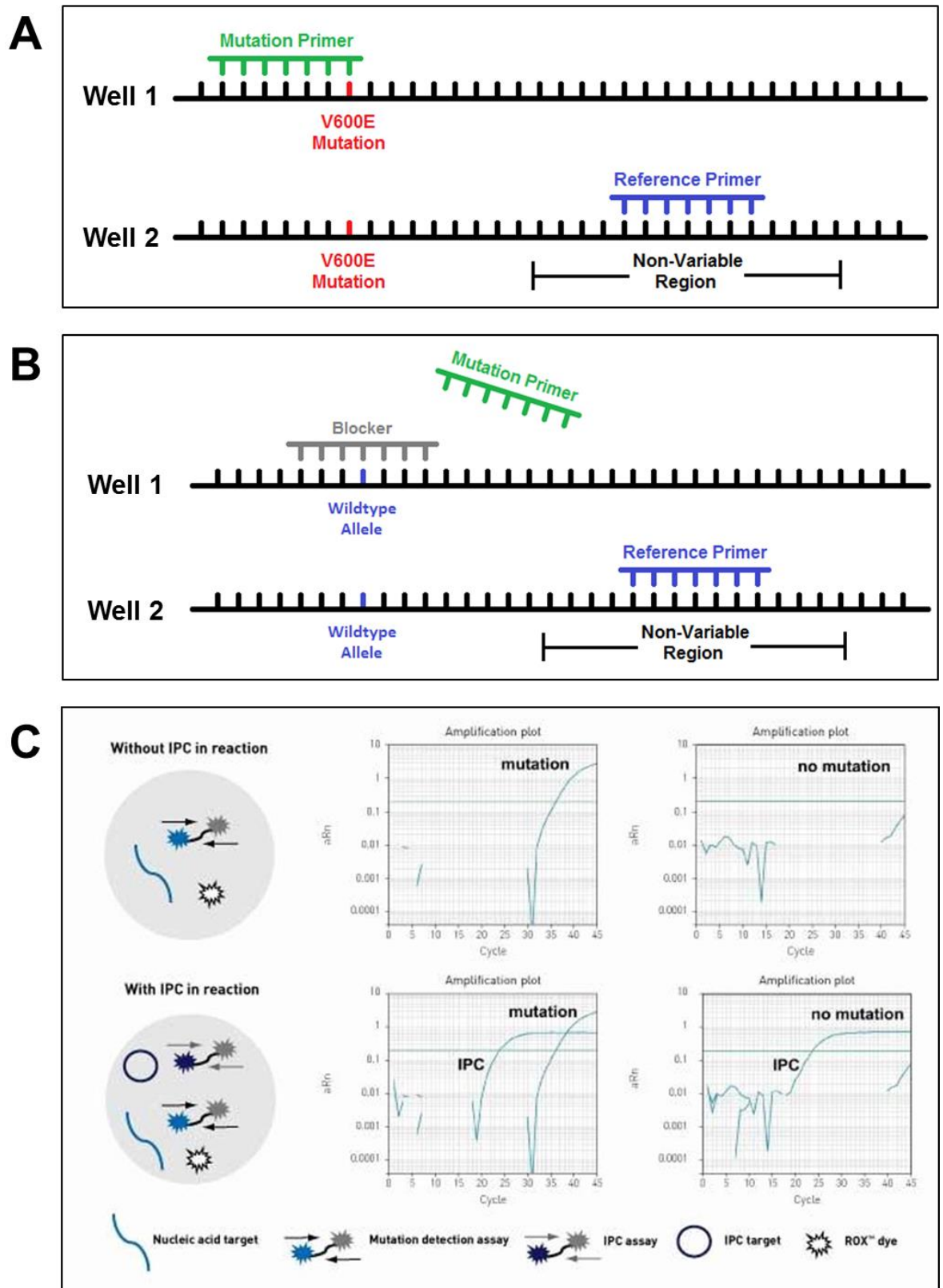
Figure 4.3. Mutation Detection RT-PCR Assay Figures

A. *For both the Qiagen and Life Technologies assays, DNA is split between two PCR wells; well one contains a mutation primer specific for BRAF^{V600E}, well two contains a reference primer specific for a non-variable region of the BRAF gene.*

B. *The Life Technologies assay also contains a wild-type allele specific blocker within well one, which suppresses wild-type alleles, preventing non-specific amplification.*

C. *The TaqMan® Mutation Detection IPC Reagent Kit is a set of optional internal positive control reagents that can be duplexed with any TaqMan® Mutation Detection assay to provide a positive PCR control result. The IPC reagents can distinguish a mutation target negative result from a PCR failure result.(Applied Biosystems Assay Manual, 2012).*

4.5.4. *Assay Comparison*

For relative comparisons of the two different assays available at the start of this thesis, three separate standard curves were prepared (see section 4.6, page 137), with 10,000 cells at concentrations of *BRAF*^{V600E} of 0%, 0.001%, 0.01%, 0.1%, 1%, 10% and 100%. Each standard curve preparation was performed on both assays on the same day (Figure 4.4). As can be seen from the graphs, the Life Technologies assay had lower CTs for both positive mutation and reference assays, higher background CTs for mutation assays <0.1% and more consistent CTs overall. Also, the CT^{mut} of the QIAGEN assay was between 1.5 and 2.2 CTs lower than the CT^{ref}. This indicates that the mutation primer is working more efficiently than the reference primer in this assay.

The averages of the 3 observed dCT replicates for each assay were also compared to the expected dCT for each *BRAF*^{V600E} positive concentration (Figure 4.4.B). The expected dCT was derived from Equation 4.2. It can be seen that the Life Technologies assay provided dCTs which were almost exactly the same as the expected dCT curve, to a limit of 0.1% *BRAF*^{V600E} in 10,000 cells. The QIAGEN assay dCTs were not as ideal and appeared to be less stable.

$$\frac{CTMut}{CTRef} = \frac{1}{n} \qquad n = 2x \qquad \therefore x = \frac{\log n}{\log 2} \quad x = \text{expected dCT}$$

E.g. 12% *BRAF*^{V600E}+

$$\frac{12}{100} = \frac{1}{8.3} \qquad 8.3 = 2x \qquad \therefore x = \frac{\log 8.3}{\log 2} \quad x = 2.88$$

Equation 4.2. Expected dCT of the Assays

Further details of differences between the two assays are given in Table 4.1. The Qiagen assay costs £10/well, whereas the Life Technologies assay costs more at £11.63/well, plus £0.27/well if using the optional IPC reagent. This extra cost of the Life Technologies assay is

somewhat balanced by several benefits; this assay has a lower stated sensitivity at <0.1% compared to 1%, increased specificity due to the castPCR system, optional IPC, does not need technical replicates, and in our hands has fewer problems with bubbles in the reaction mix. For all of the above stated reasons, the Life Technologies assay was chosen for this project.
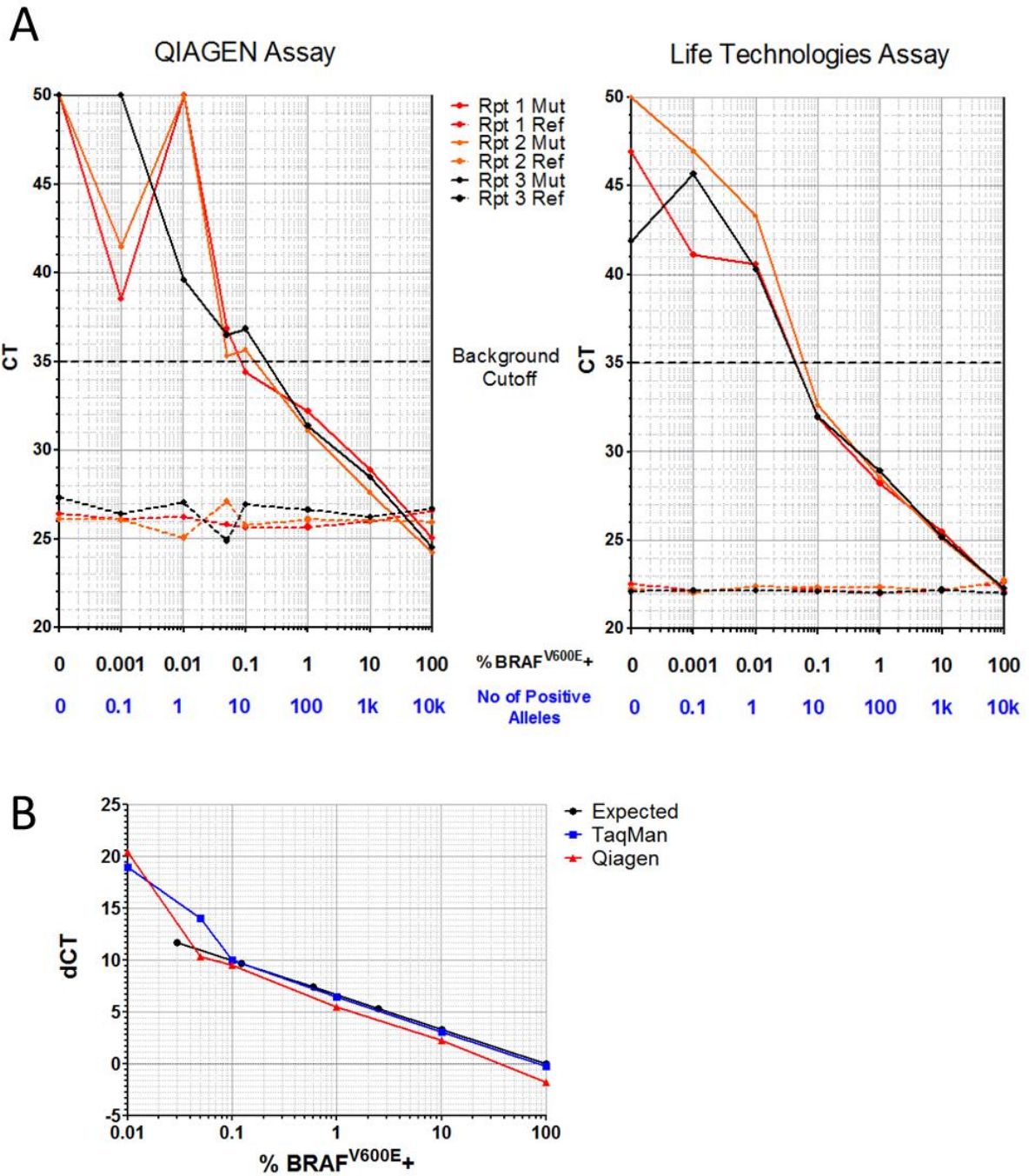
Figure 4.4. BRAF^{V600E} Mutation Allele PCR Assay Comparison

A. Comparison of three different standard curve preparations of 10,000 cells at 0%, 0.001%, 0.01%, 0.1%, 1%, 10% and 100% BRAF^{V600E} positivity run on both assays on the same day.

B. Comparison of the averages of the dCTs of the above standard curves for each assay, compared with the expected dCTs derived from Equation 4.2.

Factor	Qiagen	Life Technologies
Total Cost	£10/well	£11.63/well
Stated Sensitivity	1%	<0.1%
Specificity	No background blockers.	Increased due to castPCR blockers
Recommended DNA	5-10ng	2-20ng (2ng for 1% detection, 20ng for 0.1% detection)
Cycling Conditions	a) 10 mins, 95°C, 1 cycle b) 15 sec, 95°C then c) 1 min, 60°C, 40 cycles	a) 10 mins, 95°C, 1 cycle b) 15 sec, 92°C then c) 1 min, 58°C, 5 cycles d) 15 sec, 92°C then e) 1 min, 60°C, 5 cycles
Replicates	Triplicates Recommended	Technical replicates not required
Bubbles	Bubbles more frequent	Less bubbles, possible due to less H2O in the reaction
Other	Cannot purchase individual components of kit	IPC Kit sold separately (£0.27/well) Mutation Detection Software available

Table 4.1. BRAF^{V600E} Mutation Allele PCR Assay Comparison

A comparative summary of the two allele specific PCR assays is provided. CastPCR = Competitive Allele-Specific TaqMan® PCR.

4.5.1. *Optimal Starting Cell Numbers*

As the level of DNA was to be based on starting cell number rather than NanoDrop DNA concentration readings, it was important to assess the optimal and minimum number of cells to begin DNA preparation from. Triplicate standard curves of three separate serial dilutions were created from normal PBMCs from three different donors, originally quantified by viCell analysis. These dilutions were at the following cell numbers: 40k, 4k, 400, 40, 4 and a further dilution equivalent to 0.4 cells. DNA was extracted from each dilution using QIAGEN micro DNA extraction kits and eluted in 20µl H_2O. 5µl of the elute was run on the PCR using the reference primer so therefore CT^{ref} values for input of DNA equivalent to the extraction of 10k, 1k, 100, 10, 1 and 0.1 could be plotted (Figure 4.5).

As seen from the graph, the average CT^{refs} from these DNA preparations were almost exactly equivalent to the average CT^{refs} from the 6 replicate standard curves (see section 4.6), indicating that DNA extraction was similarly efficient for these 10k cell extractions as the 1x10^6 cell extractions for the standard curves. The highest CT^{ref} seen for wells containing DNA equivalent to 1000 cells was 27.63, so a CT^{ref} of 27 was chosen as the limit for analysis down to 0.1%. Likewise the 10k CT^{refs} were fairly consistent at ~21, so this could be used as a theoretical limit of 0.01% mutation.
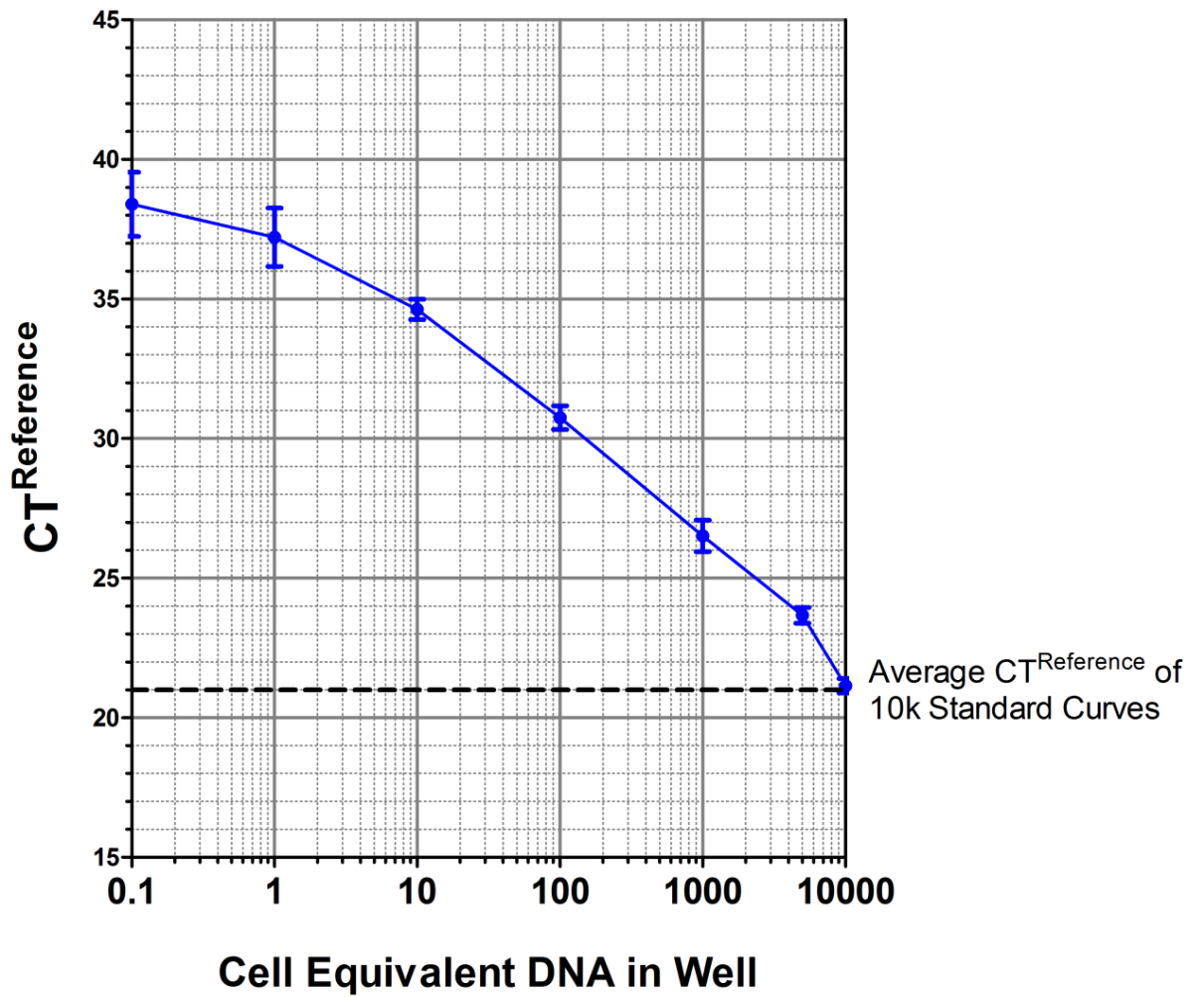
Figure 4.5. Determining Optimal Starting Cell Numbers

$CT^{Reference}$ in samples of DNA extracted from set numbers of cells and eluted into 20µl. Dashed line indicates the average $CT^{Reference}$ of 6 replicate standard curves of 10k cells.

4.6. Standard Curves

In order to have a reliable system of determining percentage positivity of samples from PCR results, standard curves were created using $BRAF^{V600E}$+ cell line A375, from the American Type Culture Collection (ATCC; www.lgcstandards-atcc.org) and a $BRAF^{V600E}$- Epstein-Barr virus transformed lymphoblastoid cell line (see section 4.7 for details of cell line preparation and culture).

Three replicate standard curves were created from preparations at various concentrations of cells (1,000/5µl, 10,000/5µl 50,000/5µl) in order to explore the ideal concentrations of cells to use in the assay (See Figure 4.6.A). As seen in the graph, the 1k curve gave reasonable dCTs down to a sensitivity of 1% (10/1000 positive cells), after this point results become less reliable. The results for the 10k and 50k curves are remarkably similar, indicating that there was no need to add DNA equivalent to more than 10k cells into the assay.

Therefore, a series of 6 replicate standard curves were created from separate DNA preparations, to extrapolate % positivity of test samples. Briefly, cells from each cell line were carefully counted using a viCell analyser (Beckman Coulter; www.beckmancoulter.com; 731050). DNA was extracted from aliquots of $1x10^7$ cells using Qiagen QIAamp DNA Mini Kits (using RNAase as directed) and eluted into 100µl H_2O. The final volume of DNA prepared from each aliquot was diluted to 5ml in H_2O to give the equivalent of 10,000 cells/5µl. DNA concentration was measured with a NanoDrop Analyser (Thermo Scientific; www.nanodrop.com) and the DNA concentration from the A375 cell line was adjusted to match that of the control DNA (approximately 1.5-fold dilution). This step is crucial due to the hypotriploid (n=62) nature of the A375 cell line. Serial 10-fold dilutions of A375 DNA into control DNA were prepared as follows: 100% mutated; 10%, 1%; 0.1%; 0.01% and 0% (100% control DNA). 5µl of each dilution was used for reference and mutation PCR assays. Aliquots of 12.5µl of each dilution were stored for use as internal controls (see section 4.8).

The final standard curve was constructed from the dCT means of the six 10,000 cell/5µl replicates using GraphPad Prism, plotting mean dCT (CT^{mut}-CT^{ref}) on the y axis and log %$BRAF^{V600E}$ on the x axis. This standard curve was used to interpolate all experimental sample values.

Results with a dCT below 10 (equivalent to the stated sensitivity of 0.1% $BRAF^{V600E}$+) were considered positive. Results with dCTs between 10 and 15 were below the stated sensitivity of the assay, though as no control $BRAF^{wt}$ samples (n=15 PBMC, n=30 LCL) had a dCT below 18, these results were considered positive outside of the quantitative range (POQR), giving a theoretical sensitivity of ~0.05%. Results with a dCT above 15 were considered negative to ensure no false positives were reported.
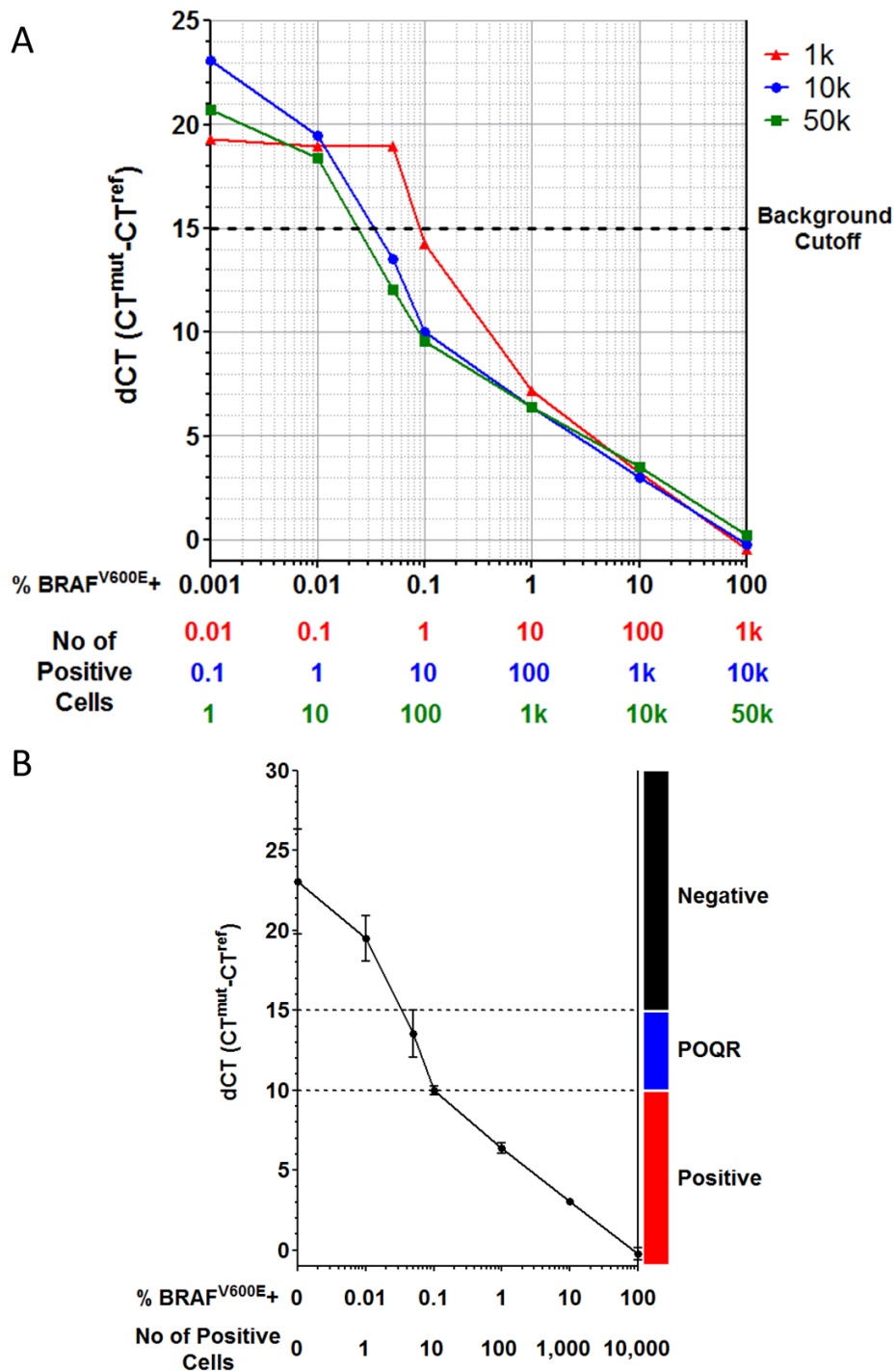
Figure 4.6. Standard Curve

Standard curves created using BRAF^{V600E}+ cell line A375 and a BRAF^{V600E}-Epstein-Barr virus (EBV) transformed lymphoblastoid cell line (LCLs).

A) Comparison of standard curves using 1k, 10k, or 50k cell DNA equivalent per well. Each curve is the average of 3 technical replicates.

B) Final standard curve using 10k cell DNA equivalent per well. The curve is the averages of 5 technical replicates. The error bars represent SD.

4.7. Cell Lines

4.7.1. *A375 BRAF^{V600E}+ Cell Line*

The BRAF^{V600E+} cell line A375 was acquired from the American Type Culture Collection (ATCC; www.lgcstandards-atcc.org) and grown in high glucose DMEM (Lonza; www.lonza.com; 4.5g/L Glucose w/ L-Glutamine 500ml) + 10% heat-inactivated foetal bovine serum (Life Technologies; http://www.lifetechnologies.com; 10270-106).

This cell line was used rather than other *BRAF^{V600E}*+ cell lines (e.g. MV522 or HCC344) as it was available in-house and the mutation has previously been validated by our colleagues (Armstrong *et al.*, 2011) using Custom Taqman SNP genotyping assays (Applera Europe BV; now Applied Biosystems; www.appliedbiosystems.com).

A375 is hypotriploid (n=62) and homozygous for *BRAF^{V600E}*. In order to ascertain the karyotype of the A375 cell line, particularly for details of chromosome 7, where the BRAF gene is located, 5 metaphases were analysed for cytogenetics. The cell line had the basic standard cytogenetic processing for adherent cells. Briefly, the cells were exposed for 18hrs with colcemid/BrDU followed by hypotonic treatment and trypsin release of cells. Cells were then harvested using standard cytogenetic process (Rooney and Czepulkowski., 1992). Metaphase preparations were banded using trypsin & Leishman stain to produce G-bands (GTL technique) and chromosome abnormalities identified were described using ISCN 2013: International System for Human Chromosome Nomenclature (Shaffer *et al.*, 2013).

There was very little heterogeneity between the 5 metaphases; Trisomy 7 and the three marker chromosomes were present in every cell. The overall karyotype was 56~60,XX,add(1)(p1?),+del(1)(q3?2),+3,+4,+5,-6,+7,+add(11)(p1?),+13,+15,+17,+3mar[cp5](Figure 4.7).

56~60,XX,add(1)(p1?),+del(1)(q3?2),+3,+4,+5,-6,+7,
+add(11)(p1?),+13,+15,+17,+3mar[cp5]
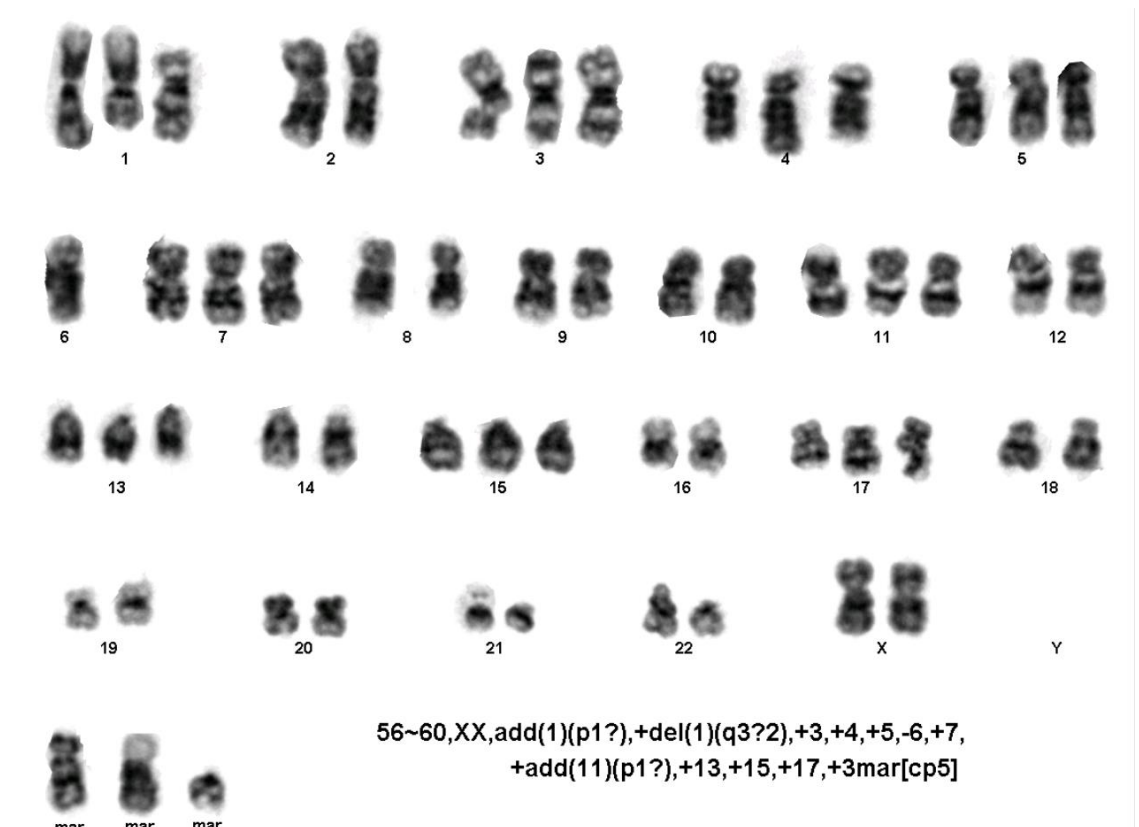
Figure 4.7 A375 Cell Line Karyotype

One of five metaphases analysed for the karyotype of the A375 cell line. There was very little heterogeneity between the metaphases, all cells had Trisomy 7 and the three marker chromosomes.

4.7.2. BRAF^V600E^-LCL Cell Line

A *BRAF^V600E^*- Epstein Barr Virus (EBV) immortalised Lymphoblastoid Cell Line (LCL) was created for use as a negative control. Briefly; 1×10^7 PBMCs were re-suspended per 1ml RF-10 media with an equivalent volume of EBV supernatant and incubated for 2 hours at 37oC in a CO_2 incubator. Cyclosporin A was added to a concentration of 1ug/ml incubated overnight as above. The cell suspension was transferred to a 24-well plate at approximately 0.6ml per well, a further 1ml 1ug/ml Cyclosporin A was added and the plate was incubated for 1 week as above. 1ml supernatant from each well was removed and replaced with 1ml fresh 1ug/ml Cyclosporin A, and the plate was incubated for 1 week as above. When the cells had started to grow, they were transferred to a 25cm2 flask in 7ml RF-10 and passaged as appropriate.

4.7.3. *A375 and LCL Cell Line Morphology*

May-Grünwald-Giemsa staining of the A375 and LCL cell lines can be seen in Figure 4.8. The majority of both cell types were large cells, up to 40µm in diameter. The A375 cell line is often described as epithelial-like (ATCC, 2014). In the cultures used in this thesis, the A375 cells appeared with large, thick dendrites and a high amount of cytoplasm containing some small vacuoles. The nuclei were irregular with one or more large nucleoli. The LCL cells used in this study were generally slightly smaller, with very fine dendrites and a lower cytoplasm:nucleus ratio. Cytoplasm often contained a high number of medium sized vacuoles. Nuclei were round or irregular with several medium sized nucleoli.
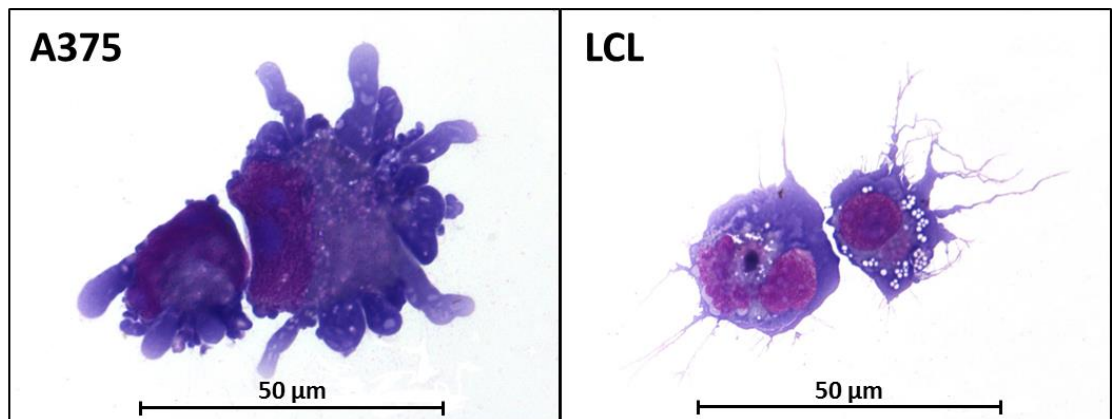
Figure 4.8 A375 and LCL Cell Line Morphologies

Cytospin slides where prepared from cells freshly taken from culture and stained with May-Grünwald-Giemsa (pH 6.8) on a Sysmex SP-1000i. Photographs were taken on a Zeiss Axioimager Z2 microscope with AxioVision 4.8 software at 100 times magnification. Scale as indicated.

4.8. Internal Controls

As well as using the IPCs provided by Life Technologies, which allow for a determination between a negative $BRAF^{V600E}$ result and a failed PCR reaction, a series of in-house IPCs were created to ensure that the mutation and reference primers used in the assay were working efficiently throughout the project. These IPCs were created alongside each replicate standard curve (see section 4.6), in order to ensure accuracy. Therefore controls of 10,000 cell equivalent DNA at 0%, 0.1% and 10% $BRAF^{V600E}$positivity, as well as a no template control (NTC) were run on each plate used to test $BRAF^{V600E}$ in patient samples. These controls were used to control for false negative results, as well as to monitor the sensitivity of the assay over time (Figure 4.9).

Over the period of this study, the controls from two preparations were used as internal controls. As can be seen from Figure 4.9.A, the CT^{ref} levels of both control sets used were reasonably consistent throughout, indicating that all of the controls had equivalent levels of total DNA. However some variation was seen in CT^{muts} in control set 1. As control set 2 had remarkably stable CT^{ref} levels in 0.1% and 10% mutation samples, it can be assumed that the variances seen in control set 1 are due to incorrect sample preparation (possibly inefficient mixing before aliquoting), rather than fluctuations in the PCR analyser. Also, at no point during PCR testing did the CT^{mut} of the 0% control (lowest CT^{mut} = 39.81) crossover with the 0.1% control (highest CT^{mut} = 38.01), see Figure 4.9.B. Therefore a sensitivity of 0.1% can be stated with confidence, and it was not necessary to normalise any patient results.

As seen in Figure 4.9.C, there was also no overlap of dCTs between 0% and 0.1% controls. Interestingly, the average dCTs of the 0.1% and 10% controls were higher than the original levels set by the standard curve averages, whereas the mean of the 0% dCTs is almost exactly the same. Standard curves were run fresh, but controls had been frozen and defrosted, so these discrepancies could indicate that $BRAF^{V600E}$ DNA may be more fragile than $BRAF^{WT}$ and thus more easily degraded during the freeze/thaw process.

4.8.1. *BRAF^{WT} Control PBMCs*

In order to determine the average background activity of the mutation primer, a total of fifteen samples of 50k *BRAF^{WT}* PBMCs were run with the *BRAF^{Mutation}* and *BRAF^{Reference}* assays, either to 40 or 50 cycles. None of these samples, or the 10k 0% *BRAF^{V600E}* control samples had a CT^{mut} below 40 cycles (see Figure 4.9.D), indicating that results for CT^{mut} below 40 can be treated as true positive results. The lowest dCT of the control PBMC samples was 13.57 when run to 40 cycles and 19.47 when run to 50 cycles (data not shown). This indicates that the ability to distinguish positive from false-positive results is greatly increased when running to 50 cycles.

4.8.2. *PCR Amplification Curve Visual Control*

After each PCR experiment, the amplification curves were examined for credibility. In order to be accepted as accurate, the curves had to be distinct with a linear amplification phase.
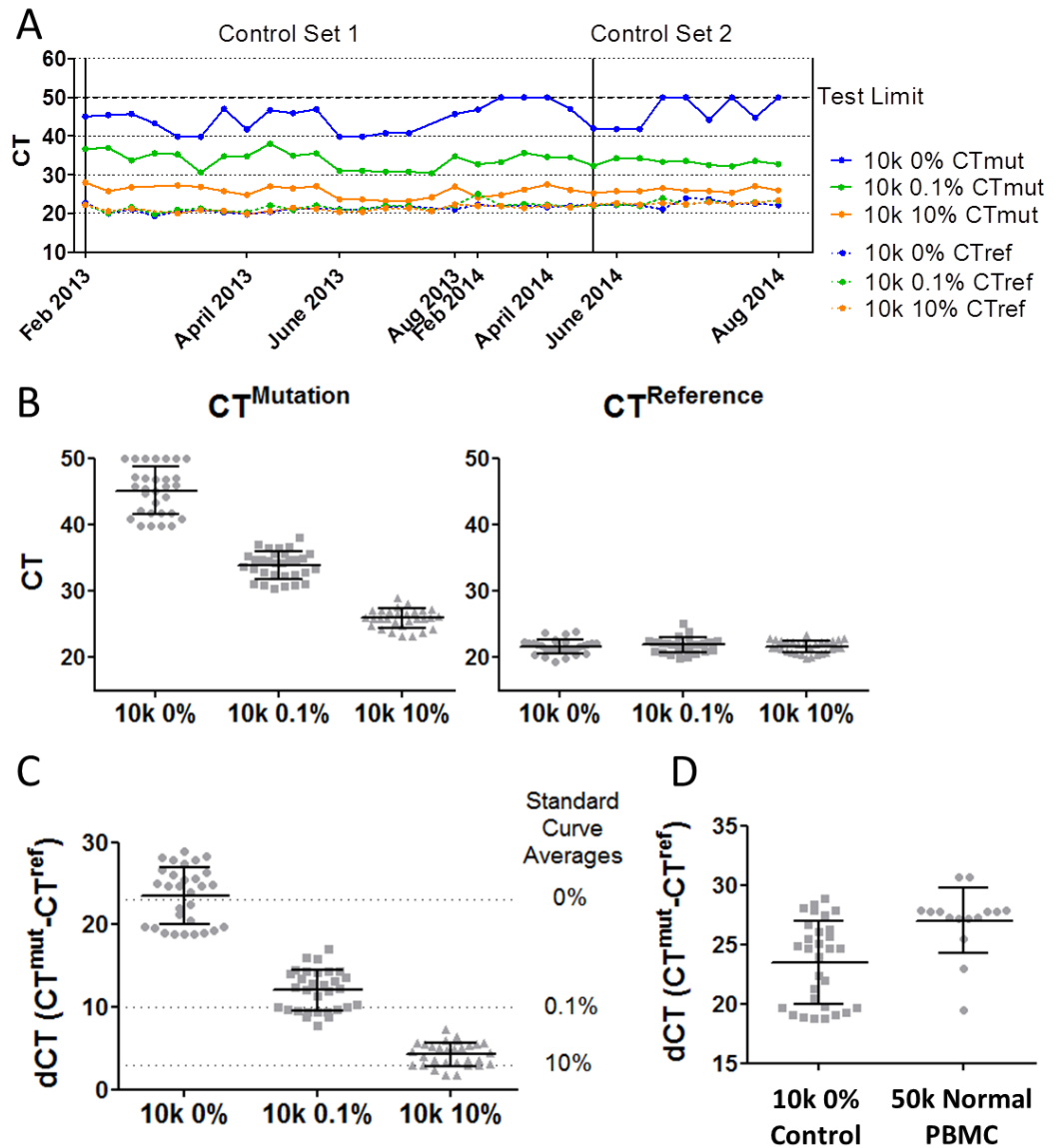
Figure 4.9 Internal Positive Controls

A) In-house internal positive control CT^{mut} and CT^{ref} results as run on each PCR plate over a period of one and a half years. Vertical black lines indicate the transition between control sets. The limit of the test was CT 50 as indicated by the wide dashed horizontal line.

B) Separate graphs of CT^{mut} and CT^{ref} as above. Error bars are means with SD.

C) dCTs ($CT^{mut} - CT^{ref}$) of all in-house controls. Horizontal dotted lines indicate the average dCTs of 6 replicate serial dilution standard curves from which the control samples were derived. Error bars are means with SD.

D) CT^{mut} results of thirty in-house 10k 0% $BRAF^{V600E}$ controls and fifteen 50k $BRAF^{wt}$ PBMC samples. At no point does the dCT result for these samples drop below 18 (Lowest CT^{mut} level was 40).

4.9. Discussions for Chapter Four

Allele-specific PCR for $BRAF^{V600E}$ was adapted to analyse fractions of peripheral blood and bone marrow mononuclear cells in a quantitative fashion. The PCR detection protocol was developed using a TaqMan® Mutation Detection Assay (www.invitrogen.com), which was found to be the most sensitive and reproducible of the two assays available at the start of this thesis. This assay involved splitting the DNA between two PCR wells, one containing a mutation primer specific for $BRAF^{V600E}$, the other containing a reference primer specific for a non-variable region of the BRAF gene. The mutation detection well also contained a Competitive Allele-Specific TaqMan PCR assay which acts as a blocker of the wild-type allele at the location of $BRAF^{V600E}$, reducing non-specific amplification.

Due to the lack of necessary sensitivity of available DNA quantification equipment (NanoDrop), DNA was estimated on original number of input cells. In order to maximize sensitivity, 10,000 nuclear equivalents of DNA (approximately 60ng) were amplified in each assay. A detection limit of 0.1% (10/10,000 cells) was confirmed by titration of the $BRAF^{V600E}$ mutated melanoma cell line A375 into BRAF wild-type EBV-transformed B cells from healthy volunteers. The DNA levels of the $BRAF^{V600E}$ positive cell A375 line were diluted down to the levels of the LCL line due to hypotriploid cytogenetics in A375.

The assay has a sensitivity of 0.1% (dCT <10, CT^{ref} cut-off 27), although it was possible to define a region of the curve that was positive outside the linear quantitative range (POQR) corresponding to 0.05-0.1% positivity (dCT 10-15, CT^{ref} cut-off 21) but for the current purpose, results in this range were treated as negative. PCR assays were run to 50 cycles, though a CT^{mut} above 40 was considered negative and CT^{ref} above 27 were considered fails. Life technologies IPC kits, in-house controls (10,000 cells, 0%, 0.1%, 10% mutation) and visual examination of all PCR curves were used to ensure validity of results.

Chapter 5. Tracking $BRAF^{V600E}$ in Histiocytic Disorders

5.1. Introduction

In Chapter 3, I examined a known bone marrow haematopoietic disorder (AML) and its implications on the dendritic cell/monocyte system. In this chapter I will relate a known dendritic cell/monocyte system disorder (Histiocytosis) to the bone marrow haematopoietic system.

The work in this chapter concerns comparative flow cytometry of the histiocytic disorder, Langerhans cell histiocytosis (LCH) and Erdheim Chester disease (ECD), as well as hairy cell leukaemia (HCL). Also, allele-specific PCR assays are used to determine cells which contain the $BRAF^{V600E}$ mutation, and which may potentially be precursors of the diseases.

5.1.1. *Chapter Aims*

1) To compare the distribution of $BRAF^{V600E}$ in peripheral blood mononuclear cells (PBMC) of LCH, ECD and HCL.

2) To test whether $BRAF^{V600E}$ is present in the bone marrow (BM) haematopoietic stem cell.

5.1.2. *Langerhans Cell Histiocytosis and Erdheim Chester Disease*

Langerhans Cell Histiocytosis (LCH) and Erdheim Chester Disease (ECD) are rare histiocytic disorders with highly distinct pathological and clinical features. LCH cells express langerin, CD1a and Birbeck granules and cause granulomatous lesions predominantly in the bone, epithelia and haematopoietic organs (Nezelof *et al.*, 1973; Allen *et al.*, 2010). ECD lesions show abundant CD68+ foamy macrophages and affect the distal long bones, skin (xanthelasmata) and cardiovascular tissue (Haroche *et al.*, 2012b). LCH is found in both children and adults while ECD is predominantly found in adults. Both diseases can lead to organ impairment, disability and death.

5.1.3. *The Origin of LCH/ECD Cells*

LCH cells have a number of similarities with epidermal Langerhans Cells (LCs) including Birbeck Granules, Langerin and CD1a expression(Nezelof *et al.*, 1973), but the relationship between LCH cells and LCs or LC precursors remains undefined. LCH cells have a different gene expression pattern to LCs (Allen *et al.*, 2010; Hutter *et al.*, 2012), also mouse studies have shown that langerin is not specific to LCs (Ginhoux *et al.*, 2007) and indeed langerin and CD1a can be expressed by other subsets of human DCs(Bigley *et al.*, 2014). It has been shown that LC-like cells can be formed from CD34+ BM progenitor cells (Caux *et al.*, 1992; Strobl *et al.*, 1997; Ratzinger *et al.*, 2004; Klechevsky *et al.*, 2008) and circulating monocytes(Geissmann *et al.*, 2001; Hutter *et al.*, 2012), so it is possible that LCH cells may originate in the bone marrow or from circulating myeloid cells.

As ECD cells have a macrophage-like appearance, they are thought to be derived from circulating myeloid cells, though as both LCH and ECD can coexist in individual patients (Hervier *et al.*, 2014), another possibility would be that both diseases may originate from an early stem cell with the ability to produce both cell types.

5.1.4. *Genetic Mutations in Histiocytosis*

Recent observations indicate that the histiocytes in both diseases harbour single monoallelic mutations of BRAF, MEK, RAS or other regulatory/survival pathway members that presumably prevent the resolution of inflammation and allow a progressive accumulation of abnormal cells. 50-70% of LCH and ECD lesions harbour $BRAF^{V600E}$ mutations (Badalian-Very *et al.*, 2010; Haroche *et al.*, 2012b). Two BRAF wild-type ECD patients have since been shown to express the $NRAS^{Q61R}$ mutation (Aitken *et al.*, 2014; Emile *et al.*, 2014). The ability to detect pathogenic mutations at high sensitivity allows several important questions to be addressed:

5.1.5. *Chapter Hypotheses*

1) Allele specific PCR of the *BRAF^{V600E}* mutation in peripheral blood and bone marrow will identify potential precursor cells of the histiocytic disorders LCH and ECD.

2) The distribution of *BRAF^{V600E}* alleles will correlate with the type of histiocytosis and other clinical variables.

3) Allele specific PCR of the *BRAF^{V600E}* mutation will be useful for diagnosis and/or disease monitoring of patients with LCH and ECD.

5.2. Materials and Methods for Chapter Five

5.2.1. *Patient Samples and Clinical Data*

Blood, skin, bone marrow and surplus biopsy material was obtained from LCH, ECD and HCL patients. Blood and skin was obtained from healthy volunteers or patients undergoing plastic surgery. All samples were acquired with ethical approval from the Newcastle and North Tyneside Research Ethics Committee (Appendix 8). Patients with histiocytosis were referred to a local clinic and gave consent for ethically approved use of their clinical data. Disease activity was assessed by biopsy at diagnosis or subsequently by physical and radiological examination including magnetic resonance imaging and positron emission tomography. See Figure 5.1 for patient recruitment and Table 5.1 for detailed patient clinical characteristics.
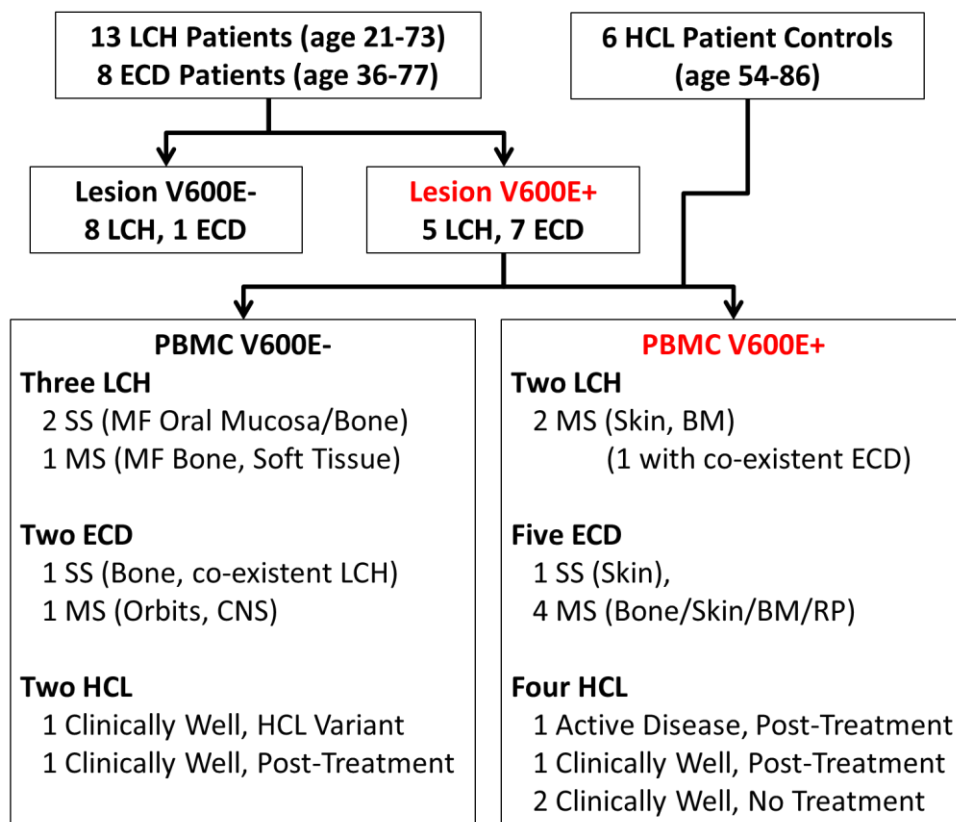
Figure 5.1. Patient Recruitment

LCH = Langerhans Cell Histiocytosis; ECD = Erdheim Chester Disease; HCL = Hairy Cell Leukaemia; SS = Single-System; MS = Multi-System; MF = Multi-Focal; BM = Bone Marrow; RP = Retroperitoneum; CNS = Central Nervous System.

Patient	Age	Gender	Disease	Type	Affected Organs	DI	Smoker	Clinical BRAF Result	Lesion	PBMC Research Result	Diagnosis to PBMC testing (yrs)	Active Disease	Treatment
A2951	73	M	LCH	MS	Skin, LN, GI, BM	N	N	Pos (IHC)	Pos (skin)	Pos 3.30%	<1	Skin, BM, LN, GI	2 Weeks Post Dexamethosome + Etoposide
A2712	59	F	LCH/ECD	MS	RP (ECD), BM (ECD+LCH), skin (LCH)	N	N	Pos (PCR)	Pos (BM, Skin)	Pos 0.33%	1	Skin, BM, Retroperitoneum	1 Month Post 2nd Cladribine
A2568	21	F	LCH	MS	MF bone (femur, ileum, skull), soft tissue	Y	Y	Pos (PCR)	NT	Neg	1	No	Maintenance 6MP and MTX
A2570	49	M	LCH	SS	MF Oral (mucosa and gums)	N	N	Pos (PCR)	Pos (Gum)	Neg	6	Oral	Topical Steroid
A2567	46	M	LCH	SS	MF bone	Y	N	Pos (PCR)	NT	Neg	14	No	12 Months Post Cladribine
A6924	42	F	LCH	MS	MF bone, lung	N	Y	Neg (PCR)	NT	Neg	15	Bone	NSAIDS
A7058	50	F	LCH	MS	UF bone (skull), CNS	Y	Y	Neg (PCR)	NT	Neg	1	No	None
A2970	35	M	LCH	MS	MF bone (mandible, ileum), oral (mandible, maxilla, teeth), CNS meninges	N	N	Neg (PCR)	NT	Neg	2	No	None
A2853	31	F	LCH	MS	UFl bone (skull), skin, liver, CNS	Y	N	Neg (PCR)	NT	Neg	7	Neuro-degenerative	None
A2564	44	M	LCH	MS	Bone (skull), middle ear, oral	N	Y	Neg (PCR)	NT	Neg	2	No	None
A2955	43	M	LCH	MS	MF Bone (skull), lung, LN	N	Y	Neg (PCR)	NT	Neg	<1	Yes	None
A2766	33	M	LCH	SS	Inguinal LN	N	N	Neg (PCR)	NT	Neg	<1	No	None
A2565	56	F	LCH	SS	UF Bone (skull)	N	N	Neg (PCR)	NT	Neg	45	No	None
A2867	28	M	ECD/LCH	SS	ECD bone	N	N	Pos (PCR)	NT	Neg	<1	Bone	Pegalated IFNa
A7343	72	F	ECD	MS	Bones and retroperitoneum	N	N	Pos (PCR)	Pos**	Pos 0.01%*	10	Yes	Post Vinblastine, Prednisone, AKT Inhibitor
A7346	77	F	ECD	SS	Skin	N	N	Pos (PCR)	Pos**	Pos 0.19%	2	Yes	Post Vinblastine Prednisone
A7347	36	F	ECD	MS	Bone, lung	N	N	Pos (PCR)	Pos**	Pos 0.14%	1	Yes	Post Vemurafenib
A7348	56	M	ECD	MS	Bones, retroperiteonum, brain	N	N	Pos (PCR)	Pos**	Pos 5.48 %	2	Yes	On Vemurafenib
A7349	75	M	ECD	MS	Bones, aorta, retroperitoneum	N	N	Pos (PCR)	Pos**	Pos 0.03%*	7	Yes	On Vemurafenib
A7350	51	M	ECD	MS	Orbits, CNS	N	N	Pos (PCR)	Pos**	Neg	<1	Yes	None
A7344	67	M	ECD	MS	Skin, aorta, orbits, CNS, dura, Bone marrow, retroperitoneum	N	N	BRAF Neg NRAS^{Q61R} mutation	Neg	Neg	1	Yes	None
A2123	65	M	HCL	Classic	BM, Blood	N	N	Pos	NT	Pos 2.11%	6	Clinically Well WBC 1.5x10^9/L	4 years Post Cladribine
A7044	68	F	HCL	Classic	BM, Blood	N	N	Pos	NT	Pos 1.08%	1	Clinically Well WBC 2.9x10^9/L	None
A7073	86	M	HCL	Classic	BM, Blood, Spleen	N	N	Pos	NT	Pos 3.70%	27	Active Disease WBC 13.4x10^9/L	27 Years Post Splenectomy & Interferon
A7264	67	M	HCL	Classic	BM, Blood	N	N	Pos	Pos (BM)	Pos 14.0%	<1	Clinically Well WBC 8.7x10^9/L	None
A7220	54	M	HCL	Variant	BM, Blood	N	N	Neg	NT	Neg	18	Clinically Well WBC 8.6x10^9/L	None
A7199	54	M	HCL	Classic	BM, Blood	N	N	Pos	NT	Neg	<1	Clinically Well WBC 4.0x10^9/L	2 Months Post Cladribine

Table 5.1. Patient Clinical Characteristics - *A review of the common clinical features of the cohort is presented.*

Age: age at time of PBMC testing for BRAF^{V600E} .   *Risk: risk organ involvement (haematopoietic, spleen, liver, lung)*

Time to test: time from diagnosis to BRAF^{V600E} testing   Treatment: Treatment received up to the time of BRAF^{V600E} testing

DI: Diabetes Insipidus; LCH, Langerhans Cell Histiocytosis;   ECD, Erdheim Chester Disease; MS, Multisystem; SS, Single System;

MF, Multifocal; UF, Unifocal; IHC, Immunohistochemistry; PCR, Polymerase Chain Reaction.

* Although PBMC BRAF result was below 0.1%, further fractionation revealed positivity >0.1%. ** Lesion BRAF testing at collection site

5.2.1. *Lesional BRAF^{V600E} testing*

Genomic DNA from formalin-fixed paraffin embedded LCH lesions was extracted using Promega Maxwell Tissue DNA Purification Kits (Promega; www.promega.co.uk) and the region flanking codon 600amplified by PCR. Amplicons were purified and genotyped by primer extension for the c.1799T>A, p.Val600Glu (V600E) mutation using the Sequenom iPlex protocol (Sequenom; www.sequenom.com). The extension products were detected by MALDI-TOF Sequenom mass spectroscopy using a Sequenom Typer 4.0.

5.2.2. *BRAF^{V600E} allele-specific PCR*

Peripheral blood and bone marrow cell subsets were sorted as previously described (see section2.4.2, pg. 71). Genomic DNA from sorted cells and neutrophil pellets (see section2.3.2, pg. 69), LCH lesion cells or control cell lines was extracted using Qiagen micro-kits (Qiagen; www.qiagen.com). BRAF mutation and reference qPCR was performed with competitive allele-specific TaqMan® mutation detection assays: Mutation Allele Assay: BRAF_476_mu 4465804 Hs00000111_mu; Gene Reference Assay - BRAF_rf - 4465807 Hs00000172_rf, according to the manufacturer's instructions (Life Technologies; www.lifetechnologies.com). A standard curve was derived from 6 serial dilutions of genomic DNA from *BRAF^{V600E}* melanoma cell line A375and EBV-transformed LCLs. 0%, 0.1% and 10% V600E mutation controls were performed with every run. The percentage of *BRAF^{V600E}*mutation was determined from the dCT (CT^{reference}-CT^{mutant}) using the standard curve. Graphs were plotted with Prism Version 5.0 (GraphPad Software, Inc.).For further details of this methodology, see Chapter 4.

5.2.3. *Sanger Sequencing*

Genomic DNA was amplified from the pellets of 90-3,600 sorted cells using the REPLI-g Mini Kit (Qiagen; www.qiagen.com) according to manufacturers' instructions. The amplified DNA was then diluted 1:20 and 3 μl of the diluted DNA was then used as a template in the PCR reaction. All reactions were performed on a 2720Thermal Cycler (Applied

Biosystems; www.appliedbiosystems.com). The cycling conditions were as follows: initial denaturation for 15 min at 95 °C, followed by 35 cycles of: 30 secs at 95 °C, 30 secs at annealing temperature of 55 °C and 1 min at 72 °C with a final extension for 10 min at 72 °C. The reaction volume of 30 µl contained DNA, 1× PCR Buffer containing 1.5mM $MgCl_2$ (Qiagen), 0.25 mM dNTPs, 0.5 µM each primer and 1U HotStar Taq DNA Polymerase (Qiagen). The nucleotide sequences of the primers used for PCR amplification of the $NRAS^{Q61R}$ mutation were the following: NRAS Forward 5'- GTGGTAACCTCATTTCCCCA-3' and NRAS Reverse 5'- GGGACAAACCAGATAGGCAG with a specific product size of around 400 bp. PCR products were visualised on a 2% (w/v) agarose gel with added ethidium bromide and then prepared for sequencing. The clean-up reaction contained 15 µl of PCR product, 30 U Exonuclease I (Thermo Scientific; www.thermoscientific.com) and 3 U FastAP Thermosensitive Alkaline Phosphatase (Thermo Scientific). The reactions were then incubated at 37 °C for 30 mins. The reactions were stopped by heating the mixture at 85 °C for 15 mins. This clean-up reaction removes unincorporated primers and degrades unincorporated nucleotides. The resulting PCR products were then ready to use for sequencing without additional purification. Around 80 ng (20 µl) of purified PCR product was sent to SourceBioscience (www.sourcebioscience.com) for Sanger Sequencing along with 100 µl of NRAS forward and reverse primer at 3.2 µM concentration. The sequencing traces were analysed using Chromas Lite v2.1 software (www.chromas-lite.software.informer.com).Chromatogram peaks were then measured and the relative peak height percentage was calculated using the peak height of one allele divided by the sum of the peak heights of both alleles.

5.2.4. *Statistical methods*

Kruskal-Wallis non-parametric tests (not assuming Gaussian distributions) with Dunns' multiple column comparison post-tests and/or Fisher's exact tests were performed using GraphPad Prism 5.0 software.

5.3. Results

All analysis was performed after some patients had received cytoreductive therapy but no patients were on active treatment. No statistical differences were seen in treated or untreated patients.

5.3.1. *Langerhans Cell Histiocytosis Patients*

Over the period 2011-2013, 13 cases of adult LCH were referred to the Northern Centre for Cancer Care (NCCC), Newcastle, UK. Five patients had $BRAF^{V600E}$+ lesions, 3 with multi-system (MS) LCH and 2 with single system (SS) LCH bone disease. One of the $BRAF^{V600E}$+MS patients was subsequently found to have advanced aortitis due to co-existent ECD (A2712).

In agreement with the BRAF status defined by primer extension assays (Table 5.1), allele specific PCR confirmed the presence of $BRAF^{V600E}$ in lesions from 2 of the 3 multisystem LCH patients. Unfractionated PBMC contained mutated BRAF at 0.33 and 3.29% in A2712 and A2951, respectively.

The third MS LCH patient with quiescent disease on maintenance chemotherapy had no detectable mutated BRAF in the blood; 2 other patients with $BRAF^{V600E}$+ SS LCH, 1 active at the time of evaluation, were also $BRAF^{V600E}$ negative in blood. $BRAF^{V600E}$ mutation was not detected in the blood of the 8 patients with $BRAF^{V600E}$ negative lesions. The association between positive $BRAF^{V600E}$ in peripheral blood and active MS LCH in this small sample did not reach significance (p= 0.067 by Fisher's exact test).

LCH cells and lymphocytes from a skin lesion of patient A2712 were sorted and tested for $BRAF^{V600E}$. Results showed 46.24% mutational alleles in the LCH cells, and only 0.51% in the T Cells, which translate as at least 92.5% positivity of LCH cells and <1% $BRAF^{V600E}$ in lymphocytes.
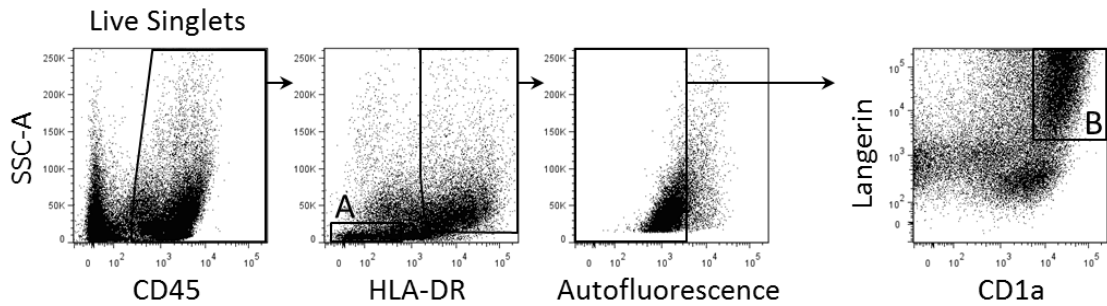
Figure 5.2. Lesional Cell Sorting in LCH Patient A2712

Gating strategy for cells in the LCH lesion of patient A2712. Leukocytes were isolated by expression of CD45. Antigen presenting cells were gated by expression of HLA-DR and T cells were sorted by low SSC-A and lack of HLA-DR expression (A). Macrophages were excluded by autofluorescence. LC/LCH cells were sorted by high expression of both CD1a and Langerin (B).

5.3.2. *Erdheim Chester Disease Patients*

Over the period 2013-2014, samples were taken from 7 cases of ECD who were patients at the Memorial Sloan Kettering Cancer Centre (MSKCC), New York, USA. One further patient was referred to the NCCC, presenting with skeletal ECD and was found to have pathological evidence of LCH confined to bone (A2867).

Seven patients, including the patient with co-existent LCH had $BRAF^{V600E}$+ lesions, 5 with multi-system (MS) ECD and 2 with single system (SS) skin or bone disease. The lesion $BRAF^{V600E}$-patient was previously shown to be positive for an $NRAS^{Q61R}$ mutation, by the group at MSKCC (Emile *et al.*, 2014).

$BRAF^{V600E}$ mutation was detected in the whole PBMC of 5/7 lesion$BRAF^{V600E}$+ECD patients, however two of these patients (A7343 and A7349) were positive outside of the quantitative range (POQR). In these two patients, the mutation was detected above the limit of detection of 0.1% when subsets were enriched. The SS ECD patient with co-existent LCH was negative for $BRAF^{V600E}$in the PBMC. The final patient, who had ECD restricted to the central nervous system (CNS) and orbits, was

negative for $BRAF^{V600E}$in the PBMC, and enriched fractions did not reach levels above POQR (highest level in Lineage-DR- cells; 0.03%).See Figure 5.1 for patient recruitment and Table 5.1 for detailed patient clinical characteristics.

5.3.3. *Hairy Cell Leukaemia Patients*

Over the period 2013-2014, 5 cases of HCL and 1 HCL variant were referred to a clinic in the North of England. Four patients had $BRAF^{V600E}$+ cells in the peripheral blood, one patient who had previously had cladribine treatment and the HCL variant patient were both $BRAF^{V600E}$ negative. See Figure 5.1 for patient recruitment and Table 5.1 for detailed patient clinical characteristics.

5.3.4. *Peripheral Blood Diagnostic Full Blood Count (FBC) Profiles*

In order to ascertain whether there were any abnormal expansions or deficiencies in blood cell counts, Full Blood Count (FBC) results from each patient at the time of peripheral blood sample collection for this project, were gathered from the diagnostic laboratories. The LCH patients showed heterogeneity in terms of white blood cell counts (WBC) as well as in neutrophil counts. The two multi-system LCH patients showed an obvious increase in total WBC and monocyte fractions and a slight or severe thrombocytopenia. The majority of LCH patients were within the 95% CI for total lymphocyte counts, whereas all of the ECD patients were low or below the 95% CI. All of the ECD patients were also within the 95% CI for platelets. HCL patients were variable for all cell types, with general decreases in monocytes and platelets. Haemoglobin levels were low for nearly all patients of all groups, the ECD and HCL means being slightly lower than the LCH means.
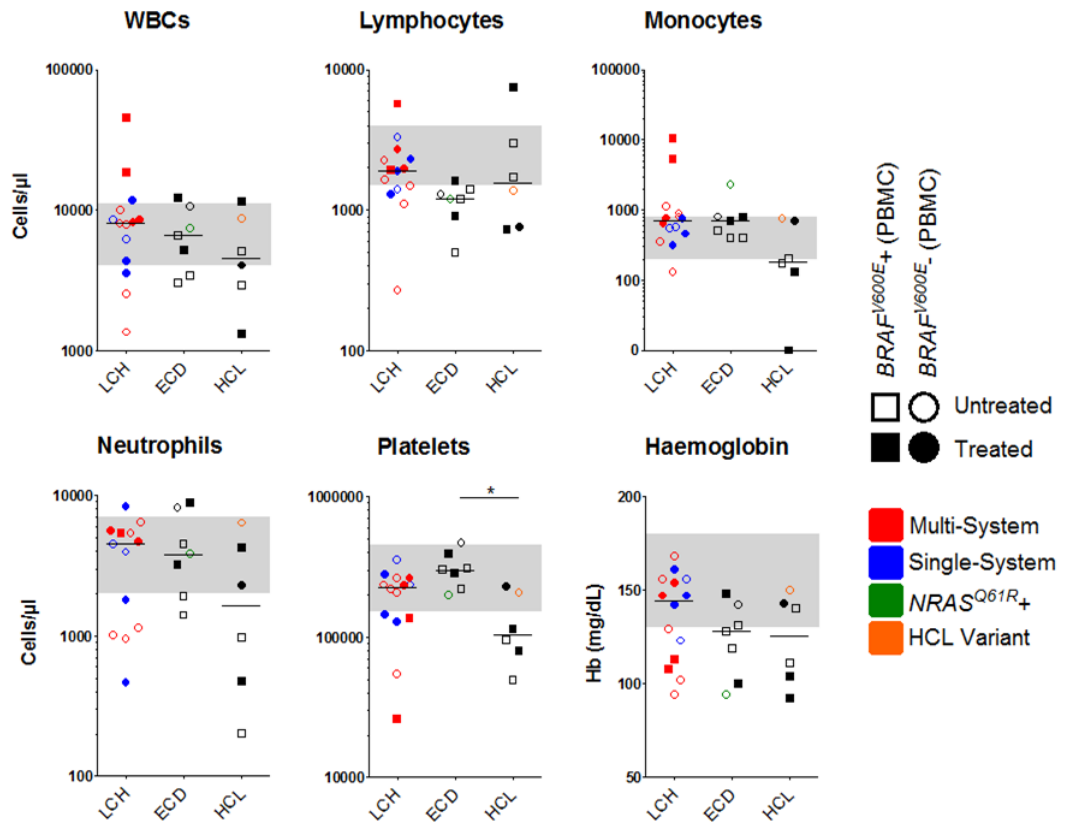
Figure 5.3. Peripheral Blood Diagnostic Full Blood Count Profiles

Comparison of automated diagnostic blood counts of patients with LCH (n=13), ECD (n=7) and HCL (n=6). Grey line is the standard range. Analysis was performed as previously described. WBC = White Blood Cells; Hb = Haemoglobin. Error bars show medians. Statistical analysis with Kruskal-Wallis non-parametric tests and Dunns' multiple column comparison post-tests. * p <0.05; ** p <0.01.

5.3.5. *Peripheral Blood Mononuclear Cell Phenotypes*

The FBC analysis given in section 5.3.4, page 156 gives comparative results on bulk cell types such as lymphocytes and monocytes, however, I have previously shown in this thesis that these cell types can be subdivided into several subsets. Therefore, flow cytometric profiling of the subsets was performed to analyse whether any specific subset expansions or deficiencies were present which were potentially masked in the bulk cell profiling.

Absolute counts of CD14+ and CD14+16+ monocytes and total CD11c+ mDCs were increased in some patients with LCH, compared with healthy controls, notably those with active disease, whereas CD16+ monocytes appeared relatively depressed. Conversely in ECD, CD16+ monocytes were more often within the 95% Confidence Interval (CI) of the normal range and CD14+ monocytes were relatively diminished.

CD141+ mDCs and CD123+ pDCs were not substantially increased in either LCH or ECD patients although CD123+ pDC levels were heterogeneous. CD141+ mDC levels appeared to be deficient in the ECD patients, however patient numbers with data for this cell fraction were more limited. As expected, HCL patients had an almost total deficiency in all myeloid subsets.

Lymphocyte counts were more heterogeneous than myeloid counts in the patient groups. In LCH; T, B and NK cells were not increased. In HCL, lymph counts varied considerably and were increased in some cases. In ECD, lymphocyte counts were decreased in all of the $BRAF^{V600E}$ patients, but relatively normal in the $NRAS^{Q61R}$ patient. Patient A2712 was B cell deficient but had received prior therapy with cladribine.

All analyses were performed when patients were not receiving active therapy although some had previously received cytotoxic drugs.
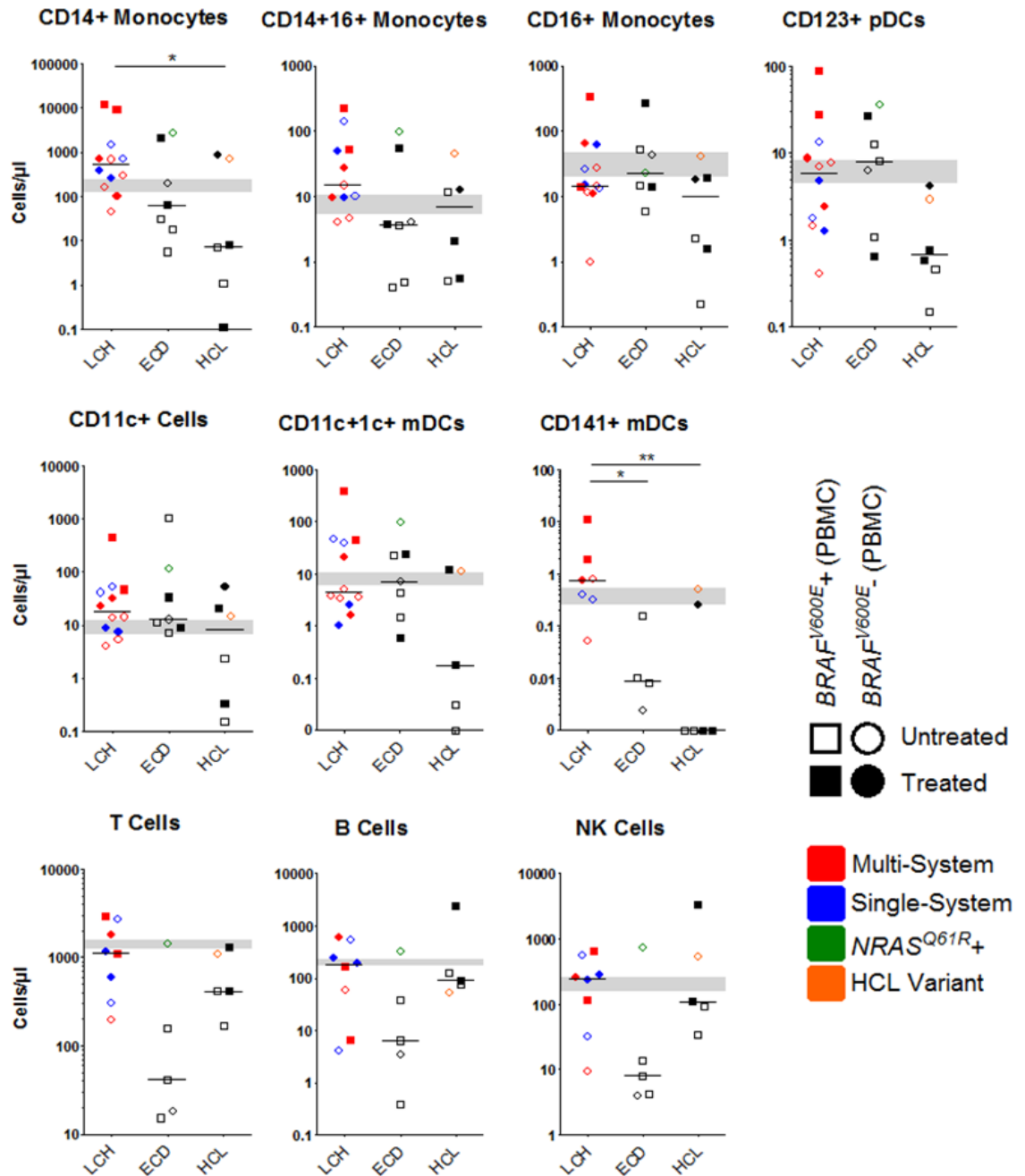
Figure 5.4. Peripheral Blood Mononuclear Cell Phenotype Profile

Comparison of controls (n=21, grey bars, indicating 95% CI) and patients with LCH (n=13), ECD (n=7) and HCL (n=6) by TruCOUNT analysis. Analysis was performed as previously described. pDC: plasmacytoid Dendritic Cell; mDC myeloid Dendritic Cell. Error bars show medians. Statistical analysis with Kruskal-Wallis non-parametric tests and Dunns' multiple column comparison post-tests. * p=<0.05; ** p=<0.01. CD123+ pDCs in HCL and T cells in ECD were statistically reduced from normal (p=<0.05 for both).

5.3.6. *Peripheral Blood Cell BRAF^{V600E} Mutation Profiling*

Having demonstrated mutated BRAF in the peripheral blood (section 5.2.2, page 152), I sought to map these alleles to a specific cellular compartment. Peripheral blood was separated into mononuclear fractions and granulocytes. Mononuclear cells were further divided into CD3+ T cells, CD19+ B cells and HLA-DR+ Lineage- cells, the last composed of monocytes and dendritic cells, (Figure 5.5.A). There was no evidence of abnormal populations of mononuclear cells in these patients and no cells bearing LCH markers CD1a and Langerin or HCL marker CD103 (Figure 5.5.B-C)
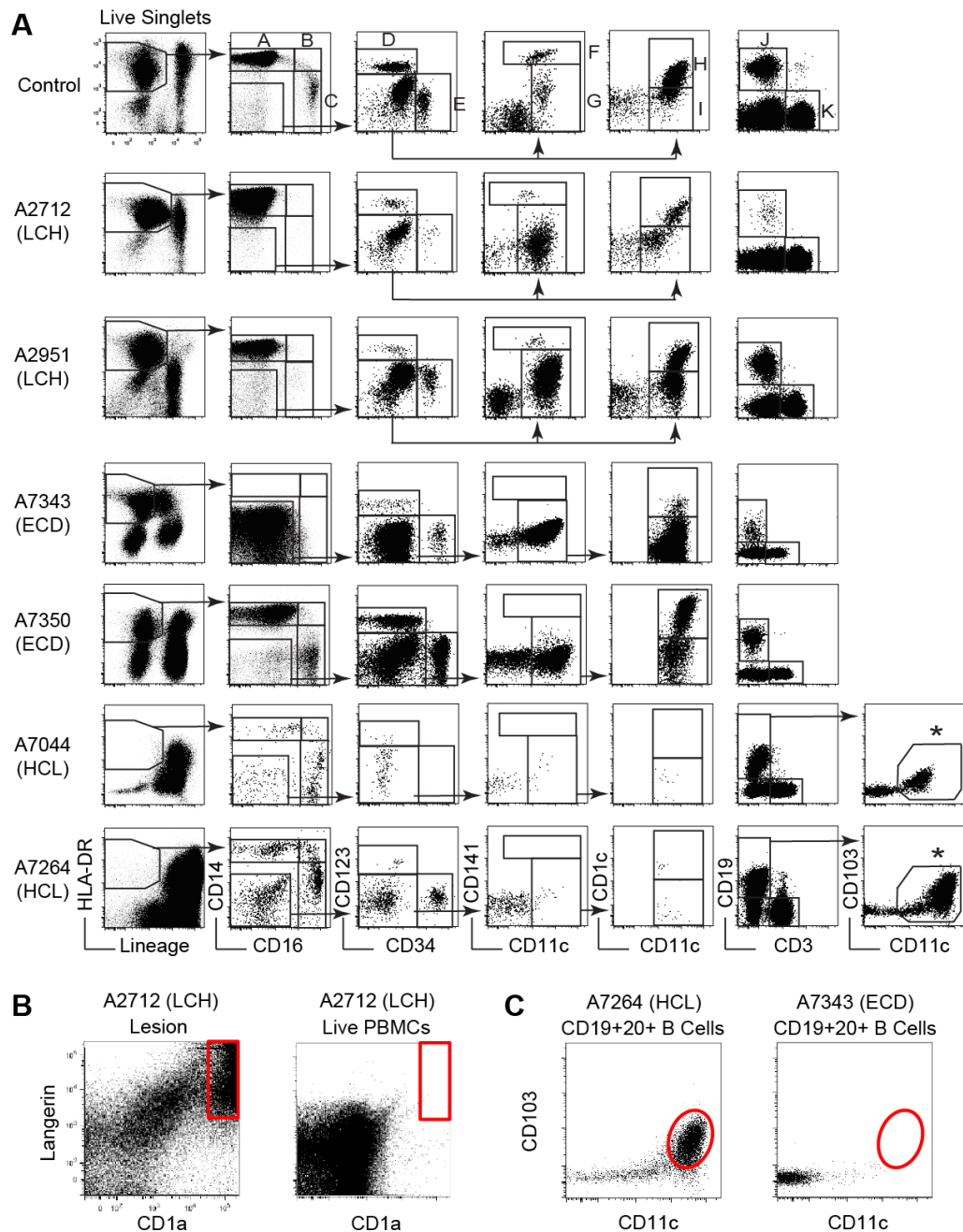
Figure 5.5. Peripheral Blood Gating Strategies

A. Mononuclear cell profiling of BRAF^{V600E} MS LCH patients compared with control. Lineage contained CD3/CD19/CD20/CD56. The CD3 vs. CD19 plot was derived from a separate tube. Populations: A: CD14+ monocytes; B: CD14+16+ monocytes; C: CD16+ monocytes; D: CD123+ pDCs; E: CD34+ progenitors; F: CD141+ mDCs; G: CD11c+ mDCs; H: CD1c+ mDCs; I: residual CD11c+ cells; J: CD19+ B cells; K: CD3+ T cells; *: HCL cells.

B. Absence of circulating Langerin+ or CD1a+ cells in patients with MS LCH. Lesional cells from the skin and PBMC of patient A2712 shown for comparison.

C. Absence of HCL cells in LCH/ECD patients. CD19+20+ B cell-like cells of HCL patient A7264 and ECD patient A7343 shown for comparison.

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5.3.6.1. *Comparative Gating Strategies*

A recent study found mutated BRAF in CD14+ and CD14- fractions of CD11c+ cells (Berres *et al.*, 2014). As these populations are heterogeneous, I sought to resolve the fractions in more detail, to find the exact cell types harbouring the mutation. The CD11+CD14+ component contains classical and intermediate monocytes while the CD11c+14- population comprises CD16+ monocytes, CD1c+ DCs and residual CD1c- CD11c+ cells (often counted with CD1c+ DCs as 'CD11c+ mDCs'). The gating strategy used in the aforementioned study, and the gating strategy used in this thesis is compared in Figure 5.6.
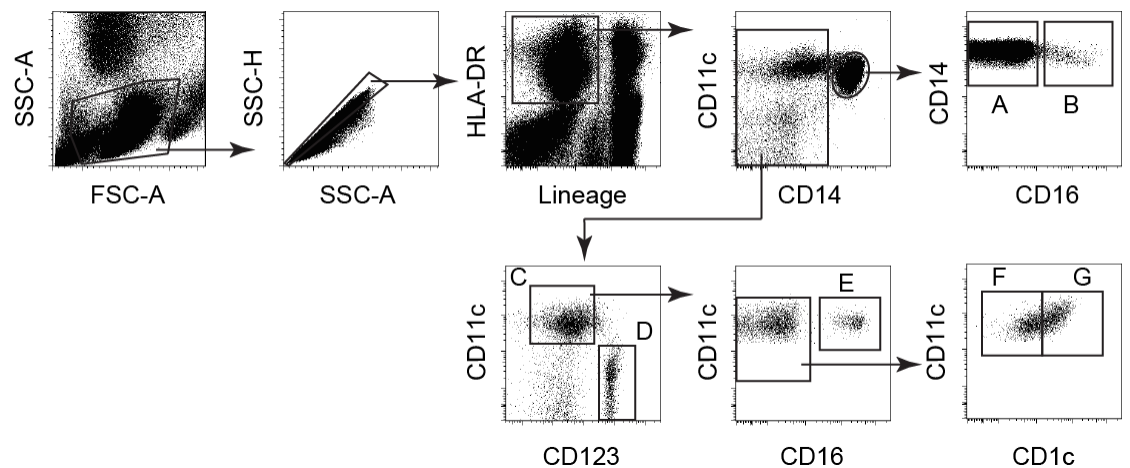
Figure 5.6. Comparative Analysis of Berres et al Gating Strategy

Comparison of the gating strategy used in this thesis with the study of Berres et al (Berres et al., 2014). When HLA-DR+ Lineage- cells are displayed as a 2D plot of CD11c vs. CD14, CD11c+ CD14+ cells contain two subsets of monocytes: (A) CD14+ classical monocytes; and (B) CD14+CD16+ intermediate monocytes. CD14- cells contain CD11c+ myeloid cells (C) and CD123+ pDCs (D). The CD11c+ myeloid cells from this gate may be divided into three further subsets, the CD16+ non-classical monocyte (E) residual CD11c+ mDCs (F) and CD1c+ mDCs (G).

5.3.1. *Peripheral Blood May-Grünwald-Giemsa Stain Morphology*

In order to evaluate if there were any circulating cells with abnormal morphology in the disease types, cytospins of each sorted mononuclear cell subtype were made and stained with May-Grünwald-Giemsa. Normal samples were processed in a quicker time than disease patients due to constraints between clinic times and flow cytometry sorter availability (Max 6 hours for normal, 18 hours for LCH and HCL), and ECD cytospins were from frozen material received from another site.

Evaluation of the cytospins showed no evidence of abnormal populations in either LCH or ECD patients (Figure 5.7). HCL also showed no abnormalities in the morphology of the normal cell subtypes. This absence of abnormal cells suggested that mutated BRAF was harboured by leukocytes with a normal phenotype. There may however be differences in cellular size, the LCH patient's monocytes and mDCs, particularly the CD16+ monocytes appeared to be larger than the normal cells, and both the ECD and HCL cells appeared to be smaller and more activated. The small cell sizes in the ECD patient could be an artefact of the freeze/thaw process. These observations are only exploratory; many more patients would have to be assessed to determine if these are common morphologies.

HCL cells were sorted from patient A7264 by positivity staining for CD19, 20, 11c and 103. The majority of these cells were medium sized and had cytoplasmic projections with a characteristic hair-like appearance. The cells had irregular cell membranes containing a moderate amount of cytoplasm and round or oval shaped nuclei with well-defined borders and overly large nucleoli. A smaller fraction of cells were round in appearance, lacking projections and containing several large vacuoles in the cytoplasm.

LCH cells were sorted from a skin lesion of patient A2712 by expression of CD1a and langerin. There was heterogeneity in these cells for staining intensity and cellular size. The majority of cells were large in comparison to circulating DCs, with no or varied amounts of cytoplasmic

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projections which could be fine like mature DCs, or thicker, similar to the $BRAF^{V600E}$+ cell line A375 (Figure 4.8, pg. 142), or possibly veiled like immature DCs. Although large in comparison with normal cells, the nuclei of the LCH cells were small in comparison with their size, and were irregularly shaped with prominent nucleoli. The cells tended to contain a large amount of cytoplasm containing several small vacuoles.
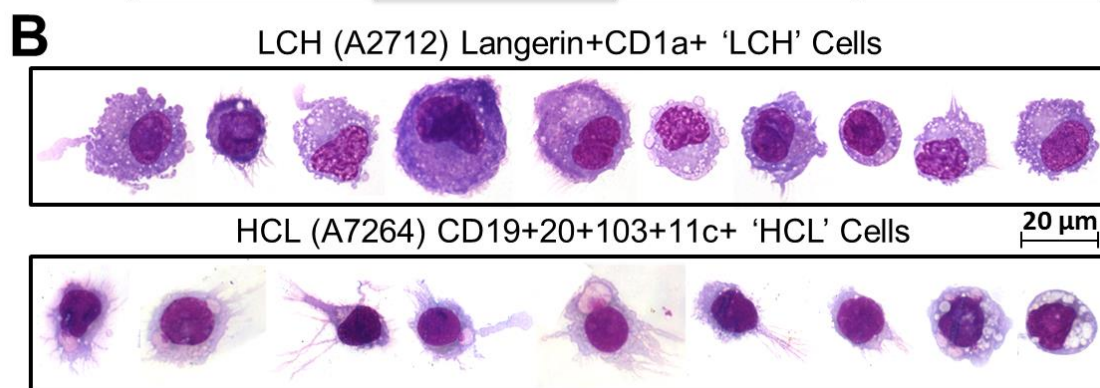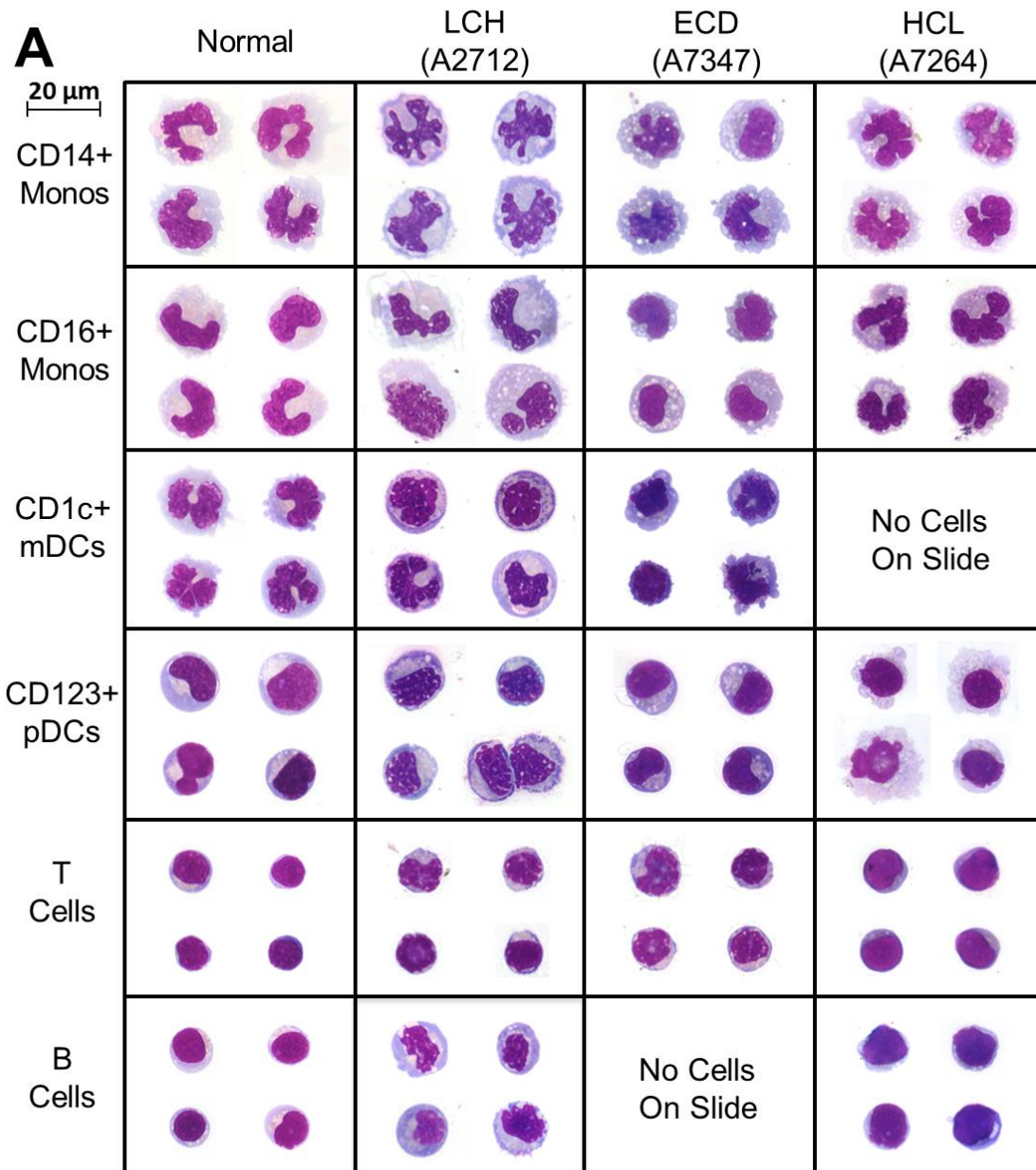
Figure 5.7. Cytology of Sorted Mononuclear Fractions

Cytospin slides were prepared from sorted cells and stained with May-Grünwald-Giemsa (pH 6.8) on a Sysmex SP-1000i. Photographs were taken on a Zeiss Axioimager Z2 microscope with AxioVision 4.8 software at 100 times magnification. Scale as indicated.

5.3.2. *Peripheral Blood Mononuclear Cell BRAF^{V600E} Profiles*

The cellular subsets from all patients displaying *BRAF*^{V600E} positivity in the PBMCs, were flow cytometrically sorted as in Figure 5.4, and tested for *BRAF*^{V600E} by the PCR methods developed in Chapter 4 in order to ascertain the specific identity of the cells containing the mutation.

As was expected, in HCL the majority of mutated alleles were seen in the CD11c+CD103+ HCL cells, with some positivity seen in the CD11c-103- B Cells and NK cells of some patients (Figure 5.8).

In both MS LCH patients *BRAF*^{V600E} was greatly enriched in all fractions of monocytes and CD1c+ mDCs. Neutrophils and T cells were negative but pDCs and B cells were also low positive in A2951. Although some positivity was seen in the CD14+ monocytes, the greatest enrichment of mutated BRAF was found in CD16+ and CD14+CD16+ monocytes even though these were minor populations relative to CD14+ monocytes (Figure 5.8). Residual CD1c- CD11c+ cells were also positive in A2951, but at a lower level than CD1c+ DCs.

T cells were negative for the mutation in all patients, and pDCs, B cells and NKs were negative or positive to a very low level in all the LCH and ECD patients. Surprisingly, no *BRAF*^{V600E} was seen in ECD patients in any of the subsets corresponding to those which were *BRAF*^{V600E}+ in either LCH or HCL.

To gain further insight into the potential of monocytes or mDCs to contribute to peripheral LCH cells, the product of *BRAF*^{V600E} allele frequencies and the abundance of each cell fraction were calculated. This demonstrated that 95-98% of the mutated BRAF signal was contained in CD14+, CD14+CD16+ and CD16+ monocytes. The total *BRAF*^{V600E} contribution of CD1c+ mDCs was <2%.

		CD14+ Mono	CD16+ Mono	CD1c+ mDC	CD123+ pDC	T	B	NK	HCL
HCL	A2123	0.00	0.00	0.00	0.00	0.00	0.05	0.40	22.85
	A7044	0.00	0.00	0.00	0.00	0.00	0.35	1.06	73.65
	A7264	0.00	0.00	0.00	0.00	0.03	0.29	0.01	29.34
	A7073	nt	nt	nt	nt	0.01	26.87	0.10	38.21
LCH	A2712	0.14	4.07	0.11	0.06	0.01	0.00	0.00	n/a
	A2951	4.82	45.61	4.96	0.90	0.03	0.16	0.00	n/a
ECD	A7343	0.00	0.03	0.00	nt	nt	0.00	0.00	n/a
	A7346	0.00	0.01	0.00	0.00	0.00	nt	nt	n/a
	A7347	0.00	0.00	0.00	0.01	0.00	0.01	nt	n/a
	A7350	0.00	0.04	0.00	0.00	0.03	0.00	0.00	n/a

Figure 5.8. Peripheral Blood HLA-DR+Lineage- Subsets % Mutation

The % distribution of BRAF^{V600E} among peripheral blood cell subsets.

nt = not tested (due to low cell numbers); n/a = not applicable.

5.3.3. *Peripheral Blood Lineage/DR Fractions BRAF^{V600E} Profiles*

In order to exclude any *BRAF^{V600E}* positive but unidentified mononuclear progenitors, the distribution of *BRAF^{V600E}* was analysed across the entire mononuclear population according to four quadrants defined by the HLA-DR vs. lineage plot (Figure 5.14).

As expected, in HCL patients the majority of mutated alleles (68-99%) were localised within the HLA-DR+Lineage+ fraction, which is mostly composed of B Cells. The remaining mutated alleles were located in the HLA-DR-Lineage+ fraction, and are most likely HCL cells which have leaked into this fraction.

In multisystem LCH, *BRAF^{V600E}* was primarily localised to monocytes and CD11c+ mDCs. Overall, 78-94% of all *BRAF^{V600E}* alleles were found in HLA-DR+, lineage- cells. Only a few percent of *BRAF^{V600E}* signal was recovered in the HLA-DR- lineage- gate or lineage+ fractions, indicating that monocytes and DCs were the major carriers.

In contrast, in ECD, *BRAF^{V600E}* was undetectable in monocytes and 80-82% of mutated alleles were recovered from the HLA-DR- lineage- quadrant. Further analysis localised these alleles to CD33+ early myeloid cells. Thus differences in disease phenotype were reflected in distinct patterns of involvement of mononuclear cells.

The HLA-DR-Lineage- fraction of two further ECD patients was analysed (see Figure 4.8 for gating strategy) revealing the presence of most *BRAF^{V600E}* alleles in a population of CD33+ early myeloid cells (Figure 5.8). Low levels of positivity were seen in other Lin-DR- subsets including eosinophils and cells which bore the appearance of innate lymph cells.
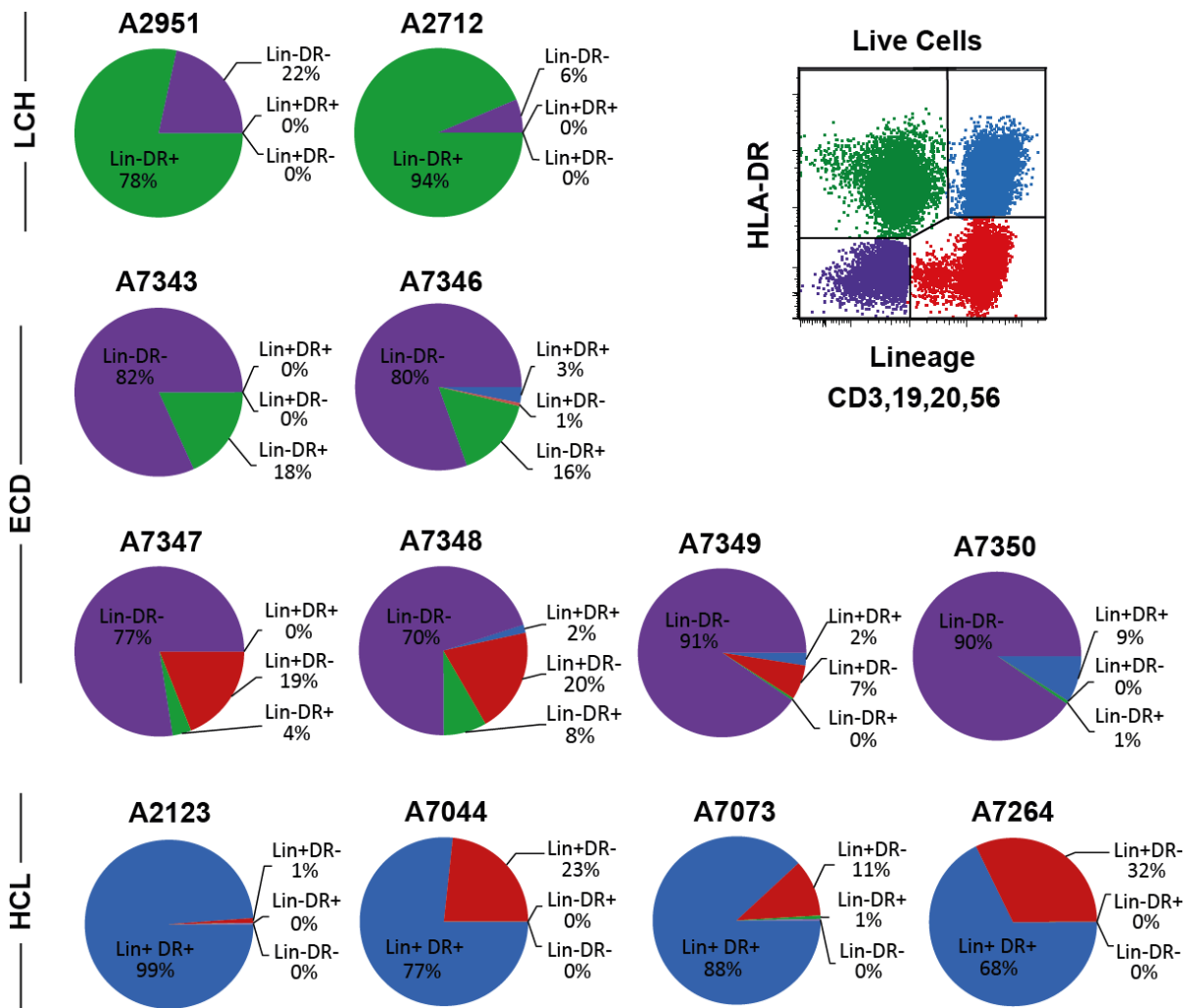
Figure 5.9. Peripheral Blood Lineage/HLA-DR Subsets % Mutation

The distribution BRAF^{V600E} in sorted HLA-DR-Lineage- PBMC of LCH, ECD and HCL patients. Pie chart colours relate directly to Lineage/DR plot inset (control sample); Green = Lineage-DR+; Blue = Lineage+DR+; Red = Lineage+DR-; Purple = Lineage-DR-.

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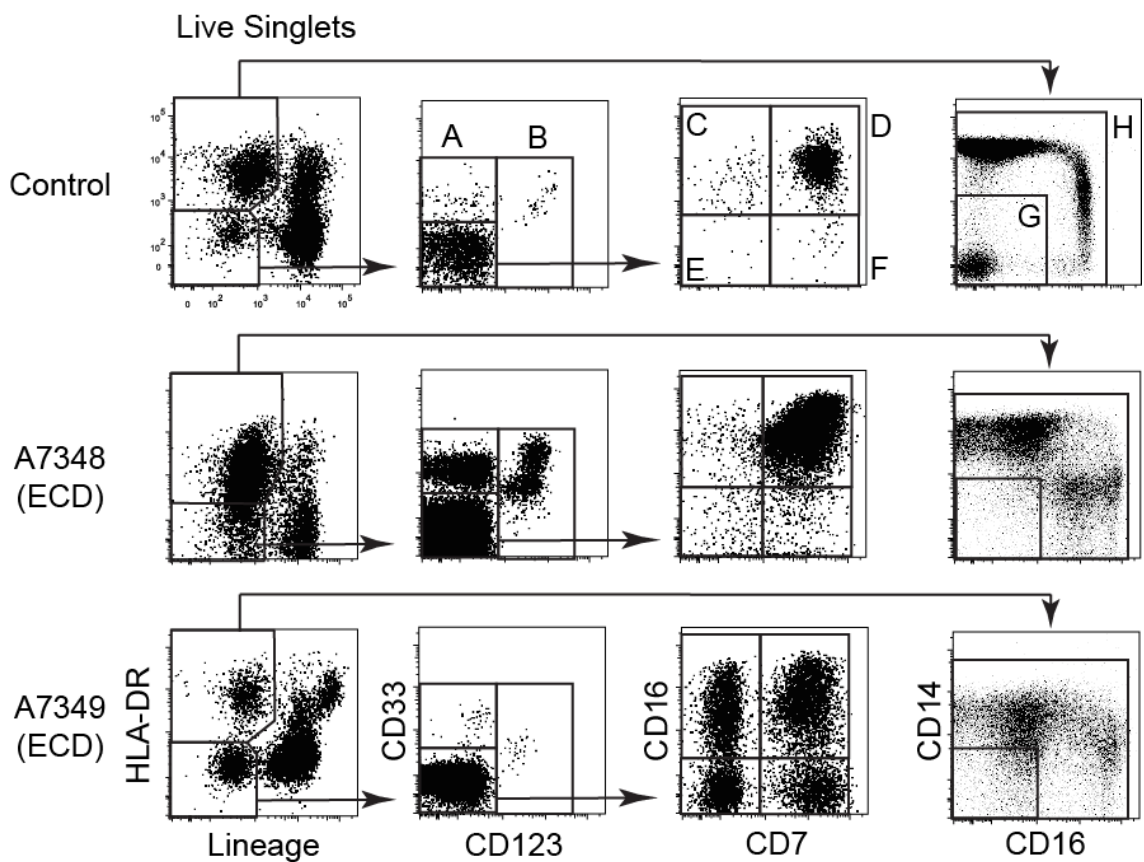
Figure 5.10. Peripheral Blood HLA-DR-Lineage- Subset Sort Gating

Gating strategy for subsets of the HLA-DR-Lineage- fraction from two ECD patients compared with a control. The HLA-DR-Lineage- fraction of live cells is gated and split by expression of CD33 and CD123 into three subsets: CD33+ Myeloid (A); CD123+ Basophils (B) and a double negative fraction which is thought to be comprised mostly of innate lymph cells. This double negative fraction was further split by CD16 and CD7 into four sub-fractions (C-F). The HLA-DR+Lineage- fraction was split between CD14-CD16- dendritic cells (H) and a combined gate of CD14+ and CD16+ monocytes (G).
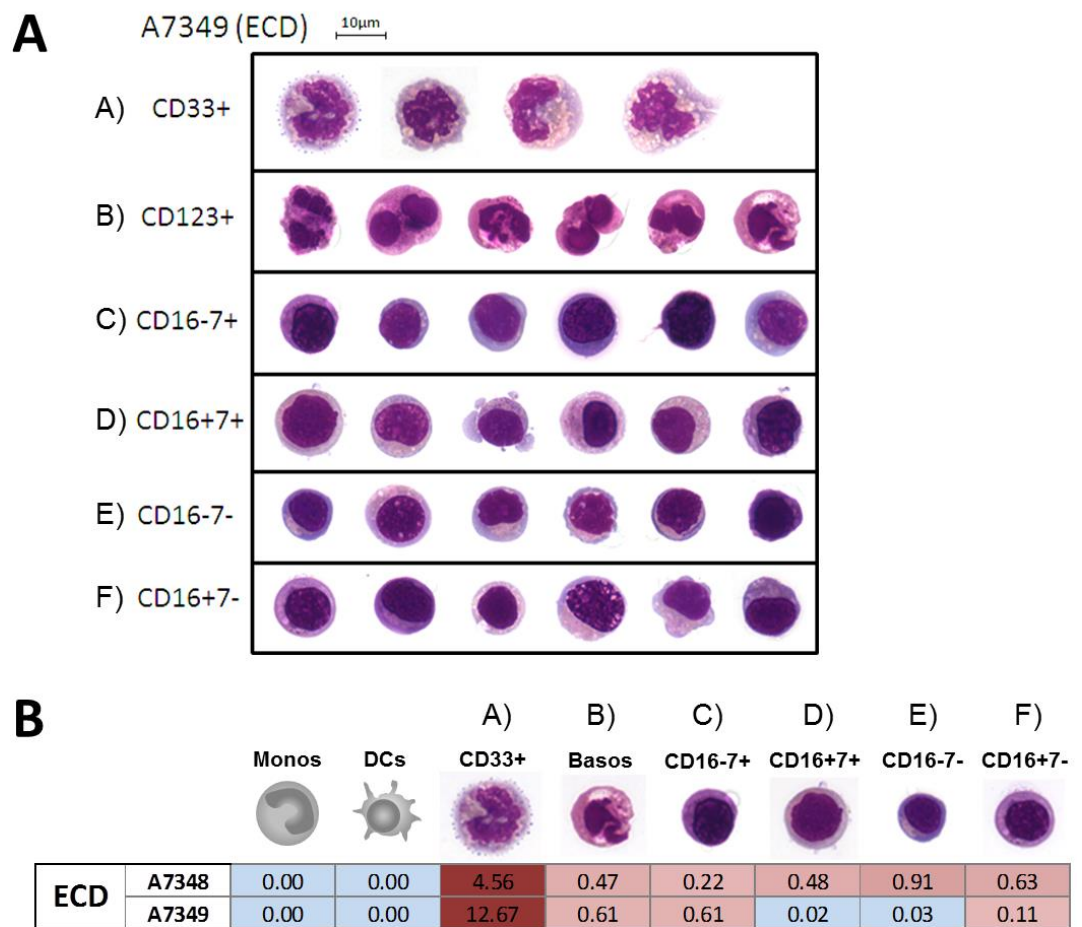
Figure 5.11. Peripheral Blood HLA-DR-Lineage- Subset Results

A. Cytospin slides were prepared from HLA-DR-Lineage- fractions of patient A7349 and stained with May-Grünwald-Giemsa (pH 6.8) on a Sysmex SP-1000i. Photographs were taken on a Zeiss Axioimager Z2 microscope with AxioVision 4.8 software at 100 times magnification. Scale as indicated.

B. The % distribution of $BRAF^{V600E}$ mutation among HLA-DR-Lineage- peripheral blood cell subsets. Monos = CD14+ & CD16+ Monocytes; DCs = Dendritic cells, i.e. All HLA-DR+Lineage-CD14-CD16- cells.

5.3.4. *Bone Marrow Progenitor Phenotypic Profiling*

In order to evaluate the presence or absence of LCH cells in patient bone marrow, and the levels of normal progenitor cells, samples of this tissue were run on the bone marrow progenitor cell flow cytometric profiling protocols developed earlier in this thesis (section 2.6.3, page 79), with the additional inclusion of CD1a and Langerin antibodies to test for aberrant LCH cells.

In keeping with pathological examination (data not shown), bone marrow specimens of the MS LCH patients contained CD1a+ Langerin+ LCH cells by flow cytometry. Notably, these and any CD14+ cells that might contain $BRAF^{V600E}$ were localised exclusively to the CD34- fraction of bone marrow mononuclear cells (Figure 5.12.A). The CD34+ BM progenitor population was divided into CD38 high and low fractions and subsequently analysed according to expression of CD10, CD45RA, CD123 and CD90 to capture hematopoietic stem cells (HSC) and committed progenitor fractions (Figure 5.12.B).

The frequency of CD34+ cells was not increased in the MS LCH patients, but there was a relative expansion of the HSC and the myeloid fractions; common myeloid progenitors (CMP) and granulocyte macrophage progenitors (GMP) megakaryocyte/erythroid progenitors (MEP) and the uncharacterised $CD123^{high}$ fraction (Figure 5.13). At the time of analysis, both patients had received prior cytoreductive chemotherapy. Multi-lymphoid progenitors (MLP) were decreased in the older patient (A2951) and B/NK progenitors were markedly decreased in the patient lacking peripheral B cells who had previously received cladribine (A2712).
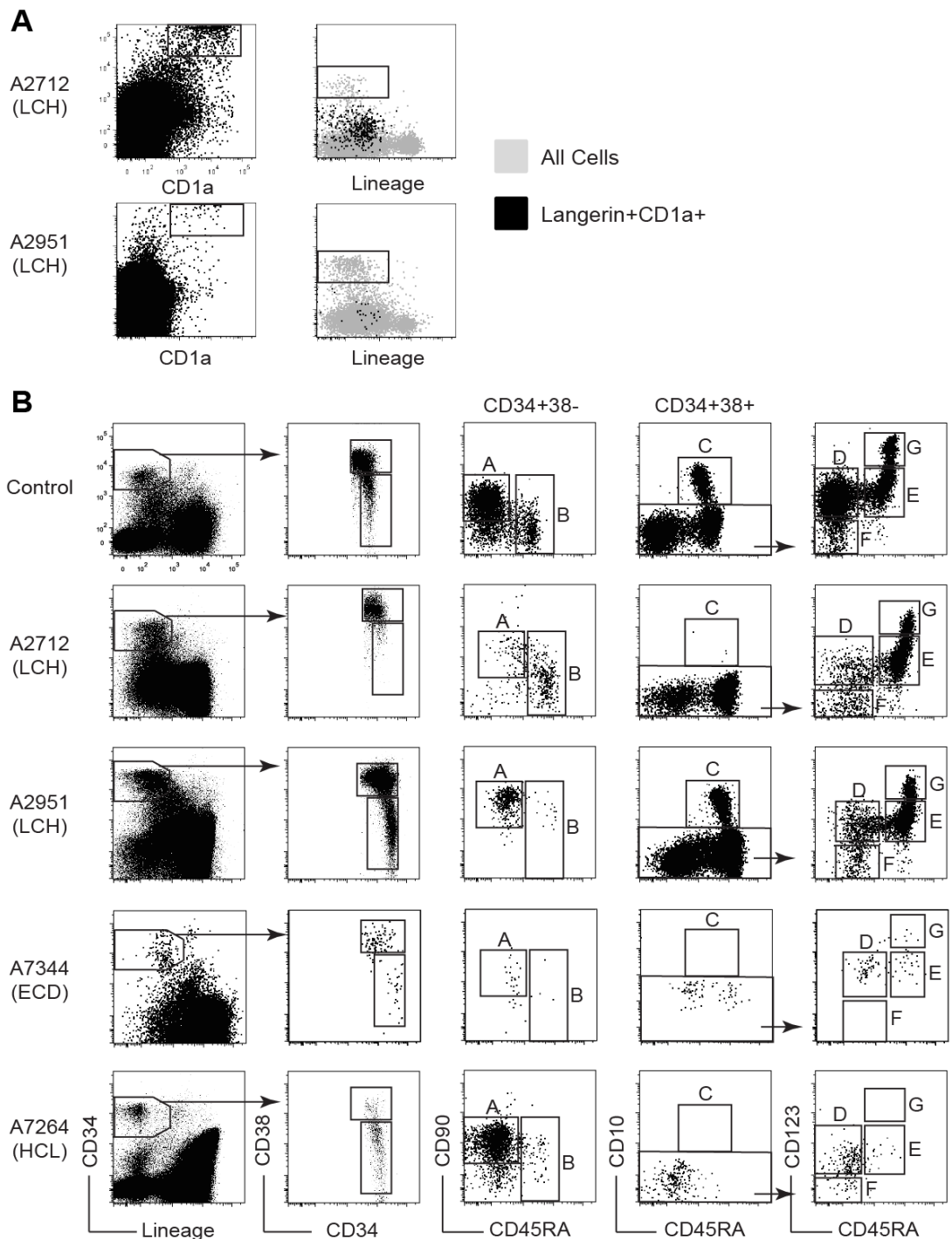
Figure 5.12. Bone Marrow Progenitor Cell Profile of LCH Patients

A. Langerin$^+$CD1a$^+$ LCH cells were detected by flow cytometry in both patients with active MS LCH (left panels). These cells were excluded from the CD34$^+$ gates used to analyse progenitors (right panels).

B. Phenotype of BM from BRAF^{V600E}/NRAS^{Q61R} patients compared with healthy control. Populations: A: Hematopoietic stem cells (HSC); B: Multi-lymphoid progenitors (MLP); C: B/NK cell progenitors (B/NK); D: common myeloid progenitors (CMP); E: granulocyte/macrophage progenitors (GMP); F: megakaryocyte/erythroid progenitors (MEP); G: CD123^{high} population.
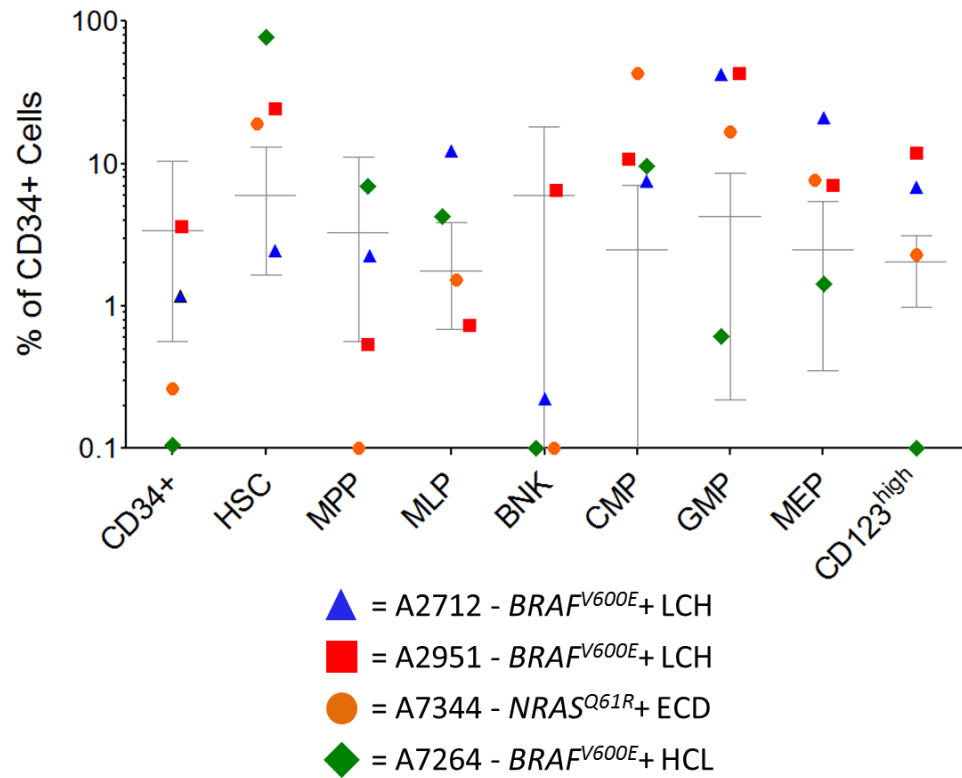
Figure 5.13. Bone Marrow Progenitor Cell Profile of LCH Patients

BM CD34$^+$ progenitor profiles in four patients; two BRAF^{V600E}+ MS LCH, one NRAS^{Q61R}+ ECD and one BRAF^{V600E}+ HCL. Grey error bars demonstrate the median and normal range in healthy controls (n=21) for comparison. CD34+ cells are expressed as % of total live cells, all other cell types are expressed as % of CD34+ cells. BM aspirate was obtained in parallel with the peripheral blood shown previously.

5.3.5. *Bone Marrow Progenitor BRAF^{V600E} Profile*

To explore the existence of long lived *BRAF^{V600E}* + progenitors that could differentiate into monocytes with *BRAF^{V600E}* mutation, bone marrow CD34+ cells from the two MS LCH patients were sorted and analysed by PCR for mutated BRAF. *BRAF^{V600E}* was present in HSC, CMP and GMP. MLPs which have lymphoid, monocyte and DC potential *in vitro* contained a low level of 0.2% *BRAF^{V600E}* in A2951.

BM was available from one of the HCL patients, but there were very few CD34+ cells present. The HCL patient had decreased levels of BNK, GMP, MEP and the uncharacterised CD123^{high} fraction, with relative increases in HSCs and MLP (Figure 5.13), though the only subset in high enough numbers for*BRAF^{V600E}* mutation testing was the HSCs, which were found to be positive to 0.57%.

5.3.5.1. *NRAS^{Q61R} Mutations in ECD*

Unfortunately no BM was available from any of the *BRAF^{V600E}*+ ECD patients, however BM from the ECD patient with an *NRAS^{Q61R}* mutation was analysed. This patient had a relatively low level of BM CD34+ cells, with reduced numbers of MPP and BNK and relatively increased levels of CMPs, GMPs and MEPs. The CD123^{high} fraction was within the normal range.

In this patient, the BM progenitor subtypes were sorted and DNA extracted as previously described, then was amplified and analysed by Sanger Sequencing for the *NRAS^{Q61R}* mutation. The results from the sequencing showed a similar profile to the *BRAF^{V600E}*+ profile in LCH, with positivity in the HSCs, CMPs and GMPs (Figure 5.14), however the population with the highest degree of mutation was the GMPs rather than the CMPs as was seen in LCH.

A.

		HSC	CMP	GMP	MLP	B/NK	HCL
BRAF *V600E* — **HCL**	A7264	0.57	nt	nt	nt	nt	33.37
LCH	A2712	0.47	4.56	0.61	0.00	0.00	n/a
	A2951	0.61	12.67	1.33	0.20	0.00	n/a
NRAS^{Q61R} — **ECD**	A7344	29.00	21.00	63.00	0.00	0.00	n/a

B.
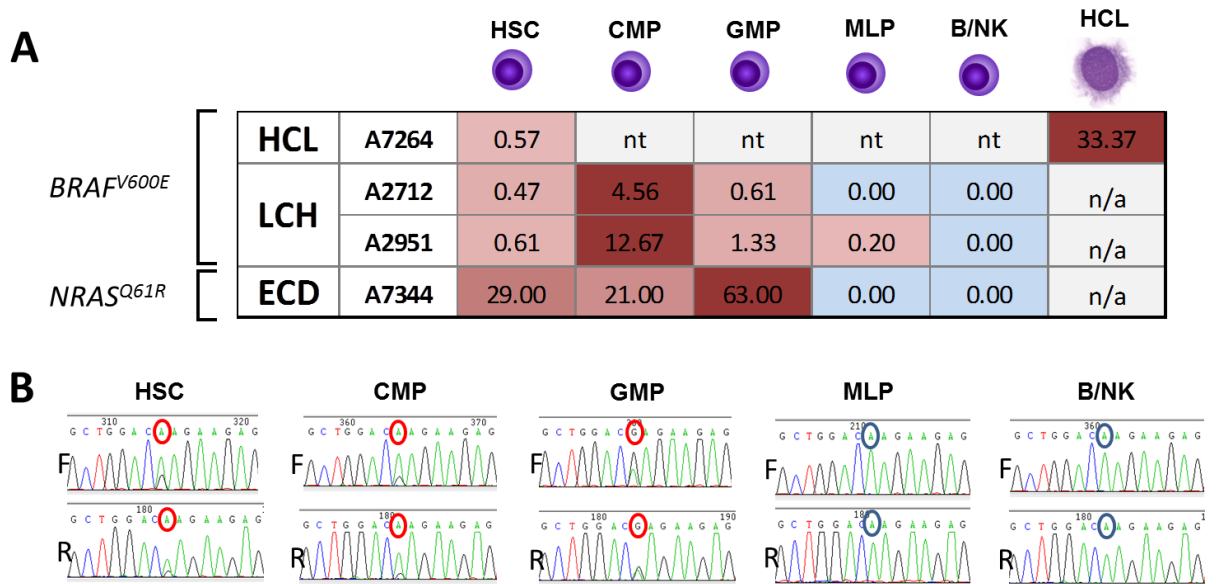
Figure 5.14. Peripheral Blood Subset % Mutation

A. The % distribution of $BRAF^{V600E}$ among bone marrow progenitor cell subsets. nt = not tested (due to low cell numbers); n/a = not applicable. BM aspirate was obtained in parallel with the peripheral blood shown previously.

B. Detection of $NRAS^{Q61R}$ by Sanger sequencing. Red circles indicate positivity, blue circles indicate negativity.

5.4. Discussions for Chapter Five

This chapter describes a high resolution mapping of the $BRAF^{V600E}$+ haematopoietic clonal disease cells in adult patients with LCH, ECD or HCL using the techniques established in Chapter 4, alongside multi-parameter flow cytometric peripheral blood and bone marrow phenotyping.

5.4.1. *Phenotyping*

Consistent with previous reports, no CD1a+ or Langerin+ cells were detected in the peripheral blood (Rolland *et al.*, 2005; Senechal *et al.*, 2007; Satoh *et al.*, 2012; Berres *et al.*, 2014). Likewise, the LCH and ECD patients did not have any detectable circulating CD11c+103+ HCL cells.

Patients with MS LCH had relatively expanded myelopoiesis with a reactive phenotype, but cytological examination of the blood and bone marrow excluded any evidence of leukemic transformation. Expansion of blood CD11c+ mDCs in LCH has been previously but not uniformly reported (Rolland *et al.*, 2005; Senechal *et al.*, 2007). Possible differences were seen between the LCH and ECD patients, in general the $BRAF^{V600E}$+ECD patients had less expanded myelopoiesis, with possible deficiencies in CD141+ mDCs and lymphoid cell types, whereas the $NRAS^{Q61R}$+patient was more in keeping with the MS LCH, with expanded monocytes and CD11c+ mDCs, and relatively normal or slightly expanded lymphoid cell numbers. As expected in HCL, the majority of patients had reductions in all myeloid cell types, with variable numbers and increases in B and NK cells. Unfortunately due to the low number of recruited patients, it is hard to draw any further conclusions from the PBMC phenotyping results.

Similarly, few BM samples were available for the study. BM from the two PBMC $BRAF^{V600E}$+ MS patients showed normal levels of CD34+ progenitors, but with a myeloid skewed phenotype including CMP, GMP, MEP and the uncharacterised CD123^{high} fraction. The older patient who had previously had cytoreductive cladribine treatment had decreased levels of BNK progenitors. BM from the $NRAS^{Q61R}$ patient showed reduced

CD34+ and MPP progenitor numbers, but a similar profile of increased myeloid progenitors CMP, GMP and MEP, but with normal numbers of CD123^{high} cells. Due to the fact that the BM from HCL patients is characteristically fibrotic (Burke, 1978), only one sample was available for analysis. This patient had extremely low levels of CD34+ progenitors, mostly comprised of HSC, with low levels of myeloid progenitors and complete absence of CD123^{high} cells.

5.4.2. PBMC BRAF^{V600E}

Due to the increased numbers of circulating monocytes and CD1c+ myeloid DCs in the MS LCH patients, it could be inferred that they might contribute to the pathogenesis of LCH, however this requires more than phenotypic correlation. I therefore looked to patients with detectable BRAF mutation in their peripheral blood to determine whether CD11c myeloid DCs or monocytes were part of the abnormal clonal myelopoiesis and might therefore contribute to LCH pathology.

A number of previous reports did not find mutated BRAF in the peripheral blood or bone marrow of patients with LCH (Sahm *et al.*, 2012; Satoh *et al.*, 2012). One reason may be that this appears restricted to MS patients with active disease, which are a relatively small subset of patients particularly among adults (Berres *et al.*, 2014). Another factor is that sufficiently sensitive techniques must be used such as allele-specific PCR, rather than primer extension or next generation sequencing, to detect mutated alleles below 1% abundance (Satoh *et al.*, 2012).

Data on *BRAF^{V600E}* in ECD lesions and peripheral blood are even more controversial, possibly dependant on the specificity of techniques used. One study puts the percentage positivity of ECD lesions at 54% with pyrosequencing (Haroche *et al.*, 2012b).However a later study shows that 67% of patients are positive by pyrosequencing but 100% using the immunohistochemistry antibody VE1, followed by detecting the mutation in 100% of patient's PBMC by droplet delay PCR (ddPCR) or pyrosequencing coupled to locked nucleic acid PCR (LNA-PCR) (Cangi *et*

al., 2014). In my hands I have seen the VE1 antibody to have non-specific, granular staining in cells such as lymph node macrophages and sebaceous gland cells (data not shown), and is therefore not useful for the specific identification of LCH or ECD cells.

In order to deal with these potential problems, I used a commercially available mutation detection PCR assay, modifying the amount of input DNA to ensure that sufficient genome equivalents were analysed to detect as few as 0.1% mutated alleles.

In keeping with a recent report in children(Berres *et al.*, 2014), *BRAF*^{V600E} was only detected in the peripheral blood of LCH patients with active MS or 'high risk disease' involving liver, spleen or bone marrow. Therefore I would speculate that there is a correlation between MS LCH and circulating *BRAF*^{V600E}, though due to limitations in patient numbers I cannot say that this is a certainty. If this is the case though, it could go some way to explaining the differences between MS and SS disease, the former arising from the BM via a circulating precursor, the latter arising from a localised tissue precursor.

BRAF^{V600E} mutation was detected in the PBMC of 2/3 patients with multi-system (MS) LCH, 5/8 ECD patients and 4/6 HCL patients. Of the two negative HCL patients, one has variant HCL, which is known to be *BRAF*^{WT} in all cases (Tiacci *et al.*, 2011), the other patient was clinically well (WBC 4.0x10^9/L) and had received cytotoxic cladribine treatment two months previously. Patients with single system LCH or BRAF wild-type lesions did not have detectable *BRAF*^{V600E}in their peripheral blood.

In the study by Berres *et al*, mutant BRAF was found in both CD14+ and CD14-CD11c+ populations in childhood LCH (Berres *et al.*, 2014). In this thesis, similar results were obtained in adults showing that CD14+ monocytes are a substantial reservoir of *BRAF*^{V600E}. It was also possible to resolve the distribution of *BRAF*^{V600E} within CD14-CD11c+ cells in more detail. Results here indicate that the major mutated component in LCH is contained in non-classical CD16+ monocytes. CD1c+ mDCs, although

BRAF^*V600E*+, are relatively rare and contributed less than 2% of *BRAF*^*V600E*signal detectable in the HLA-DR+Lineage- population. The cell types seen to contain the *BRAF*^*V600E* mutation all have a phagocytic capacity, so a false positive result may have arisen from phagocytosis of aberrant LCH cells. However, I have seen that the peripheral blood contains no evidence of LCH cells, so this is unlikely.

In ECD, the major mutated component was a CD33+ early myeloid cell and in HCL the majority of mutation was seen in the CD103+11c+ HCL cells or CD103-11c- normal B cells. The HLA-DR-Lineage- quadrant is the least characterised fraction of cells; it will be well worth studying this quadrant in ECD to learn more about the difference between LCH and ECD.

The occurrence of mutated BRAF in multiple lineages in multisystem LCH is anticipated on the grounds that, in order to sustain clonal myelopoiesis, the mutation must occur in a sufficiently primitive population to be self-renewing. BRAF mutations that occur in non-self-renewing hematopoietic compartments may only be transiently detectable in the peripheral blood or in an isolated lesion, which is self-healing, as others have observed (Satoh *et al.*, 2012; Kansal *et al.*, 2013). In the lung, presence or absence of mutated BRAF has recently been used to indicate the clonality of LCH in this site (Yousem *et al.*, 2013). The level of BRAF mutation in PBMC was low. This is in keeping with abnormal haematopoiesis occurring in parallel with physiological haematopoiesis and suggests that LCH lesions form because the few percent of mutated precursor cells have enhanced survival mechanisms in tissues.

The fact that in ECD the mutated BRAF is most restricted to what appears to be an early myeloid cell, suggests that the cells in ECD may be arrested at an earlier differentiation time point than those in LCH and more driven towards a macrophage like phenotype. It would be interesting to explore whether any *BRAF*^*V600E* can be seen in the HLA-DR-CD33+ of MS LCH patients, although if this is the case, the population is likely to be very small in comparison to the *BRAF*^*V600E*+ cells in the HLA-DR+Lineage-

fraction, as the mutation levels in the HLA-DR-Lineage- fraction of LCH accounted for only 1-2% of the total mutation load. It would also be interesting to explore the HLA-DR-CD33+ fraction of ECD patients in greater detail, to see if they already express, or can be induced to express CD68 like the infiltrating macrophage-like cells in ECD.

5.4.3. BM BRAF^{V600E}

The consistent finding of $BRAF^{V600E}$ in the peripheral blood of both MS LCH patients at several time points implies the existence of self-renewing myeloid progenitors with mutated BRAF. Although the bone marrow contained cells with LCH phenotype and CD14+ cells carrying mutated BRAF, it was possible to exclude these and to demonstrate that $BRAF^{V600E}$ was present in early myeloid progenitors including the GMP, CMP and HSC, with a bias towards the CMP fraction. These findings add further support to recent data showing that unfractionated CD34+ progenitors carrying the BRAF mutation from children with MS LCH may be expanded *in vitro* into myeloid DC and monocyte colonies. In this study it was proposed that MS LCH arises from self-renewing BM progenitors that produce circulating precursor cells but SS LCH evolves by mutation of myeloid progenitors in the periphery (Berres *et al.*, 2014). Unfortunately colony forming unit assays could not be repeated in this thesis due to low progenitor cell numbers. It is difficult to exclude low level BM and blood involvement in SS disease, but the threshold of detection afforded by allele-specific PCR appears to yield a distinction that correlates well with clinical observations.

Bone marrow from $BRAF^{V600E}$+ ECD patients was not available, though BM from an ECD patient carrying the $NRAS^{Q61R}$ mutation revealed a similar profile of positivity to LCH, with the mutation detected in HSCs, CMPs and GMPs, with a skewing towards the GMPs. Similarly in HCL, one patient showed $BRAF^{V600E}$ mutation in the CD34+38-45RA-90+ haematopoietic stem cells (HSCs), echoing recent data showing $BRAF^{V600E}$ positivity in the HSCs of five HCL patients (Chung *et al.*, 2014). These results indicate that at least some subtypes of each disease could

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have origins as early as the haematopoietic stem cell. It would certainly be interesting to examine the other BM progenitor subsets in HCL to see whether the profile is different to LCH and ECD.

5.4.4. *Lesional BRAF^{V600E}*

Analysis of the skin lesion in LCH patient A2712 confirmed previous results that close to 100% of cells with characteristic LCH phenotype are mutated (Badalian-Very *et al.*, 2010; Sahm *et al.*, 2012; Satoh *et al.*, 2012; Berres *et al.*, 2014). The relatively low and stable abundance of *BRAF^{V600E}* in blood and bone marrow subsets is perhaps surprising given the context of a multi-system disease. Comparison with the 100% frequency in lesional LCH cells implies that *BRAF^{V600E}* does not confer a selective advantage within the progenitor or precursor compartments, but rather operates to promote myeloid cell recruitment, proliferation or survival by pathways that are only activated at an inflammatory nidus in the periphery. From this perspective, MS LCH is unlike a leukemic process in which driver mutations invariably cause clonal dominance of haematopoiesis and tissue infiltration is a relatively rare secondary phenomenon.

Lesions in LCH are made up of an admixed selection of LCH cells, T cells, eosinophils, macrophages and dendritic cells. Unfortunately, in this study there was limited availability of lesional material to sort, and therefore only the LCH and T cells of one patient were tested for the mutation. The previous study by Berres *et al* stated that the eosinophils In the lesion were not positive for the mutation (Berres *et al.*, 2014) and it is interesting to note that eosinophils have not been seen in mouse LCH (Steiner *et al.*, 2008).Therefore it seems possible that eosinophils in LCH are a skin-specific immune response rather than a direct contributor to pathobiology, unlike in Hodgkin's lymphoma, where eosinophils are known to play an important role (Pinto *et al.*, 1997).

5.4.5. *Summary*

Using techniques established earlier in this thesis, I have successfully derived peripheral blood and bone marrow phenotypic

profiles and clonal lineages of the histiocytic disorders Langerhans Cell Histiocytosis and Erdheim Chester Disease, as well as reproducing results seen previously in Hairy Cell Leukaemia. However, as several subsets were seen to express the $BRAF^{V600E}$ mutation, the exact identity of possible LCH and ECD precursor cells is yet to be discovered. Also, I cannot say with certainty that any of the $BRAF^{V600E}$+ circulating subsets directly contribute to the development of the disease cells.

Prospective studies are now required to test whether therapeutic decisions can be made according to $BRAF^{V600E}$ testing in the peripheral blood. Adults with high risk MS LCH are rare and may be difficult to treat but the results here illustrate the biological features in common with MS LCH in children. The presence of $BRAF^{V600E}$ in hematopoietic stem cells and poor outcome with myelosuppressive chemotherapy suggest that targeted therapy may be beneficial. The patients described in this study did not access clinical trials of BRAF inhibitors owing to exclusion due to performance status or personal preference. The two patients with PBMC $BRAF^{V600E}$+ MS LCH had aggressive disease with only partial responses to chemotherapy. $BRAF^{V600E}$ levels were monitored throughout treatment and remained detectable in the peripheral blood throughout with substantial burdens in the PBMC and BM shortly before death (data not shown). Taken together, the results obtained in patients of all ages suggest that the BRAF status of an LCH or ECD lesion and testing for $BRAF^{V600E}$ in the blood and BM may assist the delivery of optimal therapy for LCH and ECD.

Faced with several possible myeloid precursor populations, *in vitro* differentiation models may help to define the potential of different $BRAF^{V600E}$+cells to form LCH or ECD cells. Langerin expression may be induced *in vitro* on both CD14+ monocytes and CD34+ progenitor fractions (Caux *et al.*, 1992; Strobl *et al.*, 1996; Geissmann *et al.*, 1998; Ratzinger *et al.*, 2004; Klechevsky *et al.*, 2008; Bauer *et al.*, 2012; Hutter *et al.*, 2012). It is unclear whether or not CD34+ progenitors transition through a monocyte differentiation stage during this process. GM-CSF, IL-4, TNF-α and TGF-β, factors that induce langerin *in vitro*, have all been

reported in LCH lesions (Annels *et al.*, 2003; Fleming *et al.*, 2003). However, several studies have confirmed the role of cell signalling through CD40, Notch and other receptors that are less often studied in *in vitro* models of LC differentiation (Egeler *et al.*, 2000; Hoshino *et al.*, 2005; Hutter *et al.*, 2012). These models are consistent with the potential of CD14+ monocytes to form LCH-like cells but also demonstrate that Langerin expression is not an exclusive property.

Chapter 6. Peripheral Blood DC Langerhans Cell Potential

6.1. Introduction

The work in this chapter involves the *in vitro* culture of flow cytometry sorted mononuclear subsets from control peripheral blood (PB), in an attempt to determine which, if any, subsets can be induced to bear a phenotype similar to Langerhans cells, and thus, also similar to the pathologic cells in Langerhans Cell Histiocytosis (LCH).

6.1.1. *Chapter Aims*

1) To analyse negative and positive methods of DC and monocyte isolation, to ascertain the true composition of the isolated cells.

2) To find if it is possible to stimulate CD1c+ mDCs or other DCs and monocytes of the blood to bear the features of Langerhans cells *in vitro*.

6.1.2. *On the Origin of LCH Cells*

The key markers of LCH cells are surface expression of langerin and CD1a and internal presence of Birbeck granules (BGs). These pathologic cells are thought to be related to the Langerhans cells (LCs) resident in the epidermis, which also bear all three markers (Birbeck *et al.*, 1961; Nezelof *et al.*, 1973; Harrist *et al.*, 1983; Rousseau-Merck *et al.*, 1983; Schuler *et al.*, 1983; Valladeau *et al.*, 2000). However, the definitive origin of the LCH cells is still unclear.

Although murine LCs are self-renewing in the steady state (Merad *et al.*, 2002), during inflammation, they are replaced by recruited cells that differentiate and express Langerin and CD1a following inflammation (Ginhoux *et al.*, 2006; Nagao *et al.*, 2012; Seré *et al.*, 2012).Observations in humans also confirm that LCs can be self-maintained (Czernielewski and Demarchez, 1987; Bigley *et al.*, 2011; Kanitakis *et al.*, 2011) or replaced by bone marrow-derived cells in the context of transplantation and inflammation (Perreault *et al.*, 1985; Collin *et al.*, 2006; Kanitakis *et*

al., 2011; Mielcarek *et al.*, 2014). The nature of bone marrow derived LC precursors that repopulate the epidermis following inflammation is incompletely defined. Experiments in mice with clodronate depletion and bead-labelling suggest a monocyte origin but do not completely exclude other precursors (Ginhoux *et al.*, 2006). It was therefore highly relevant to explore in more detail the capacity of blood borne cells to become LCs, since dysregulated differentiation in this pathway might explain the origin of LCH.

The experiments in this chapter were prompted by three recent observations. Firstly the discovery of the *BRAF*^{V600E} mutation in the peripheral blood CD14+ monocytes and CD14-CD11c+ cells and bone marrow myeloid progenitors of multi-system Langerhans cell histiocytosis (LCH) patients(Berres *et al.*, 2014), which may be precursors of the classic LCH disease cells. Secondly the serendipitous observation that langerin was induced on mononuclear cells isolated from blood bank leukocyte filters held at room temperature overnight. These *in vitro* observations suggest that CD34+ progenitors, monocytes and/or mDCs have the capacity to form LCH-like cells. Finally, the observation that CD1c+ mDCs in tissues express a low level of langerin and that blood CD1c+ mDCs contain langerin mRNA (Bigley *et al.*, 2014). Therefore, it may be possible that LCH cells have an origin in circulating monocytes or CD1c+ mDCs or even further back in the haematopoietic system in BM progenitor cells.

In humans, langerin+ cells can be made *in vitro* from monocytes (Geissmann *et al.*, 1998; Hoshino *et al.*, 2005; Hutter *et al.*, 2012), suggesting a potential precursor role. However, experiments with CD34+ progenitors also demonstrate the existence of an LC-differentiation pathway that appears independent of CD14+ monocytes (Caux *et al.*, 1996; Strunk *et al.*, 1996). More recent studies with murine models have shown that expression of *BRAF*^{V600E} in myeloid haematopoietic progenitors drives a disease phenotype similar to low-risk LCH, whereas expression in differentiated myeloid cells recapitulated severe high-risk LCH.

Another report suggested the existence of CD3/7/14/16/19 negative LC precursors in human blood, and although noted to express CD1c, these were incorrectly described as expressing CD1a (Ito *et al.*, 1999; Mason. D *et al.*, 2000; MacDonald *et al.*, 2002). For a summary of historical langerin induction experiments (See Table 1.5, page 64). A previous study has shown an increase in CD11c+ cells in the PB of LCH patients (Rolland *et al.*, 2005), although this finding is disputed in a study showing no increases (Senechal *et al.*, 2007). Previously in this thesis it was seen that CD11c+ cells, and specifically the CD11c+1c+ mDCs were increased in LCH patients, particularly those with multi-system disease.

More recent observations in mice lacking LCs due to Id2 knockout or conditional ablation show that LC repopulation occurs in two waves (Nagao *et al.*, 2012; Seré *et al.*, 2012). The epidermis is initially infiltrated by a short term precursor with low langerin expression and features in common with monocytes, followed by a long term precursor that acquires the full phenotype of LCs, including self-renewal capacity (Nagao *et al.*, 2012; Seré *et al.*, 2012). It is therefore possible that more than one type of circulating myeloid cell could be the originators of LCH cells.

Historical reports where LCs, containing Birbeck granules have been derived from monocytes, used CD3,7,19,45RA,56 negative selection from PBMC with the now discontinued Monocyte Isolation Kit I from Miltenyi Biotec (http://www.miltenyibiotec.com) (Geissmann *et al.*, 1998; Mohamadzadeh *et al.*, 2001; Guironnet *et al.*, 2002). In the HuDC lab, methods of positive selection are employed which have the ability to distinguish two/three different monocyte subsets and at least two different DC subsets. It is therefore important to find how relatable these methods are, and what is truly isolated by each technique.

6.1.3. *Chapter Hypotheses*

1) Previous reports demonstrating LC-like cell differentiation from monocytes, may have been contaminated by non-monocyte circulating dendritic cells.

2) Monocytes and CD1c+ DCs both form Langerin+ cells in response to defined *in vitro* stimuli.

6.2. Materials and Methods for Chapter Six

6.2.1. *Cell Culture*

CD14+ monocytes, CD16+ monocytes, CD1c+ myeloid DCs (mDCs) and CD123+ plasmacytoid DCs (pDCs) were sorted from PBMC (see section 2.4.2, pg. 71) obtained from healthy volunteers under local ethical approval (Appendix 8). Sorting was performed with an ARIA Fusion (Becton Dickinson; B-D) using previously described protocols. 10,000 cells were cultured in RPMI with 10% FBS or X-Vivo in 100µl. Supplements were added at the following concentrations: GM-CSF: 50ng/ml; TGFβ: 10ng/ml; BMP7: 200ng/ml. Cultures were maintained for 3-14 days and supplemented with fresh cytokines on day 4, 8 and 12.

6.2.2. *Langerhans Cell Isolation*

Normal skin was obtained from mammoplasty and breast reconstruction surgery, obtained from healthy volunteers under local ethical approval (Appendix 8). Fresh Langerhans cells were isolated as previously described (Haniffa *et al.*, 2012). LCs were isolated from epidermal sheets separated from whole skin keratome sections with dispase (Invitrogen) 1mg/ml incubated at 37^oC for 90 minutes in RPMI and subsequently digested with collagenase (Worthington Type IV) 1.6 mg/ml incubated for 12 hours at 37^oC in RPMI with 10% foetal bovine serum (FBS). Viability was >90% by DAPI exclusion (Sigma). Langerhans cells were FACS sorted by lack of autofluorescence and expression of CD45+HLA-DR+CD1a^{high}Langerin^{high}.

6.2.3. *Electron Microscopy*

Cells were fixed for electron microscopy (EM) according to standard protocols in 2% glutaraldehyde, then pelleted, dehydrated and fixed in resin (reagents from TAAB Lab, Aldermarston, UK). Ultrathin sections were cut with a diamond knife RMC MT-XL ultramicrotome and examined with a Philips CM 100 Compustage (FEI) Transmission Electron Microscope. Images were collected with an AMT CCD camera (Deben).

6.3. Results

6.3.1. *Spontaneous Langerin Induction*

The experiments in this chapter were prompted by the serendipitous observation that langerin was spontaneously induced on mononuclear cells isolated from blood bank leukocyte filters held at room temperature overnight (Figure 6.1). It was therefore decided to see which particular cells were undergoing this spontaneous induction.
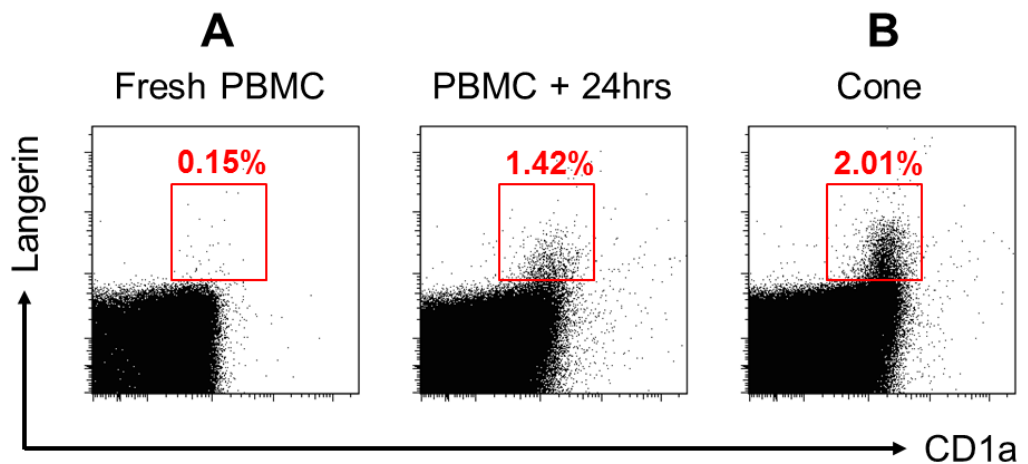
Figure 6.1. Spontaneous Langerin Induction in PBMCs

Examples of spontaneous langerin induction:
  A. *Peripheral blood mononuclear cells (PBMCs), 24hours after density centrifugation separation.*
  B. *Blood bank leukocyte filters (Cone) held at room temperature overnight.*

6.3.2. *Comparative Flow Cytometry*

Previous studies on the production of LC-like cells from circulating cell subset have used a variety of initial isolation methods (See Table 1.5, page 64). The use of negative selection techniques may not enrich a truly heterogeneous cell population, which could lead to the identification of LC-precursors which are in fact contaminants in the original preparation. Therefore I sought to determine which subsets have been isolated by negative isolation methods in previous studies.

In order to compare the exact cell types isolated by negative and positive monocyte and DC isolation methods currently in use; three replicate samples were stained with either negative 'untouched monocyte' selection markers CD3,7,19,45RA,56 or HuDC lineage markers CD3,19,20,56. Monocyte and DC markers CD14,16,123,11c,1c were stained in separate channels to give a downstream gating strategy of the cells isolated.

As seen in Figure 6.2, the cells negatively selected with the 'untouched monocyte' method contain not just classical CD14+ monocytes (~90% of isolated cells), but also CD16+ non-classical monocytes (~5%), CD11c+1c+ myeloid DCs (mDCs; ~3%) and CD123+ plasmacytoid DCs (pDCs; <1%).
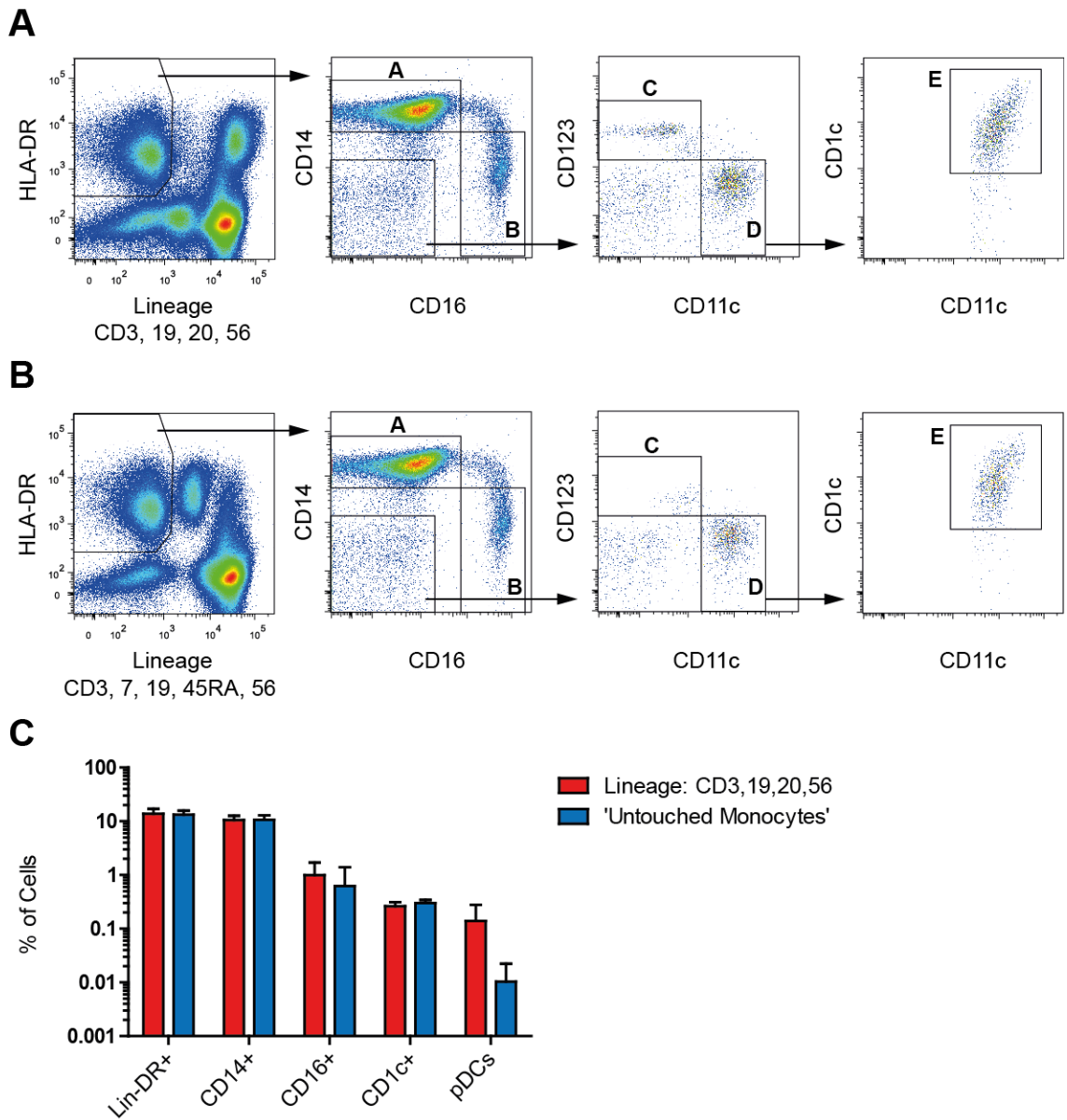
Figure 6.2. Comparative Gating Strategies

A. PBMC were sorted into fractions indicated using the sequential gating shown. A: CD14+ monocytes; B: CD16+ monocytes; C: CD123+ pDCs; D: CD1c+ mDCs.

B. Illustrative gating of PBMCs using a lineage cocktail as described in an 'untouched' monocyte isolation kit, containing CD3,7,19,45RA,56. CD123+ pDCs are relatively depleted by the inclusion of CD7 and CD45RA but CD1c+ mDCs remain.

C. Frequency of monocyte and DC subsets as in A) above compared with the frequency of each subset present in PBMCs analysed with a standard lineage cocktail of CD3,19,20,56. Mean of three experiments +/- SEM is shown. CD1c+ mDCs are not significantly depleted by the protocol (Wilcoxon Rank Sum test p = 1.00).

6.3.3. *TGF-β AND Bone Morphogenetic Protein 7*

Prompted by a previous observation that CD1c+ mDCs in tissues express a low level of langerin and that blood CD1c+ mDCs contain langerin mRNA (Bigley *et al.*, 2014), DC and monocyte fractions of human PBMC were cultured in conditions that induce langerin in progenitor cells, including TGF AND BMP7. This induced a rapid up-regulation of langerin and CD1a dual expression by CD1c+ mDCs, peaking within 3 days (Figure 6.3, Figure 6.4). Langerin$^+$ cells also appeared in CD14$^+$ and CD16$^+$ monocytes but the level of expression was lower and did not increase further by day 7.

From 10,000 sorted CD1c+ mDCs (counted by the sorter) 5,000-7,000 cells were recovered the next day and 2,000-3,000 at the end of the culture period (6,000-7,000 of monocytes at day 7) (Figure 6.5.A). There was no increase in cell numbers in any culture. Induction of a CD1a+ langerin^{high} population was restricted to CD1c+ DCs treated with TGFβ, BMP7 or both (Figure 6.5.B). No significant synergy was observed between TGFβ and BMP7. No langerin induction was seen in medium with serum alone, but serum-free medium with supplements also failed to induce any expression. Exhaustive testing of different serum free media was not conducted.
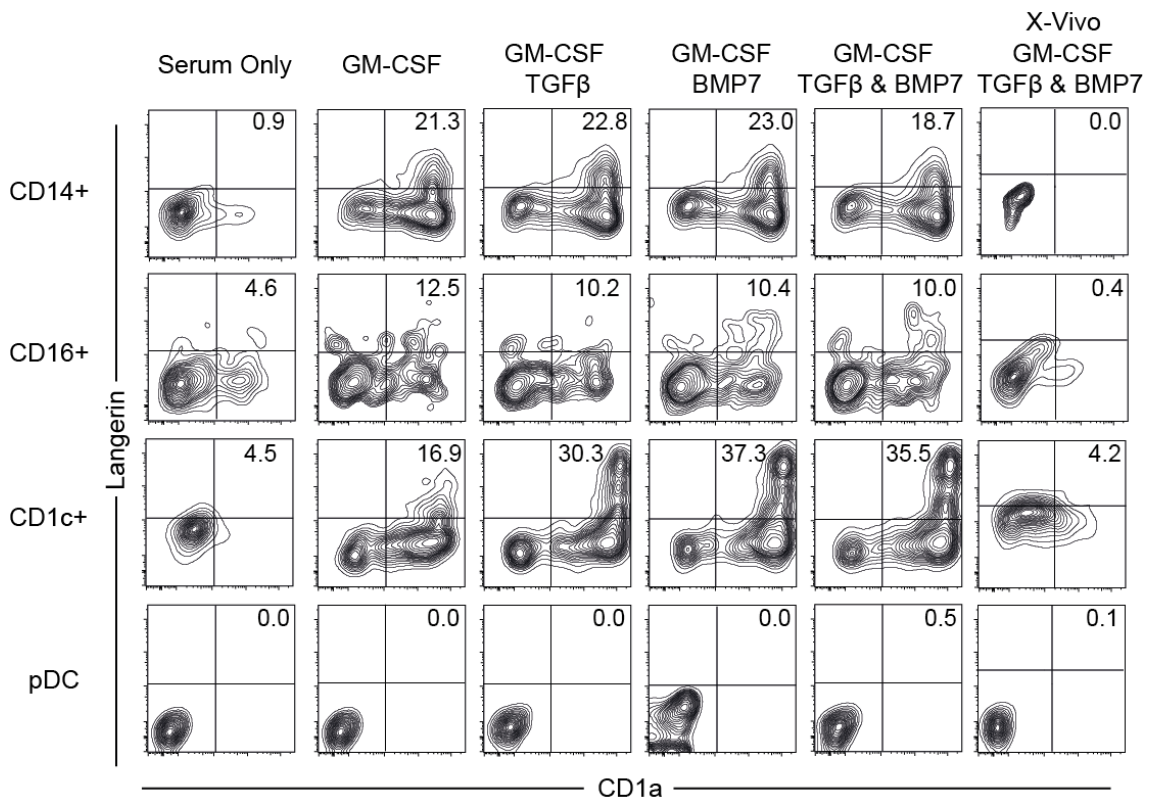
Figure 6.3. Raw Flow Data from 3 Day Cultures

Sorted cells cultured for 3 days in conditions as indicated showing expression of CD1a and extracellular langerin. The experiment was repeated five times except for the panels with X-Vivo which were repeated three times. Four subsets of cells were collected from one donor, different in each experiment.
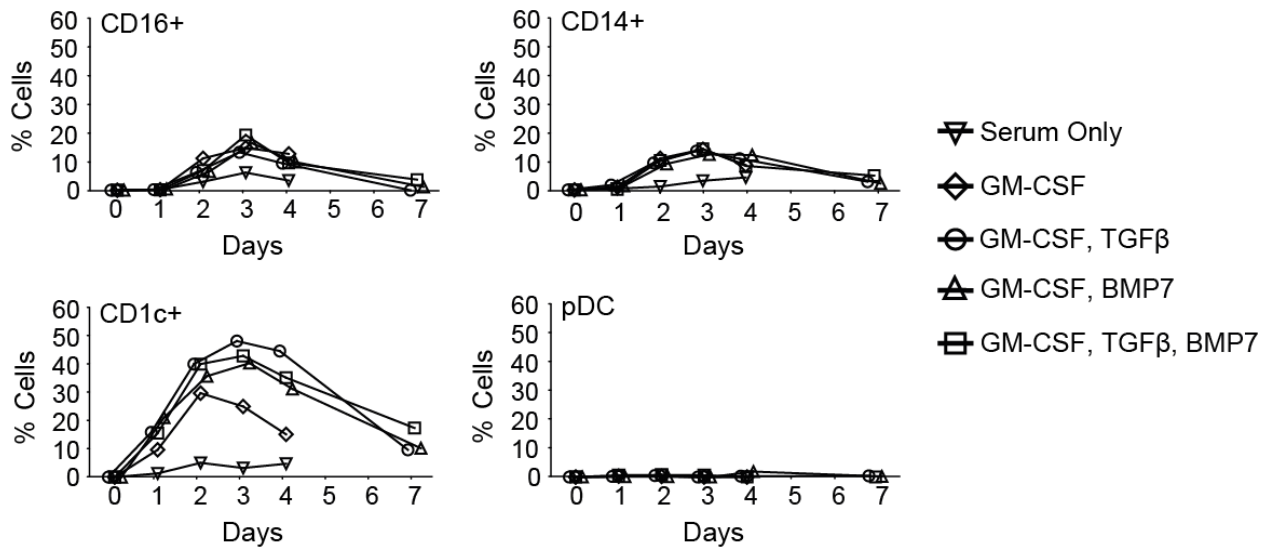
Figure 6.4. Expression of Langerin and CD1a by Monocytes and DCs

A. *Time course of expression of CD1a and langerin double-positive cells. Showing a rapid upregulation of langerin and CD1a in CD1c+ mDCs and CD14+ and CD16+ monocytes within 72h, peaking at 3 days and a gradual decline in the percentage of positive cells up to 7 days of culture. In CD1c+ mDC cultures with TGFβ or BMP7 a langerin high, EpCam+ fraction was observed, which was unseen in any of the other cell subsets. Mean +/- SEM of five experiments with different donors.*
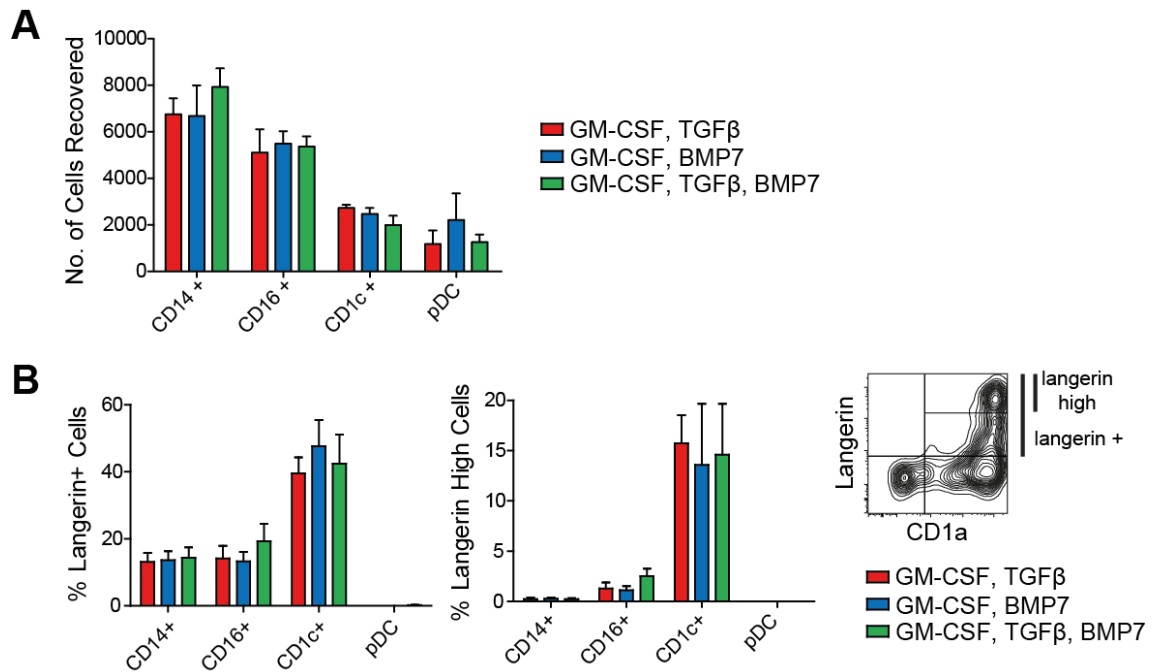
Figure 6.5. Day 7 Cell Recovery and Langerin/CD1a Dual Positivity

A. Recovery of viable cells under each condition at 7 days of culture, estimated by the total number of DAPI-negative cells recorded when the culture was analysed and run to dryness on the cytometer. 10,000 cells were added to each well as counted by the sorter but typically resulting in 6,000-8,000 viable cells at the start of the culture. There were no statistically significant differences between each condition for a given subset of cells.

B. Percentage of langerin+ cells derived from GM-CSF+TGFβ (red bars), GM-CSF+BMP7 (blue bars) and GM-CSF+TGFβ and BMP7 (green bars) after 3 days of culture (upper plot). Percentage of langerin^{high} cells derived from GM-CSF+TGFβ (red bars), GM-CSF+BMP7 (blue bars) and GM-CSF+TGFβ and BMP7 (green bars) after 3 days of culture (lower plot). Mean +/- SEM of five experiments with different donors. Gating of langerin+ and langerin^{high} cells is illustrated using CD1c+ mDCs incubated with GM-CSF and BMP7 as an example. There were no statistically significant differences between each condition for a given subset of cells. p = <0.01 compared with corresponding CD14+ monocyte culture. Differences in langerin induction between monocyte subsets and pDCs for a given culture condition, were not significant.

6.3.4. *May-Grünwald-Giemsa Stain Morphology*

As the general morphology of LCs is quite different to circulating monocytes and DCs, representative examples of each cultured cell type were cytospun and stained with May-Grünwald-Giemsa stain at days 0, 1, 3 and 7, to examine any morphological changes (Figure 6.6.A). Cytological development did not differ substantially between subsets of monocytes and CD1c+ mDCs, although CD1c+ mDCs developed a higher nucleus to cytoplasmic ratio and CD16+ monocytes remained smaller.

Lower resolution images of groups of cells on the cytospins were taken of 7 day cultures to represent an overview of cell morphology variation, and for comparison with freshly isolated Langerhans cells (Figure 6.6.B). CD1c+ mDCs were large after *in vitro* culture but developed pale pink perinuclear staining and vacuole, similar to primary LCs.
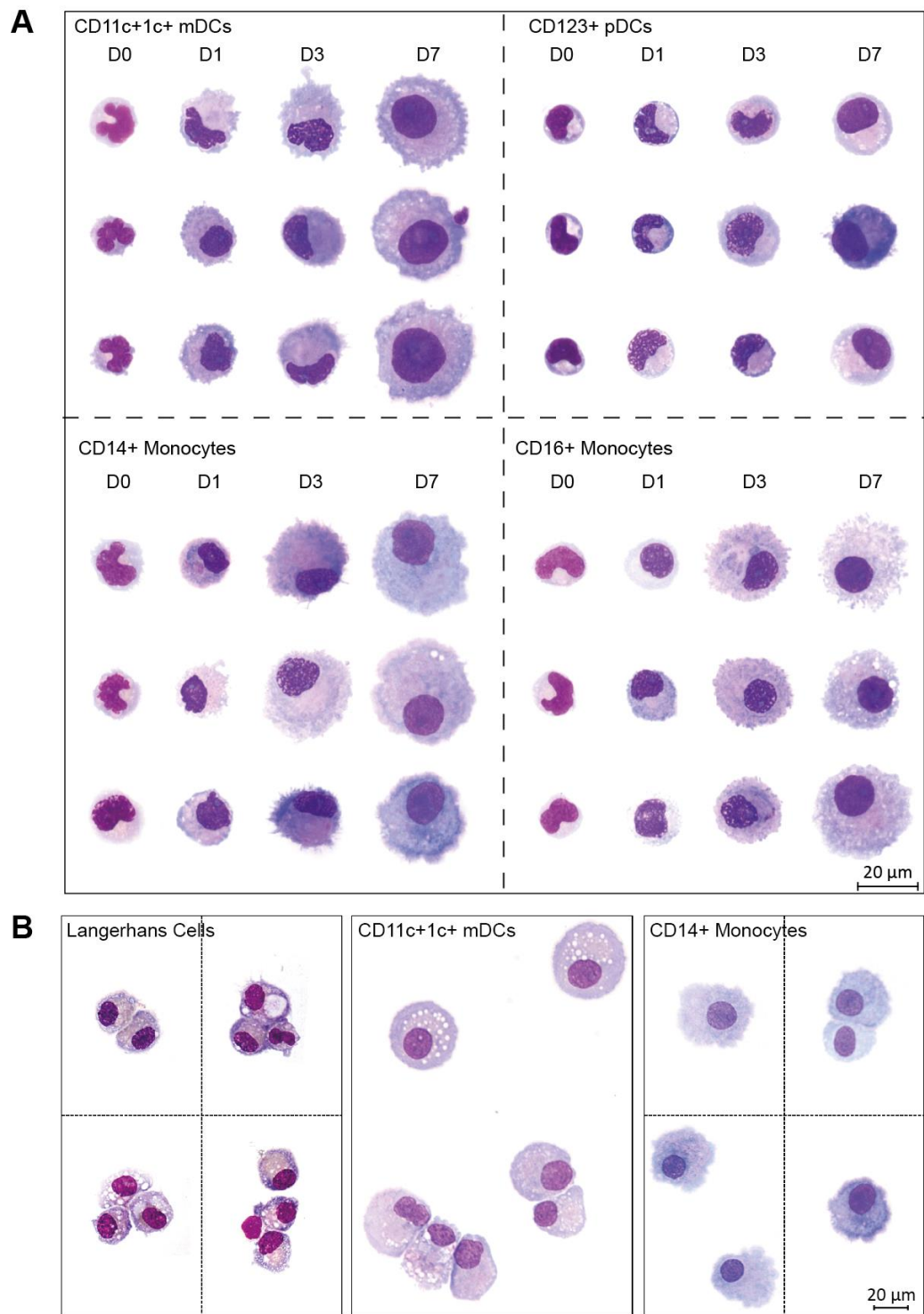
Figure 6.6. May-Grünwald-Giemsa Stain Morphology

A. Representative examples of Giemsa-stained cell fractions at intervals in cell culture (From GM-CSF + BMP7 culture).

B. Giemsa-stained fields of cells showing freshly isolated epidermal Langerhans cells, compared with 7 day cultures of CD1c+ mDCs and CD14+ monocytes in GM-CSF, TGFβ and BMP7. CD1c+ mDCs were large after in vitro culture but developed pale pink perinuclear staining and vacuoles, similar to primary LCs.

6.3.5. *Cell Surface Marker Characterisation*

In order to determine the presence or absence of LC-associated markers CD1a, Langerin, EpCAM and E-Cadherin on the cultured cells, flow cytometry was performed on days 0 and 7 of CD1c+ mDC cultures. Expression of HLA-DR and CD83 were also assessed to determine the levels of maturation in the cells.

This showed induction of EpCAM, E-Cadherin and low expression of CD11b, similar to primary LCs. Langerin+CD1c+ mDCs remained immature, with lower expression of HLA-DR and CD83 compared to freshly isolated LCs (Figure 6.7.A). The expression of EpCAM was highest on langerin^{high} cells derived from CD1c+ mDCs; in contrast, langerin+ cells derived from CD14+ monocytes had low EpCAM and higher CD11b expression (Figure 6.7.B). There was no expression of CD83 on the CD1c+ mDC cultures, showing that they are still less mature than the CD83+ LCs.
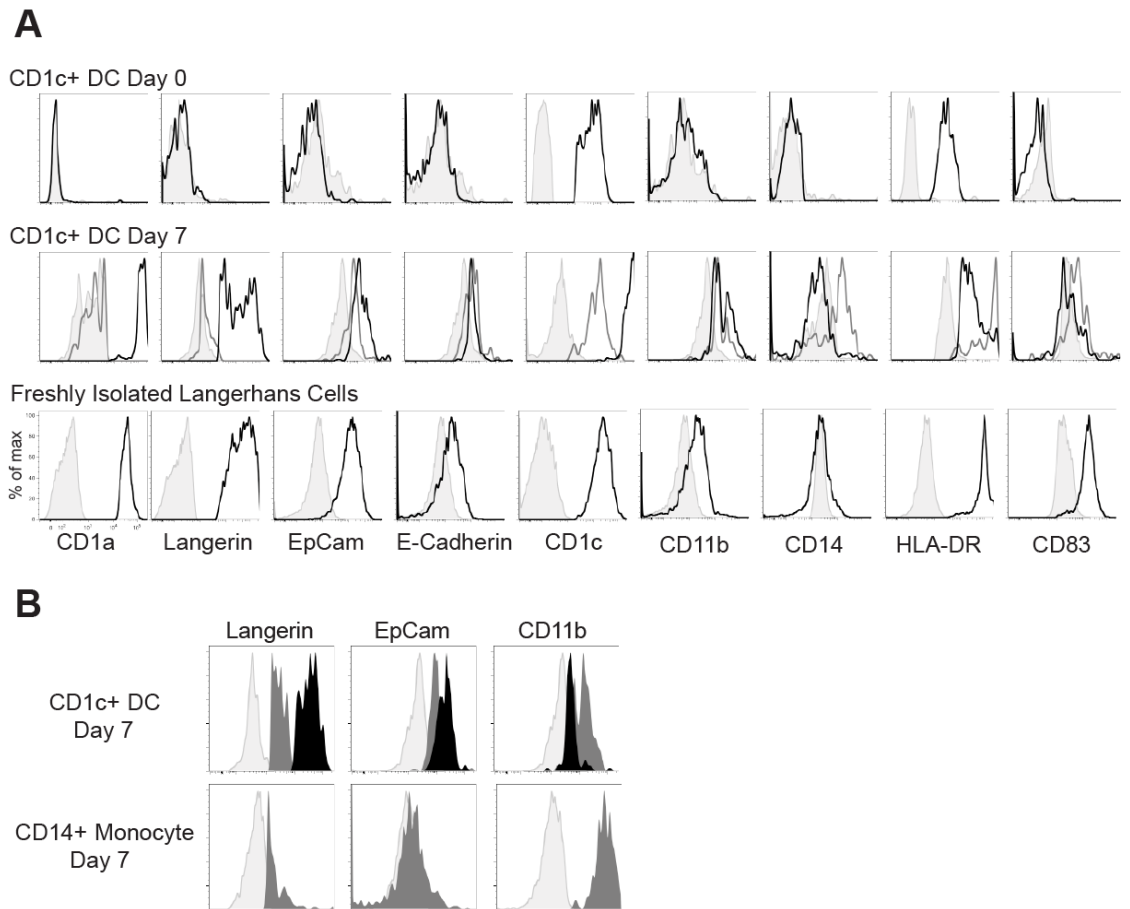
Figure 6.7. Cell Surface Marker Characterisation of LC-Like Cells

A. Cell surface marker characterisation of LC-like cells obtained after 7 days of culture. Shaded histograms: isotype controls: grey line CD1a and Langerin double negative cells; black line CD1a+ Langerin+ cells. Similar results were obtained with GM-CSF and TGFβ, BMP7 or both together in three different donors; one of three experiments performed with GM-CSF, TGFβ and BMP7 is shown.

B. Comparison of Langerin, EpCAM and CD11b expression in CD1c+ mDCs and CD14+ monocytes. Light grey: isotype; mid grey CD1a+ Langerin low cells; black: CD1a+ Langerin high cells. One of three experiments performed with GM-CSF, TGFβ and BMP7 is shown.

6.3.6. *Birbeck Granules*

LCs are most typically identified by their expression of Langerin and CD1a, but the most defining feature is the presence of Birbeck granules (BGs). Therefore, day 3 cultures of all cell subsets treated with GM-CSF and TGFβ/BMP7 were harvested for EM (all cells), in order to determine the presence or absence of BGs. No BGs were seen in ultrathin sections of any culture (>50 cells viewed). Day 7, 10 and 14 cultures of CD1c+ mDCs, CD14$^+$ monocytes and CD16+ monocytes treated with GM-CSF and BMP7 were harvested for EM (all cells). At day 7, BGs were observed in 12/20 ultrathin sections of CD1c+ mDCs with many sections containing more than 10 granules. At day 10, only 1/20 ultrathin sections of CD1c+ mDCs contained BGs (six BGs in total), and by day 14, no BGs were seen. In contrast, BGs were rarely found in cultures of CD14$^+$ monocytes (a single granule in 20 cell sections at day 7), and none were seen in cultures of CD16+ monocytes (Figure 6.8).
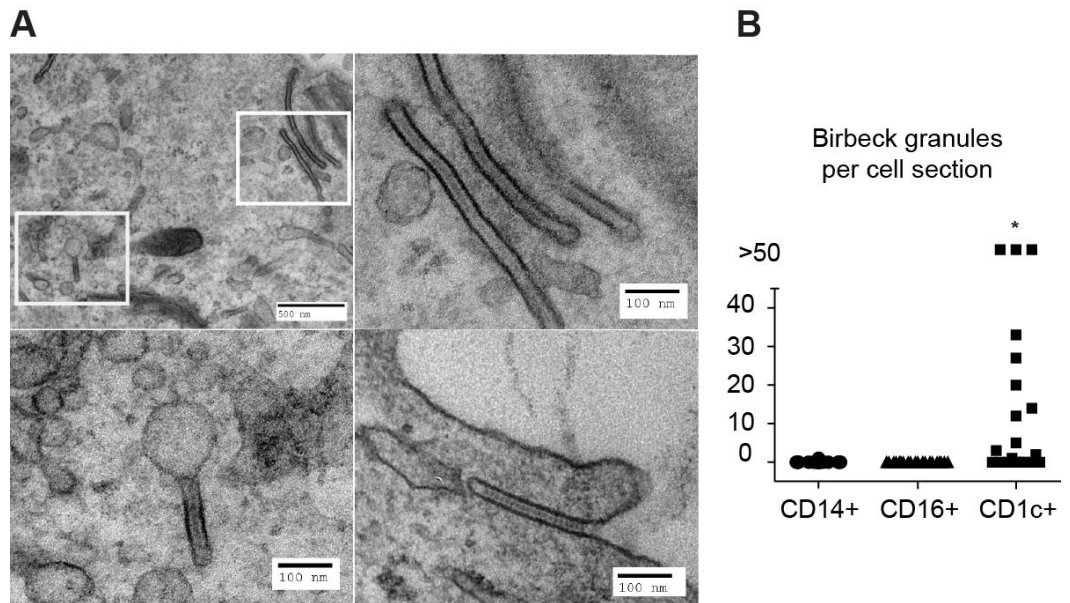
Figure 6.8. Formation of Birbeck granules in cultures of CD1c+ mDCs

A. EM images of Birbeck granules in CD1c+ mDCs cultured for 7 days showing classical tennis-racket morphology, pentalaminar structure and formation by endocytosis. Insets in the top left panel are displayed beneath and to the right. The experiment was performed twice with 2 different donors with GM-CSF and BMP7. The entire culture was processed for EM.

B. Comparison of the number of Birbeck granules per cell section between CD14+ monocytes and CD1c+ mDC cultures on day 7. * $p = <0.01$.

6.3.7. *Electron Microscopy – 'Thumbprint' Organelles*

During the EM evaluation of the presence of BGs, other atypical granules were observed in almost 100% of the CD1c+ mDCs at day 7, hereafter named 'thumbprint' organelles due to their characteristic thumbprint appearance on day 7. When re-viewing the other time points, it was seen that these atypical thumbprint organelles could be tracked in size throughout the days of culture (Figure 6.9). On day 2, the thumbprint organelles were roughly circular, approximately 100-200nm in diameter, with irregularly spaced circular internal striations/lamellar. Between days 4 and 10, the thumbprint organelles became more irregular in shape, but with a larger number of more regularly spaced internal striations/lamellar, sometimes with a central area of clear cytoplasm. On day 14, no thumbprint organelles were visible, though larger organelles were apparent which were split into long, coiled lines, which resembled BGs in that they had a central linear density, but were much longer than a characteristic BG. Cell numbers at day 14 were greatly reduced; however, the expanded thumbprint organelles were seen in all remaining cells.
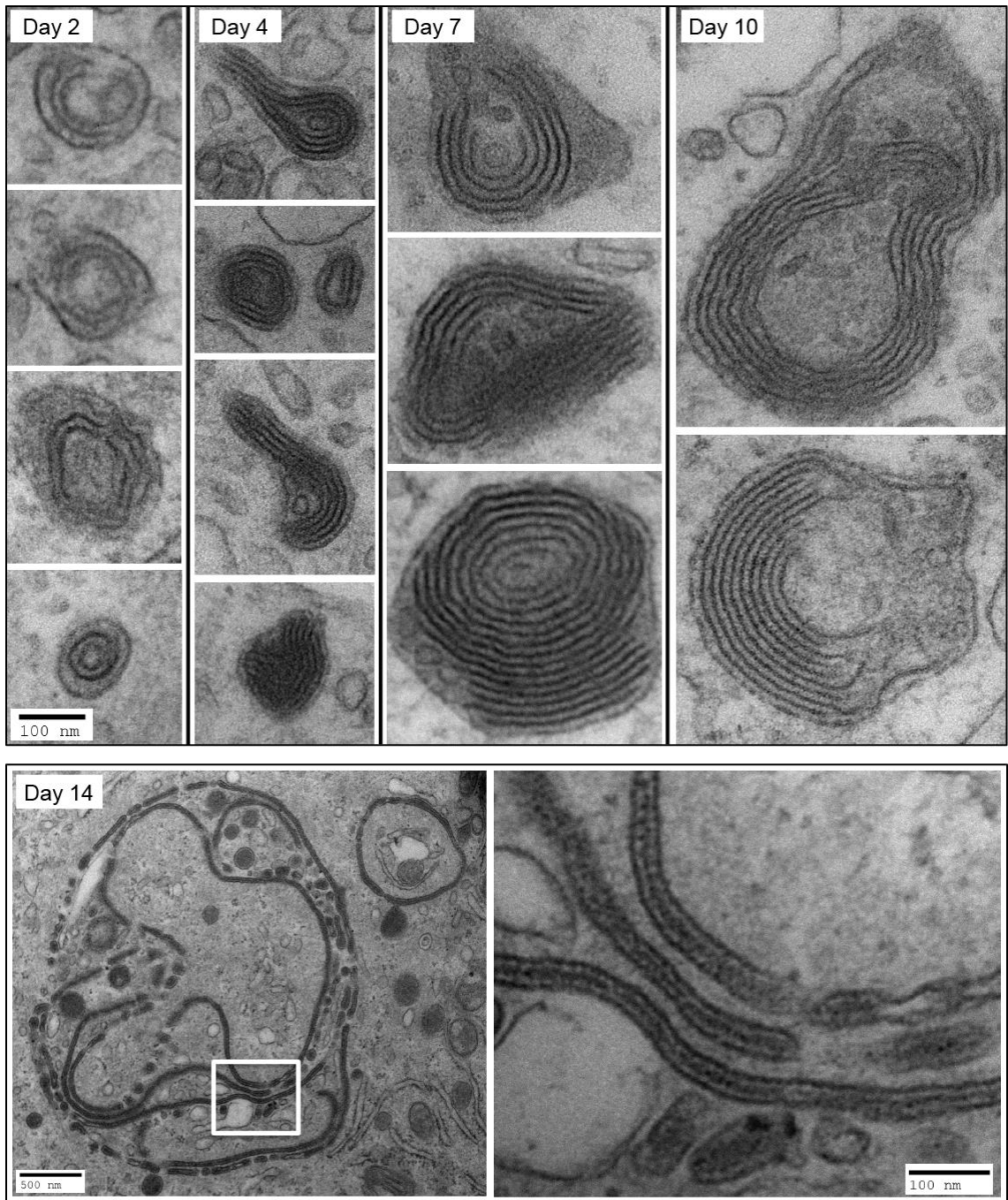
Figure 6.9. Unidentified 'Thumbprint' Organelles

EM images of unidentified 'thumbprint' organelles in CD1c+ mDCs cultured for 2-14 days in RF-10 with GM-CSF and BMP7. Organelles had a pentalaminar structure and appeared to grow in size between day 2 and day 14. The experiment was performed twice with 2 different donors with GM-CSF and BMP7. The entire culture was processed for EM.

6.4. Discussions for Chapter Six

The results in this chapter indicate that there are several possible pathways to the generation of langerin+ cells in humans and that CD1c+ mDCs but not CD14+ monocytes, generate a high yield of langerin[high] CD1a+ EpCAM+ cells with Birbeck granules under the conditions examined. Results also show that BMP7 can affect LC-like differentiation with equal efficiency to TGFβ.

GM-CSF, TGFβ and BMP7 are found in epithelial tissues *in vivo* where similar differentiation may occur (Greter *et al.*, 2012; Yasmin *et al.*, 2013a). Previous experiments on CD34+ progenitors have highlighted the difference between a TGFβ-dependent pathway of LC generation from CD1a+ intermediates versus the TGFβ-independent generation of monocyte-derived DCs that are unable to express langerin(Caux *et al.*, 1992; Strobl *et al.*, 1996; Ratzinger *et al.*, 2004; Klechevsky *et al.*, 2008; Yasmin *et al.*, 2013a). It is likely that this dichotomy anticipated the differential ability of CD1c+ mDCs and monocytes to generate LCs with high langerin, CD1a and EpCAM expression and Birbeck granules that I now observe.

Recent data in mice describing two waves of repopulation of the LC compartment show an early monocyte-like EpCAM- langerin[low] wave succeeded by a myeloid precursor of unknown origin(Nagao *et al.*, 2012; Seré *et al.*, 2012). In situ studies also show that LC repopulation after inflammation occurs with similar kinetics in humans(Kaplan *et al.*, 1987). CD1c+ mDCs have properties that make them candidates for the second-wave LC precursor (such as high numbers of epidermal sheet dendrites, high expression of langerin, EpCAM and CD1a, and low expression of CD11b). The nearest homologue to the CD1c+ blood DC in mice is the circulating pre-cDC (Naik *et al.*, 2006; Liu *et al.*, 2009). It will be interesting to determine whether this fraction contains the long term precursor of murine LCs.

These are *in vitro* experiments, using serum, as in recent publications describing DC differentiation (Balan *et al.*, 2014; Martinez-Cingolani *et al.*, 2014). There is a possibility that serum can be involved in the differentiation. For this reason, experiments were also performed without serum, using X-vivo

instead of RPMI with 10% bovine serum. After sorting, viability was approximately 50% lower in serum-free medium and I did not observe any Langerin induction and only low level CD1a expression.

These data help to explain two puzzling claims from other publications in this field. Firstly, that LCs with BGs can be produced from CD14+ monocytes (Geissmann *et al.*, 1998; Mohamadzadeh *et al.*, 2001; Guironnet *et al.*, 2002), may be contaminated with up to 3% CD1c+ mDCs when using the 'untouched monocyte' method of cell isolation. These contaminating cells may therefore contribute to the high expression of Langerin and Birbeck granules reported. Where monocytes have been isolated by positive selection using CD14 antibodies, there is lower langerin expression as described here (Hutter *et al.*, 2012).

Secondly, the finding that there are CD1a+ 'LC precursors' in human blood (Ito *et al.*, 1999). Blood DCs were reported to express CD1a due to incorrect assignment of clone BB5 as a CD1a specific antibody. It is now known that BB5 recognises CD1b/c and not CD1a (Mason. D *et al.*, 2000; MacDonald *et al.*, 2002). The non-monocyte cells in human blood that give rise to LCs in response to TGFβ and BMP7 *in vitro* are now clearly shown to be CD1c+ mDCs.

This is the first time that the thumbprint organelles have been followed during their development within cells, although it is important to note that the link between the organelles seen at each stage cannot be guaranteed. These organelles have in fact been seen previously in similar experiments, for example in a study by Grassi *et al.*, similar organelles were seen in 11-36% of monocyte-derived DCs when ficoll-derived, adherent monocytes were cultured in RF-10 with GM-CSF and IL-4 for 6-7 days, though no BGs were seen (Grassi *et al.*, 1998). Incidentally, these organelles have been noted as being pathognomonic for the congenital, self-healing form of LCH; Hashimoto-Pritzker disease (Hashimoto and Pritzker, 1973; Hicks, 2002; Hicks and Flaitz, 2005).

Later in a study by Guironnet *et al.*, cells were derived from PBMC with the 'untouched monocyte' isolation method, cultured in 'complete media',

supplemented with combinations of GM-CSF, IL-4, TGFβ. In cultures with GM-CSF and IL-4, ring shaped organelles similar to the expanded thumbprint organelles seen at day 14 of culture, were seen in ~15% of cells. In cultures with GM-CSF and TGFβ, BGs were seen in 15/101 cells, compared to 4/73 cells with the further addition of IL-4 (Guironnet *et al.*, 2002). In accordance with our data showing the lack of BG formation in CD14+ monocytes, and the presence of CD1c+ mDCs in the 'untouched monocyte' isolation method (see 6.3.1), it is likely that the 15% of the cells cultured with TGFβ which did display BGs in this paper, are actually CD1c+ mDCs not CD14+ monocytes.

More recently, in a study by Bonetti *et al*, CD133+ primitive myeloid progenitor cells were MACS separated from cord blood samples and cultured in RF-10 for 7 days with GM-CSF, TNFα, IL-4, TPO, FLT3L, SCF and TGFβ, and then until day 18 without TPO, FLT3L, SCF, but with increased TGFβ. At days 7 and at later time points, thumbprint organelles were seen (Bonetti *et al.*, 2011). No Birbeck granules were seen but a small proportion of the cells had dual expression of CD1a and langerin.

The data in this chapter show that, while monocytes are able to express langerin, when cultured with soluble ligands GM-CSF, TGFβ and BMP7, CD1c+ dendritic cells become much more LC-like with high Langerin, Birbeck granules, EpCAM and E-cadherin expression, under the same conditions. Previous observations have shown that blood CD1c+ mDCs contain langerin mRNA and that tissue CD1c+ mDCs express a low level of langerin (Bigley *et al.*, 2014). These data highlight the potential precursor function of CD1c+ mDCs and demonstrate an alternative pathway of Langerhans cell differentiation that may have relevance *in vivo*.

Chapter 7. General Discussions and Conclusions

This work explores the development of dendritic cells (DCs) in various haematological disorders including malignancies, immunodeficiencies and histiocytoses. During this thesis I have been able to relate haematopoietic disorders (AML, MDS and DCML deficiency) to the DC system, and DC disorders (histiocytosis) to the haematopoietic system.

7.1. Mononuclear Cell Development in Myeloid Malignancies

7.1.1. *Mononuclear Cell Profiles in Myeloid Malignancies*

Multiparameter flow cytometry panels were developed to allow in-depth analysis of the profiles of the mononuclear cell compartment of peripheral blood, bone marrow and other (disease) tissues. This analysis was used to uncover new information about the relationships between DC and monocyte development in pathological states.

Flow cytometry revealed several differences in mononuclear cell development between different types of bone marrow failure including DC, monocyte, B and NK lymphoid (DCML) deficiency, sporadic myelodysplastic syndrome (MDS) and acute myeloid leukaemia (AML). DCML deficiency has almost complete losses of specific cell subsets, MDS has a more generalised and less severe cellular deficiency, and AML has a remarkably preserved mononuclear differentiation, even at high levels of blasts.

7.1.2. *Comparative Phenotyping of AML Blast Cells*

One of the problems with trying to isolate distinct haematopoietic progenitor populations by cell markers is that in actuality, the expression of several markers on these fractions appear more as a continuous spectrum, rather than obviously positive and negative. This is possibly related to differentiation. This problem is exacerbated by the fact that these populations are most often comprised of very low cell numbers. A particular example is the differentiation of MEPs and CMPs. CMPs are

known to be able to differentiate into both GMPs and MEPs (Manz *et al.*, 2002). GMPs are easily distinguished by expression of CD45RA, though CMPs and MEPs, although showing different progenitor capacity (Akashi *et al.*, 2000; Manz *et al.*, 2002; Na Nakorn *et al.*, 2002; Goardon *et al.*, 2011), are difficult to isolate by flow cytometry markers. Several markers were assessed in this study for the ability to split CMP and MEP more effectively, though none were more reliable than CD123.

Also, the identification of blast cells and separation from normal mononuclear compartments can be difficult. Only ~45-68% of AML are CD34+ (Del Poeta *et al.*, 1994; Krasinskas *et al.*, 1998; Legrand *et al.*, 2000; Thalhammer-Scherrer *et al.*, 2002), and it is quite often the case that the blastic cells obscure a large portion of the flow plots due to sheer quantity, and are therefore difficult to isolate by any method. Isolation of the blastic cells could have been improved by using the markers CD33, which is positive in ~64-98% of AMLs (Del Poeta *et al.*, 1994; Khalidi *et al.*, 1998; Krasinskas *et al.*, 1998; Legrand *et al.*, 2000; Thalhammer-Scherrer *et al.*, 2002)and CD45, which is reported to be helpful in identifying blasts (Jennings and Foon, 1997; Panoskaltsis *et al.*, 2003). However, by adding these extra markers, other markers for the identification of mononuclear cell types would need to have been excluded, thus lowering the usefulness of the data.

Although further exploration of the entire haematopoietic system in BM is needed, work in this thesis has been effective at dissecting AML types by immunophenotypic analysis of progenitor cell markers, as seen in Figure 7.1, or by clustering analysis of DC-related markers.

Previous results reported by Goardon *et al*, 2011 are reproducible, showing that AML can be split into two major immunophenotypic groups (GMP/MLP type and CMP/MPP type, see Figure 7.1) based on progenitor cell markers. AML blasts can also be grouped by the DC-related immunophenotype of the blastic cells by bioinformatics. Five clusters are seen; clusters 1, 2 and 4 relate to the GMP/MLP type leukaemia and are the most heterogeneous. Cluster 5 specifically relates to blastic

plasmacytoid dendritic cell neoplasm (BPDCN), which also appears to be a GMP/MLP type leukaemia. Finally Cluster 3 relates to a leukaemia type with a 'null' phenotype for DC-related markers and correlates with the CMP/MPP type leukaemia (5/5 with available data).

Correlations were seen between the CMP/MPP; Cluster 3 type leukaemia in regards to cytogenetics; specifically 15:17 translocations and normal karyotype, although normal karyotype patients are found in both immunophenotypic types.
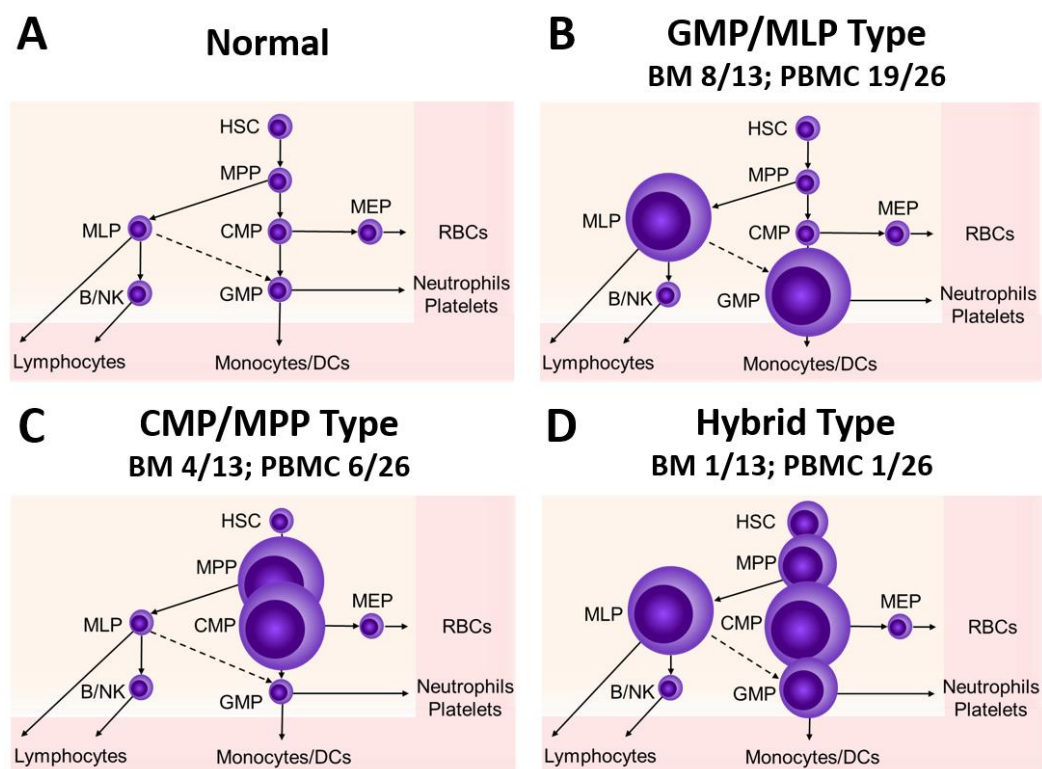
Figure 7.1. Models of Immunophenotypic AML Types

Diagrammatic representations of each immunophenotypic type of leukaemia identified in this study. Enlarged cells in the diagrams represent the expanded progenitor cell compartment. Numbers indicate the number of each type of AML patient identified by profiling in the BM and PBMC.

HSC = Haematopoietic Stem Cell; MPP = Multi-Potent Progenitor; MLP = Multi-Lymphoid Progenitor; B/NK = B/NK Cell Progenitor; CMP = Common Myeloid Progenitor; MEP = Megakaryocyte/Erythroid Progenitor; GMP = Granulocyte Macrophage Progenitor.

Even though AML patients could be grouped by the Goardon *et al* methods, or by clustering analysis, there was widespread heterogeneity in the phenotypes of the AML samples, particularly for Lineage MFIs (CD3,19,20,56) and FSC and SSC values. Also, heterogeneity was seen in the levels of the major and minor clones in the AML types. These differences may be due to specific molecular differences between the patients, or because of differences in the blast cell/normal progenitor cell composition of the expanded types. The differences seen in the lineage MFIs are interesting as these are lymphoid markers, which could imply differences in lymphoid cell potential in the blastic cells, or possibly that some of the AML patients have bi-phenotypic features of both AML and ALL. Detailed analysis of lymphoid markers may be able to further subtype the blast clusters.

Overall, it is seen that the AML immunophenotype are essentially divided by CD45RA positivity (GMP/MLP) or negativity (CMP/MPP). It is therefore clear that the addition of CD45RA into the antigen presenting cell flow cytometric panel may be very useful in the analysis of AML. CD45RA is positive on naïve and effector T cells (Sallusto *et al.*, 2004), though the role of CD45RA in myeloid cells is unclear.

I originally hypothesised that AML patients would have severe cytopenias at diagnosis due to disruption of normal haematopoiesis by the blastic cells, however the analysis in this thesis has shown that haematopoiesis in AML is surprisingly conserved. Nonetheless, data in this thesis suggests that different types of AML may have differences in the functional levels of immune cells, or specific immunodeficiencies, which may increase the risk of severe sepsis or fungal infection, responsible for the majority of AML induction therapy related deaths (Lech-Maranda *et al.*, 2010). It is therefore possible that the progenitor cell immunophenotype of leukaemia may correlate with infection and outcome. This is an important issue as induction death is now an equivalent risk to relapse death in the best risk patient groups (Lech-Maranda *et al.*, 2010).

Infection is the primary cause of death in low-risk MDS patients (30%), followed by transformation into AML (15%) and haemorrhage (13%) (Dayyani *et al.*, 2010), while high-risk MDS patients are more prone to infection (Toma *et al.*, 2012). AML patients have a lower rate of infection-related death at around 14.5% of patients (Lech-Maranda *et al.*, 2010). As seen in this thesis, although not statistically significant, there were trends for patients with MDS to have reduced immune cell numbers compared to normal controls, and to AML patients, where haematopoiesis is relatively conserved. These immune cell reductions could indicate why a higher percentage of MDS patients have an infection-related mortality than AML patients. Standard primary treatments for AML involve myeloreductive (rather than myeloablative) therapy, as such it is likely that some normal myeloid cells are present after treatment. Therefore it could be clinically significant to ascertain the extended phenotype of AML patients post-chemotherapy. Patients whose haematopoiesis remains mostly intact even after chemotherapy may have a much lower risk of infection than those who have an abolished haematopoiesis at diagnosis or post-chemotherapy. Patients who progress from MDS to AML are thought to do so due to the development of multiple secondary genetic mutations in the malignant clone (Walter *et al.*, 2012). One interesting angle for future studies would be to see if the extended phenotype and specific cytopenias in MDS patients correlate with which patients progress from MDS to AML.

7.1.3. *Minimal Residual Disease Testing*

Survival rates in acute leukaemias are improving all the time, due to new and more effective methods of diagnosis and treatment. The median survival of adult patients in 1966 was just 40 days(Medical Research Council, 1966), whereas now 50% of patients achieve a five-year survival (CancerResearchUK, 2014b). Although rates of complete remission (CR; ≤ 5% leukaemic blasts in the BM) are high (50-80%, dependant on age and gender), relapse occurs in the majority of patients within 5 years after treatment (Paietta, 2012). These relapses are likely due to the presence of minimal residual disease (MRD), the low numbers of leukaemic cells which

remain in the patient during or after therapy which are undetectable by current methods.

The most commonly used MRD testing methods include DNA and RNA based tests for cytogenetic abnormalities and multiparameter flow cytometry for abnormal cellular markers, otherwise known as Leukaemia-associated immunophenotypes (LAPs or LAIPs). DNA and RNA methods are less useful in AML than other leukaemia types, as ~40% of AML patients have normal cytogenetics (Grimwade *et al.*, 2010), though direct comparisons have shown that these methods have equivalent effectiveness (Perea *et al.*, 2006; Rossi *et al.*, 2012) and the general sensitivity level of multiparameter flow cytometry MRD is thought to be able to reach 0.01%, which is equivalent to RT-PCR (Paietta, 2012).

Evidence that multiparameter flow cytometry LAP MRD testing determined therapy leads to an improvement in AML prognosis is limited, though recent assessments have shown that multiparameter flow cytometry MRD testing is useful for overall outcome predictions and effects of maintenance strategies in patients with AML (Kern *et al.*, 2008; Buccisano *et al.*, 2012) and other leukocyte cancers such as multiple myeloma (Rawstron *et al.*, 2013). In order to be optimal for clinical use, methods of MRD testing must have the ability to be consistently and objectively applied, and to be extremely sensitive. A survey of the multiparameter flow cytometry MRD testing used for multiple myeloma revealed major heterogeneity in analysis criteria and sensitivity between centres (Flanders *et al.*, 2013). It is therefore crucial that criteria is normalised between centres with highly accurate and sensitive methods.

The reasoning behind the markers used in most LAP studies is that they are absent or found very infrequently in normal blood or BM (Feller *et al.*, 2013). These markers have been chosen by empirical methods of trial and error, whereas the methods of immunophenotyping performed in this thesis have been developed using a more rational approach, using a limited range of antigens and a classifying system based on the mature phenotypes seen in normal mononuclear cells.

The methodology described in this thesis could potentially be used for MRD testing in a complementary combination with cytogenetic analysis. The multiparameter flow cytometry panels can be used to give a high sensitivity of quantifying leukaemic cells with an abnormal phenotype, which can then be FACs sorted and tested for the particular cytogenetics seen in the patients. This would greatly increase the sensitivity of the cytogenetic analysis by specifically testing possibly leukaemic cells rather than the entire leukocyte fraction. With the development of tools to visualise high-dimensional flow cytometry onto two-dimensional plots, such as viSNE (Van der Maaten, 2008; Amir *et al.*, 2013), it may be possible to use the immunophenotyping methods developed in this thesis as a universal method of MRD testing in AML patients, similar to that which has recently been described in B-ALL (DiGiuseppe *et al.*, 2015).

7.1.4. *The Unclassified CD123^{high} Fraction of CD34+ Bone Marrow*

An interesting observation in the BM of patients relates to the CD123^{high}fraction of CD34+ cells. To date these cells have not been classified for their contribution to the haematopoietic system, although their expression of CD123 would imply that they are involved in pDC differentiation. However, in a limited number of patient samples with Blastic Plasmacytoid Dendritic Cell Neoplasm, I have seen varied expansions of the GMP, CMP and MLP fractions rather than the CD123^{high}fraction (data not shown). In this thesis, a relative expansion of the CD123^{high} and GMP fractions were seen in the LCH patients, a normal level of CD123^{high} but expansion of GMP in an ECD patient and a complete absence in an HCL patient. This could imply a role in myeloid haematopoiesis from this fraction, as it correlates with the myeloid compartment expansion in LCH, varied but averaging as normal levels of myeloid cells in ECD and an almost complete lack of myeloid cells in HCL patients. In contrast, no AML patients were seen with expansions of the CD123^{high} fraction, suggesting that CD123^{high} cells may be a later maturation stage of GMPs which AML cells are unable to differentiate into.

7.1.5. *FLT3-L as a Biomarker for Haematological Disorders*

In this thesis, it was seen that serum levels of Fms-related tyrosine kinase 3 ligand (FLT3L), were significantly increased in DCML deficiency patients, while significantly decreased in AML patients. Therefore FLT3L monitoring may be useful as a diagnostic test in this setting; excessive levels of FLT3L would identify DCML deficiency patients and FLT3L below the normal range would identify AML patients to a high sensitivity and specificity. There is a possibility that FLT3L may be used for a biomarker for DCML deficiency and AML, by means of such clinical interventions as simple as a finger prick test. This could be a useful, minimally invasive, first assessment in AML, which is predominantly a disease of the elderly, where assessment based on bone marrow examination is not always available. Additionally to a diagnostic test, it may be possible to assess levels of FLT3L over time, to monitor response to treatment.

7.2. Dendritic Cell Development in Histiocytosis

7.2.1. *Mononuclear Cell Profiles in Histiocytosis*

Flow cytometry revealed some differences in mononuclear cell development between different types of histiocytic disorder. Interesting findings in the mononuclear cell profiles of the histiocytic disorders include an apparent expansion of CD14+ monocytes in LCH and loss of lymphocytes and CD141+ mDCs in ECD patients. CD11c+1c+ mDCs were increased, as has previously been described (Rolland *et al.*, 2005), most particularly in the multi-system LCH (msLCH) patients.

7.2.2. *Tracking BRAF^{V600E} in Histiocytic Disorders*

BRAF^{V600E} mutation was detected in the myeloid lineages of the bone marrow and peripheral blood of msLCH patients using a modified mutation detection PCR assay. *BRAF^{V600E}* or *NRAS^{Q61R}* mutations detected in the myeloid lineages of the bone marrow and early myeloid precursors and innate lymphoid cells of peripheral blood in some ECD patients. As far as I am aware, this is the first time that CD1a+Langerin+

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LCH cells have been specifically sorted from LCH lesions to confirm levels of *BRAF^{V600E}* positivity and to assess morphology.

7.2.3. *Peripheral Blood DC Langerhans Cell Potential*

CD1c+ mDCs can be induced to bear a Langerhans cell (LC) like phenotype in response to GM-CSF with TGFβ and/or BMP7, suggesting a second pathway of LC differentiation through a monocyte-independent route.CD14^{+} monocytes express low langerin and do not make Birbeck granules (BGs) under the same conditions. This is contradictory to previous results where CD14+ monocytes were thought to induce langerin highly (Geissmann *et al.*, 1998; Mohamadzadeh *et al.*, 2001; Guironnet *et al.*, 2002), however these studies employed negative isolation techniques, which I have shown to likely be contaminated with CD1c+ mDCs. More recent studies where CD14+ monocytes have been positively isolated, have reported expression of Langerin and CD1a and Birbeck granules (Hoshino *et al.*, 2005; Shibasaki *et al.*, 2007; Musso *et al.*, 2008). These studies used Notch Ligand Delta-1 (with TGFβ) or Activin A (a TGFβ family member as inducers. Interestingly Notch has recently been reported to be active in LCH (Hutter *et al.*, 2012). These results indicate that CD1c+ mDCs and monocytes may both be able to become LCH like under different conditions.

7.2.4. *Possible Origins of Histiocytic Cells*

Although this study goes some way to identifying the potential precursors of LCH, ECD and HCL, there is still a lot of work to do to for complete certainty. A diagrammatic representation of the potential mutational pathways seen in the thesis for LCH, ECD and HCL is given in Figure 7.2. A caveat of this model is that even though the mutation has been seen in a number of different cell types represented, this does not necessarily prove a lineage link between the circulating cells and lesional LCH cells.

I have seen in this study what at first appear to be contradictory findings about the potential origins of LCH cells. The study by Berres *et al*, reported that CD11c+ and CD14+ circulating cells of LCH patients expressed *BRAF*^{V600E}. In this thesis I saw that although CD1c+ mDCs and CD14+ monocytes carried the *BRAF*^{V600E} mutation in LCH patients, it was the non-classical CD16+ monocytes which carried the highest level of mutation at up to 45%. Given the heterozygous nature of the mutation, this would imply that ~90% of CD16+ monocytes in that particular patient carried the mutation and therefore this cell type would be a key contender as the cell of origin in LCH.

It is tempting to speculate that the expansion of and enrichment of *BRAF*^{V600E} in monocytes in LCH patients indicates that they are the precursors of LCH cells in multisystem disease, as has been previously suggested by several authors (Geissmann *et al*., 1998; Rolland *et al*., 2005). However, there is unlikely to be a simple correlation between the frequency of *BRAF*^{V600E} and the precursor potential of a given population. Rare cells such as CD1c+ mDCs, which were seen to be increased in this and other studies (Rolland *et al*., 2005) might accumulate through strong selective mechanisms operating in the periphery while other more abundant monocyte fractions such as the non-classical monocyte might accumulate a high level of *BRAF*^{V600E} even if they are devoid of precursor potential. However I found that CD16+ monocytes were decreased in LCH patients, possibly due to migration into LCH lesions.

It was seen in chapter six, that although CD14+ and CD16+ monocytes are able to induce langerin expression with stimulation from BMP7, it is only the CD1c+ mDCs which can be induced to express langerin and CD1a to the high levels seen in LCH, at least under the conditions tested. The *in vivo* experimentation for langerin induction was performed on healthy control samples, so this does not rule out the possibility that the monocytes of LCH patients may be able to aberrantly express high levels of langerin and CD1a in the disease state. The low levels of *BRAF*^{V600E} seen in the circulating CD1c+ could indicate that the CD1c+ cells bearing the *BRAF*^{V600E} mutation have already migrated from

the blood to the lesions, whereas the monocytes bearing the mutation may be bystanders which remain in the blood. This is clearly an area which could be further explored in the future as samples become available.

In this study on adult patients, $BRAF^{V600E}$ was only seen in the circulating blood of multi-system, high-risk disease patients. This is in keeping with a recent report in children, where $BRAF^{V600E}$ was only detected in the peripheral blood of patients with active MS or 'risk disease' involving liver, spleen or bone marrow (Berres *et al.*, 2014). The expression of $BRAF^{V600E}$ in mouse models results in the formation of LCH-like lesions and LCH cells with characteristics which may be dependent on the differentiation state of the cells in which the mutation is initiated; expression in immature bone marrow progenitor cells ($BRAF^{V600E-CD11c}$ mice) leads to features of high-risk LCH, whereas expression in more mature myeloid cells ($BRAF^{V600E-Langerin}$ mice) leads to a low-risk LCH phenotype (Berres *et al.*, 2014). These findings could also give some indication as to why one mutation can lead to multiple different disease presentations such as LCH, ECD and HCL. The presenting features of the disease may be dependent on the stage of haematopoietic development at which the originating cell was at when it developed the $BRAF^{V600E}$ (or alternate MAPK pathway) mutation.

Recent work in mice investigating LC reconstitution after conditional ablation, has demonstrated that in inflammation, LCs may be reconstituted in two distinct waves; a primary short-term monocyte derived langerin-low LC, and a secondary long-term bone marrow derived langerin-high LC (Nagao *et al.*, 2012; Seré *et al.*, 2012). These data, together with the vast heterogeneity seen in LCH patients, could indicate that there may be multiple different precursor populations from which LCH cells may arise under different conditions, such as the differentiation levels of the initial cells carrying the mutation, and whether the disease is high/low risk or multi/single-system.
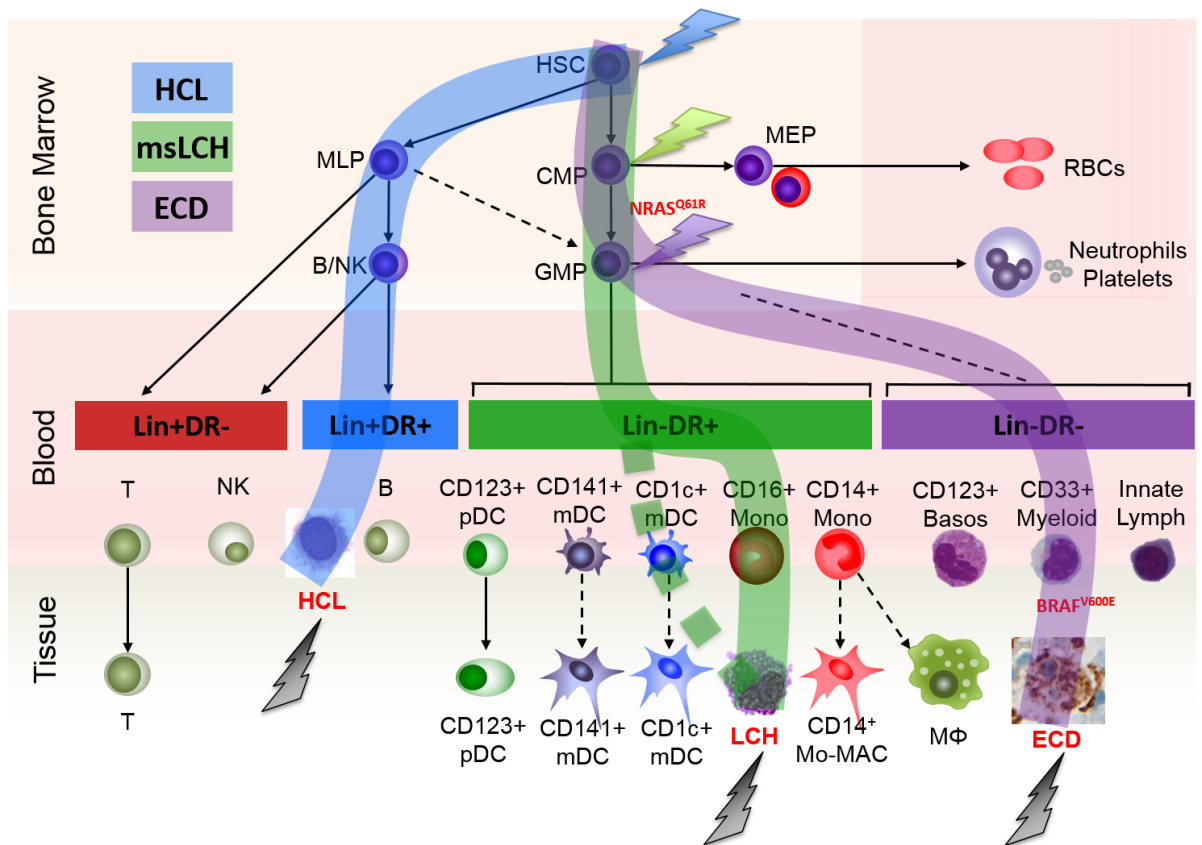
Figure 7.2. Model of Mutational Pathways in LCH, ECD and HCL

HCL = Hairy Cell Leukaemia; msLCH = multi-system Langerhans Cell Histiocytosis; ECD = Erdheim Chester Disease

Coloured Lines = Potential pathways of disease haematopoiesis; full lines are based on disease associated mutation detection (see chapter 5); dashed lines are based on in vitro differentiation studies (see chapter 6).

Coloured Lightning Symbols = Theoretical sites of disease-specific second hits.

Grey Lightning Symbols = Theoretical sites of tissue-specific second hits.

HSC = Haematopoietic Stem Cell; MLP = Multi-Lymphoid Progenitor; B/NK = B/NK Cell Progenitor; CMP = Common Myeloid Progenitor; MEP = Megakaryocyte/Erythroid Progenitor; GMP = Granulocyte Macrophage Progenitor; RBC = Red Blood Cell; T = T Cell; B = B Cell; NK = Natural Killer Cell; mDC = Myeloid Dendritic Cell; pDC = Plasmacytoid Dendritic Cell; Mono = Monocyte; Baso = Basophil; LC = Langerhans Cell; Mo-MAC = Monocyte/Macrophage; MΦ = Macrophage.

7.2.5. *Bone Marrow Origins of Histiocytosis*

Previously, $BRAF^{V600E}$ has been seen in CD34+ cells of the bone marrow of LCH patients(Berres *et al.*, 2014), and the HCSs of HCL patients (Chung *et al.*, 2014). One of the most interesting aspects of this thesis is that the disease associated mutations ($BRAF^{V600E}/NRAS^{Q61R}$) have been seen in the HSCs of selected patients of LCH, ECD and HCL. This indicates that at least some subtypes of the diseases are likely to be leukaemic disorders of early haematopoiesis.

These observations could explain some of the discrepancies in potential originating cells. A recent review speculated on CMPs as a common cellular origin in LCH and ECD (Badalian-Very, 2014), however in this thesis I have seen that the $BRAF^{V600E}$ mutation can be found as far back in haematopoiesis as the HSCs. This early stage would allow the mutation to be perpetuated into different mononuclear subset progeny which may or may not play active roles in the diseases. Recent studies have reported the possibility of more than one wave of LC recruitment (Seré *et al.*, 2012), therefore It could be possible that both CD16+ monocytes and CD1c+ mDCs play pathogenic roles in LCH.

7.2.6. *$BRAF^{V600E}$ Monitoring in Histiocytic Disorders*

It was seen in this thesis that the use of allele specific PCR is very useful in the detection of low-level $BRAF^{V600E}$ in patient samples of PBMC, BM, or tissues. This method could also be used for sequential monitoring over time, and therefore may be a useful method for minimal-residual disease testing. Recently new methods of detection of $BRAF^{V600E}$ and other related mutations have been developed for the analysis of cell free DNA in plasma and urine (Janku *et al.*, 2013; Hyman *et al.*, 2014). These methods would obviously be less intrusive to patients, and very useful for disease monitoring, but are unlikely to be useful for further elucidation of the cells of origin in these diseases.

7.2.7. *The LCH-ECD-HCL Paradox*

The presence of *BRAF*[V600E] in LCH, ECD and HCL, brings the question of how a single mutation can cause such different diseases. As seen in this thesis, the levels of mutation can be very low in the BM, but very high in lesions. This could suggest that the BRAF mutation itself is not enough to cause a high proliferation rate, which is only achieved once the cells are located in the lesions. This along with the noticeable observation that the three diseases have vastly different presentations, likely means that there must be some other pathological event that takes place to drive the specific disease phenotype which occurs.

There are variations in the median age of onset of patients; LCH is generally a childhood disease while ECD and HCL have late adult average ages of onset (with the caveat that adult LCH is thought to be underdiagnosed). This could suggest that the time of mutation acquisition is indicative of the disease type.

Few studies have been performed to examine whether additional somatic mutations are involved in pathogenesis; MAP2K1 and ARAF mutations have been implicated in whole exome sequencing studies in LCH, but they are independent of *BRAF*[V600E] (Chakraborty *et al.*, 2014; Nelson *et al.*, 2014). Another study found no somatic mutations in one ECD patient and extremely low rates of somatic mutations (median 1 mutation per sample) in all histiocytic lesions (Nelson *et al.*, 2014), which contrasts with high amounts of somatic mutations seen in most leukaemias including HCL (Tiacci *et al.*, 2011). The propensity for single somatic mutations of the MAPK pathway, with low global frequency of other somatic mutations, indicates that mutations in this pathway are crucial for the initiation of LCH and ECD. The MAPK pathway is clearly also important in HCL patients where 100% of patients carry the *BRAF*[V600E] mutation (Tiacci *et al.*, 2011), and in HCL variant patients where BRAF is wild-type but up to 50% of patients have a mutation in another member of the MAPK pathway, MAP2K1 (Waterfall *et al.*, 2014). The presentation seen in HCL may be different to that seen in LCH due to

somatic mutations appearing alongside $BRAF^{V600E}$ in the HCL patients. These mutations may potentially skew the maturation of $BRAF^{V600E}$+ cells into the more lymphoid-like HCL cells rather than the monocytes and DCs which I have seen to carry the $BRAF^{V600E}$ mutation in LCH patients in this thesis.

No recurrent cytogenetic or epigenetic abnormalities have been observed in LCH or ECD, whereas HCL patients have been seen to have recurrent cytogenetic abnormalities, particularly in chromosome 5 (Haglund *et al.*, 1994), aberrant but uniform gene expression profiles (Vanhentenrijk *et al.*, 2004) and differences in methylation status of -C-phosphate-G- (CpG) sites of DNA (Rinaldi *et al.*, 2013).

These deregulated features of HCL patients could indicate that HCL is caused by a primary somatic $BRAF^{V600E}$ mutation, with secondary cytogenetic or epigenetic changes, possibly brought on by age, whereas LCH and ECD might be caused by a primary somatic mutation related to the MEK/ERK pathway, with disease promoting events of a non-genetic cause.

One possible disease-promoting pathological event would be a role of an infectious agent, similar to the roles of HHV and EBV which have been implicated in the histiocytic disorders RDD and HLH (Luppi *et al.*, 1998; Mehraein *et al.*, 2006; Bohne *et al.*, 2013). An interaction of an infectious agent with cells carrying MEK/ERK pathway associated mutations, such as $BRAF^{V600E}/NRAS^{Q61R}$, may promote the hyper-activation of the MEK/ERK pathway in a tissue-specific manner. This could suggest why the same mutation could lead to lesions in widely dispersed and varied organs in different patients, dependant on where interaction with an infectious agent has occurred. Recent studies have potentially identified Merkel cell polyomavirus as a contributing factor in high-risk LCH pathogenesis (Murakami *et al.*, 2014; Murakami *et al.*, 2015b), while no pathogens have been associated with low-risk LCH.

It is a very likely possibility that HSCs are a heterogeneous population, thus a second possibility is that the different disease types are caused by mutations which occur at different maturation stages of the HSCs, with slightly different lineage bias. Although this study had limited patient BM samples, it was interesting to see some slight differences in the patient types. HCL patient A7264 had a large increase in HSCs, while all other BM progenitor types were reduced. Both LCH and ECD had mutations in the HSCs, CMPs and GMPs, though relative abundance differed; $BRAF^{V600E}$ was seen to be highest in the CMPs of both LCH patients, and $NRAF^{Q61R}$ was seen most highly in the GMPs of the ECD patient. Confusingly, the expansion of BM progenitor cells in LCH and ECD gave a conflicting picture, with highest expansions of GMPs in LCH patients and CMPs in the ECD patient. This could suggest that the negative effects of the mutations could become apparent at different states of maturation of progenitor cells, leading to separate disease entities. This theory has been incompletely tested at present; more BM from each patient type would have to be examined to see the relative abundance of mutation in the progenitor types.

LCH was first described from the union of three diseases; eosinophilic granuloma, Hand–Schüller–Christian disease and Letterer–Siwe disease due to related cellular morphology. The research in this thesis and from recent publications would again point to divisions in the pathology of different subtypes of LCH and also ECD. From this thesis it would seem that LCH and ECD may have a haematopoietic stem cell origin in multi-system disease, or a tissue-specific origin in single-system disease. Overall, I have seen that LCH and ECD are highly heterogeneous diseases, and that it is likely that each will be parsed back into subtypes in future studies.

7.3. Future Work

7.3.1. *Haematological Malignancies*

An interesting future goal would be to test whether the progenitor phenotype subset associates with prognosis. Currently there is a need to refine the 'intermediate' risk group of AML. In this thesis I have seen that CMP/MPP leukaemia relates to 15;17 translocations specifically, and accounts for a proportion of patients with 'normal' cytogenetics. It may be possible to split patients with normal cytogenetics by the immunophenotypic type of their blasts, and correlate this to outcome.

To strengthen this analysis, more patient samples could be collected, but also, it will be possible to combine mean fluorescence intensities (MFIs) of the markers in the mature and progenitor cell flow cytometry panels in order to strengthen the clustering of AML samples.

Another question to be answered is whether the mononuclear cells seen in the AML patients are preserved elements of normal haematopoiesis or are part of the AML clone. It may be possible to exploit the cytogenetic mutations seen within the AML cells to answer this question, by seeing if these mutations are within the differentiated mononuclear fractions. This could be achieved through the use of similar technology as discussed in this thesis for histiocytic disorders.

7.3.2. *Histiocytic Disorders*

This study has gone a long way to observing the potential cells of origin in multi-system LCH, although the origins of ECD are less clear. Further studies with more explicit analysis of the subsets in the HLA-DR-Lineage- fraction in ECD patients are needed, as well as confirmatory studies to show that the cells seen to carry the mutation can indeed bear the phenotypes ECD.

Although I have seen that CD1c+ mDCs can be induced to display an LC phenotype under the conditions tested, there may be other conditions which can induce the same phenotype in monocytes, such as

Notch ligation, and these should be explored. Also, further expression profiling and functional analysis should be performed on the LC-like cells formed to truly define the possibility of a precursor-progeny relationship between these cells and LC/LCH cells.

It is interesting to note that on day 7 of my langerin induction data, Birbeck granules (BGs) and thumbprint organelles could be observed coexisting in the same cells cultured with GM-CSF and TGFβ/BMP7 (Figure 7.3). The presence of the thumbprint organelles and BGs coexisting in the same cells does not in itself imply a relationship between the two; however, It would be interesting to study the two organelles in more detail.
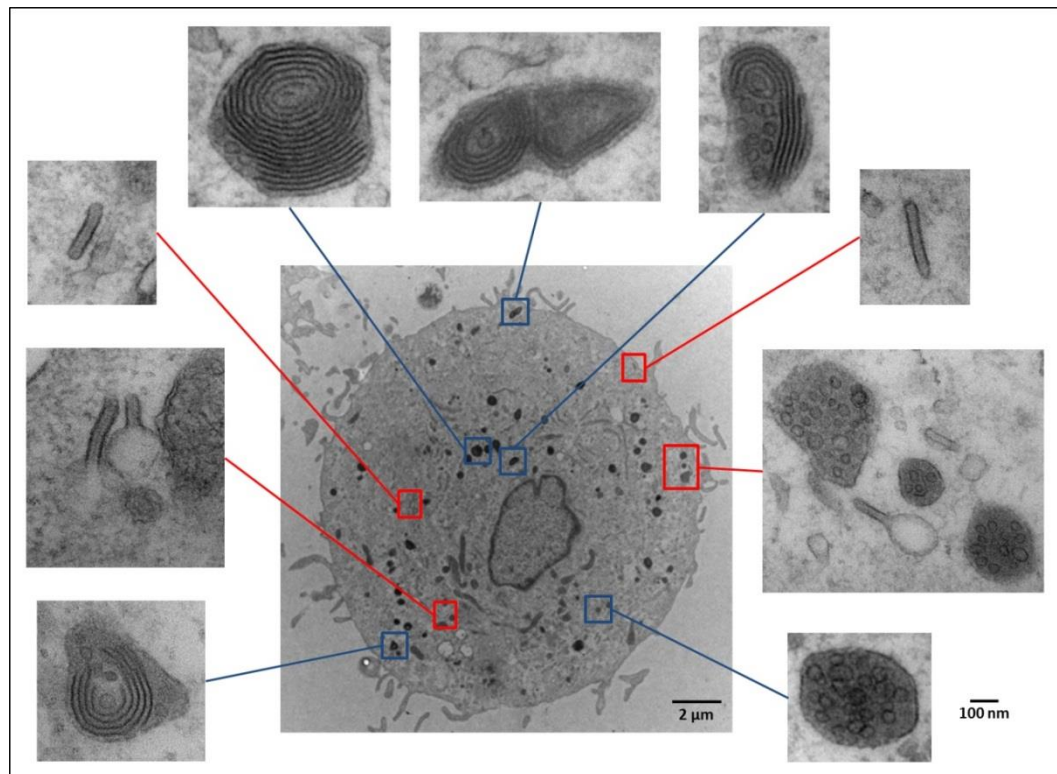
Figure 7.3. Coexistence of Birbeck Granules and Thumbprint Organelles

Example EM images of Birbeck granules and thumbprint organelles coexisting in the same CD1c+ myeloid dendritic cell at day 7 of culture. The experiment was performed twice with 2 different donors with GM-CSF and BMP7. The entire culture was processed for EM.

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HEMATOPOIESIS AND STEM CELLS

The evolution of cellular deficiency in *GATA2* mutation

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Key Points
• Diverse patient groups with *GATA2* mutation develop mononuclear cytopenia and elevated Flt3 ligand.
• Progressive cytopenias, rising Flt3 ligand, and terminal differentiation of lymphoid cells accompany clinical progression.

Constitutive heterozygous *GATA2* mutation is associated with deafness, lymphedema, mononuclear cytopenias, infection, myelodysplasia (MDS), and acute myeloid leukemia. In this study, we describe a cross-sectional analysis of 24 patients and 6 relatives with 14 different frameshift or substitution mutations of *GATA2*. A pattern of dendritic cell, monocyte, B, and natural killer (NK) lymphoid deficiency (DCML deficiency) with elevated Fms-like tyrosine kinase 3 ligand (Flt3L) was observed in all 20 patients phenotyped, including patients with Emberger syndrome, monocytopenia with *Mycobacterium avium* complex (MonoMAC), and MDS. Four unaffected relatives had a normal phenotype indicating that cellular deficiency may evolve over time or is incompletely penetrant, while 2 developed subclinical cytopenias or elevated Flt3L. Patients with *GATA2* mutation maintained higher hemoglobin, neutrophils, and platelets and were younger than controls with acquired MDS and wild-type *GATA2*. Frameshift mutations were associated with earlier age of clinical presentation than substitution mutations. Elevated Flt3L, loss of bone marrow progenitors, and clonal myelopoiesis were early signs of disease evolution. Clinical progression was associated with increasingly elevated Flt3L, depletion of transitional B cells, CD56^{bright} NK cells, naïve T cells, and accumulation of terminally differentiated NK and CD8^{+} memory T cells. These studies provide a framework for clinical and laboratory monitoring of patients with *GATA2* mutation and may inform therapeutic decision-making. (*Blood*. 2014;123(6):863-874)

Introduction

Constitutive heterozygous mutation of the *GATA2* gene causes a complex disorder of hematopoiesis with variable extramedullary defects. We have previously characterized the loss of mononuclear cells that occurred in 4 patients with immunodeficiency as a syndrome of dendritic cell (DC), monocyte, B, and natural killer (NK) lymphoid (DCML) deficiency.[1,2] Others have reported cytopenias in patients with various clinical syndromes of *GATA2* mutation. These include monocytopenia with *Mycobacterium avium* complex (monoMAC)[3-5]; lymphedema, deafness, and myelodysplasia (MDS)

(Emberger syndrome)[6,7]; and familial MDS/acute myeloid leukemia (AML).[8-11] Recent work suggests that monoMAC, lymphedema, and familial MDS/AML are all facets of *GATA2* mutation that may occur heterogeneously,[9,12] even within a single pedigree.[13] Some historical cases of familial AML are now also known to be due to *GATA2* mutation.[14-16] It is unknown whether failure of mononuclear cell development is a consequence of *GATA2* mutation in all patient groups. In particular, hereditary AML may arise without a preceding "accessory" hematopoietic phenotype.[8] Also, extramedullary

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complications such as lymphedema may or may not develop independently of hematopoietic failure.[9,17,18]

A wide range of genetic defects has been described in the *GATA2* locus including deletions, regulatory mutations, frameshift mutations, and substitutions. Extramedullary developmental defects are associated with large deletions.[9] Cohorts with lymphedema are enriched for frameshift mutations,[7] while hereditary AML has been described particularly in families with substitutions clustering in the second zinc finger.[8-10] These phenotypes are likely to be partially penetrant.[9]

Up to 50% of individuals with *GATA2* mutation develop MDS associated with fibrosis and megakaryocyte dysplasia.[4,5,10,11,19] However, many patients present with clinical problems prior to their meeting the standard criteria for MDS. Monocytopenia is a vital clue,[1,3] but mild chronic neutropenia[20] and NK deficiency[21] are also associated with *GATA2* mutation. A more precise understanding of the evolution of cellular deficiency and the progression of disease may further assist in the recognition and clinical management of this disorder.

It has previously been reported that Fms-like tyrosine kinase 3 ligand (Flt3L) is elevated in patients with DCML deficiency.[1] Flt3L is an important factor in DC development, but elevated levels have also been reported in Fanconi and aplastic anemia suggesting that hematopoietic stress is a trigger.[22,23] Further evaluation may indicate whether this is a useful marker for diagnosis and monitoring of *GATA2* mutation.

The risk of infectious complications in *GATA2* mutation is difficult to predict, but frequently remains low until the third or fourth decade. It appears from unremarkable childhood case histories, normal class-switched immunoglobulin, and a grossly intact T-cell compartment that the immune system is competent for sufficiently long to establish a level of immunologic memory. Nonetheless, the existence of a premorbid state without cytopenia has not been firmly established. Indeed, several patients have been characterized with long periods of cytopenia.[1,3] Loss of CD56^{bright} NK cells has been reported,[21] but the B-cell compartment and remaining T cells have not been examined in detail.

It has been suggested that *GATA2* mutation leads to poor risk AML.[8,11,24] Although hematopoietic stem-cell transplantation is effective in treating MDS and in resolving life-threatening infectious complications,[25] a better understanding of the trajectory of *GATA2* disease is required to optimize the treatment strategy for patients.

In this study, we present an analysis of a European cohort of patients and their relatives with *GATA2* mutation from a range of clinical backgrounds, describing in detail the evolution of cellular deficiency, the utility of Flt3L in diagnosing and monitoring disease progression, and the effects of failing mononuclear cell development upon peripheral lymphoid homeostasis.

Methods

Patients

Patients with *GATA2* mutation were referred from a wide range of clinicians suspecting a *GATA2*-related disorder. There were no specific inclusion criteria and all patients found to have *GATA2* mutation were reported. Patients with acquired MDS (World Health Organization classification: refractory cytopenia and multilineage dysplasia) were recruited from a local hematology ambulatory clinic. All MDS patients were symptomatic and some required transfusion support, but none had received high-dose cytoreductive therapy prior to testing. Direct sequencing confirmed wild-type (WT) *GATA2* coding

sequence in all cases. Patients with primary immunodeficiency disease (PID) were a heterogenous group with a history of suspected immunodeficiency and variable cellular deficiencies, were referred for investigation of possible *GATA2* mutation but were found not to have a DCML deficiency phenotype or a *GATA2* mutation. This population served as controls for further analyses performed. Blood, skin, and bone marrow (BM) aspirate surplus to diagnostic requirement was collected. Systematic collection of patient material, analysis, and collation of clinical details for publication was approved by the Newcastle and North Tyneside Research Ethics Committee 1 (Reference 08/H0906/72). Informed consent was obtained in accordance with the Declaration of Helsinki.

Flow cytometry

DCML profiling was performed as previously described.[1] Antibodies are listed in supplemental Table 1 on the *Blood* Web site. Absolute cell counts were determined by Trucount analysis (BD Biosciences). Flow cytometry data were collected using an LSRII cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR).

DNA sequencing

Peripheral blood was used as the source of DNA except for 3.II.6 (frozen muscle), 5.I.1, 7.I.1, 7.II.1, and 8.I.3 (dermal fibroblasts). Genomic DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN), while polymerase chain reaction (PCR) amplification and Sanger sequencing was performed using primers and conditions described previously.[2]

Analysis of clonality

Genomic DNA was extracted with QIAamp DNA Mini Kit and genotyping of exonic single nucleotide polymorphisms from 3 X-chromosome genes (MPP1: G/T, FHL1: G/A, and IDS: C/T) was determined using TaqMan allele-discrimination assays on an Applied Biosystems 7500 Sequence Detection System. Total RNA was isolated from neutrophil pellets and peripheral blood mononuclear cells (PBMCs) using RNeasy Micro Kit (QIAGEN), and used for assessment of clonality. Quantitative allele-specific suppressive PCR was performed on a sequence detection system (7500 platform) and allele frequency of expressed exonic single nucleotide polymorphisms was calculated as previously described.[26]

Serum biomarker screening and ELISA

The quantities of 117 serum proteins from 16 patients with *GATA2* mutation and 10 healthy adult controls were measured with MILLIPLEX Multiplex Assays (Millipore, Billerica, MA): Cytokine/Chemokine Panels I-III, Cancer Biomarker I-II, and Circulating Cytokine Receptor. Multiplex assays were processed on a MAGPIX Plate Reader (Luminex, Austin, TX), and analyzed with MILLIPLEX Analyst 5.1 software (Millipore). All samples were performed in duplicate. Significantly elevated markers were reanalyzed by enzyme-linked immunosorbent assay (ELISA) across the whole cohort. Serum ELISA was performed with quantikine human Flt3/Flk-2 ligand immunoassay, quantikine human epidermal growth factor (EGF) immunoassay, quantikine human soluble CD40 ligand (CD40L) immunoassay, quantikine human granulocyte-macrophage colony-stimulating factor (GM-CSF) immunoassay, and quantikine fibroblast growth factor (FGF) basic immunoassay (R&D Systems).

Real-time quantitative PCR

Total RNA was extracted using the RNeasy Micro Kit and treated with Dnase I. Complementary (cDNA) was synthesized using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific). Real-time PCR was performed with TaqMan Gene Expression Master Mix, and gene expression assays for *FLT3LG* (Hs00181740_m1) and glyceraldehyde-3-phosphate dehydrogenase (4352934E; Life Technologies) with Applied Biosystems 7900HT Fast Real-Time PCR System. Relative quantification of the messenger RNA (mRNA) levels was performed using glyceraldehyde-3-phosphate dehydrogenase as the reference.

Table 1. Patient cohort

Number	Kindred	Age*	cDNA	Protein	HPV = 1	Myco = 1	URTI = 1	Lung = 1	AI = 1	Clinical Score	MDS	Lymph	Cancer
1	1.I.1†	12	c.599_600insG	G200fs	+	+				2			
2	2.I.1†§	26	1061 C>T	T354M	+	+				2			
3‖	3.II.6§	34	1192 C>T	R398W	+			+		2	+		a
4	3.III.1†	22	1192 C>T	R398W	+			+		2			b
5	3.III.3	26	1192 C>T	R398W						0			
6	4.I.1†	18	c.1018-1 G>T	Δ340-381	+		+		+	3			
7	5.I.1§	40	1192 C>T	R398W	+	+			+	3	+		
8	6.I.1	18	c.594delG	G199fs	+		+	+	+	4		+	
9	6.II.1	17	c.594delG	G199fs			+			1	+		
10	6.II.2	13	c.594delG	G199fs	+					1			
11‖	7.I.1§	10	c.318_319insT	S106fs	+			+		2			
12‖	7.II.1§	10	c.318_319insT	S106fs	+					1		+	c
13	8.I.2	62	1193 G>A	R398Q						0			
14‖	8.I.3	25	1193 G>A	R398Q	+					1	mono 7		
15	8.II.1	36	1193 G>A	R398Q	+	+		+		3			
16	8.II.4	32	1193 G>A	R398Q						0			
17	8.II.5	29	1193 G>A	R398Q						0			
18	9.III.1‡	31	1061 C>T	T354M	+	+		+		3	+		
19	9.III.2‡	29	1061 C>T	T354M	+					1			
20	9.III.3‡	22	1061 C>T	T354M						0			
21	9.III.4‡	17	1061 C>T	T354M						0			
22	9.III.5‡	17	1061 C>T	T354M					+	1	tri 8		
23	10.I.1	22	c.1114 G>A	A372T	+			+		2			
24	11.I.1	8	c.257_258delGC	C85fs					+	1	mono 7		
25	12.I.1§	22	c.1018-1 G>A	Δ340-381	+	+		+	+	4	+		d
26	13.I.1	19	c.735_736insC	P245fs	+					1			
27	14.I.1	60	c.599_600insG	G200fs				+	+	2	tri 8		
			c.599_600insG	G200fs									
28	14.II.2	30	c.1168_1170del1AAG	390delK				+		1	+		
29	15.I.1	4	1081 C>T	R361C	+				+	2	+	+	e
30	16.I.1	9	c.1081-3_1031del17	A341fs	+					1		+	f

A survey of the most common clinical features of the cohort is presented. HPV (persistent infection of hands, feet, or perineum with HPV); Myco (any history of mycobacterial infection); URTI (more than 3 episodes of recurrent bacterial sinusitis, otitis, or other URTI); Lung (loss of lung volume or transfer factor <80% predicted, history of bronchiectasis, chronic bronchitis, more than one episode of pneumonia, radiologically or pathologically confirmed pulmonary alveolar proteinosis); AI (autoimmunity: arthritis, panniculitis, or autoimmune cytopenia); MDS (WHO: refractory cytopenia with multilineage dysplasia); Lymph (lymphedema); and Cancer (nonhematopoietic malignancy). The clinical score was derived by giving 1 point for each of the categories: HPV, Myco, URTI, Lung, and AI. Cytogenetics are indicated in the MDS column: mono 7, monosomy 7, tri 8, and trisomy 8. Lowercase letters indicate solid malignancy, as follows: a, cervical intra-epithelial dysplasia 3; b, ano-genital dysplasia with vulval intra-epithelial neoplasia 3; c, cervical intra-epithelial dysplasia 3; d, schwannoma/neuroma; e, cervical intra-epithelial dysplasia 3 and squamous carcinoma of vulva; and f, ano-genital dysplasia. Blank spaces indicate the absence of a clinical feature.

*Age at clinical presentation or detection of mutation, if asymptomatic.

†Patients published.[2]

‡Pedigree previously described.[14]

§Deceased.

‖Genotype and clinical phenotype only.

Statistical analysis

Analysis of variance with Bonferroni Multiple Comparisons Test was used to compare groups in most analyses. Cell count data were obviously skewed in *GATA2* deficiency, therefore, nonparametric tests were preferred (Mann-Whitney or Kruskal-Wallis with Dunn's Multiple Comparison Test).

Results

Comparison of *GATA2* mutation with acquired MDS

The cohort of 30 patients with *GATA2* mutation comprised of 16 index cases and 14 relatives. These included 4 with Emberger syndrome, 6 with "monoMAC" (3 also with MDS), 8 with MDS, 6 with other clinical syndromes, and 6 asymptomatic patients. The 6 asymptomatic patients were all related to one or more persons who had developed MDS due to point mutation of the second zinc finger (Table 1). AML had been diagnosed historically in several families, but none of the patients reported here had developed leukemia.

The most common early complications were documented from a review of the case notes, and a simple clinical score was derived (Table 1). We focused on early complications because management is less certain at this stage than when patients have already developed MDS. MDS, lymphedema, and solid malignancy were recorded, but were excluded from the clinical score.

Mononuclear cytopenia, in particular the loss of DCs, has not been systematically investigated in all presentations of *GATA2* mutation. Furthermore, it is not known how distinct the phenotype of DCML deficiency is from cytopenias that occur in acquired MDS with WT *GATA2*. To explore these issues, we performed an extended mononuclear profile on 26 of 30 patients with *GATA2* mutation, including 20 symptomatic patients with monoMAC, Emberger syndrome, or MDS, and 6 asymptomatic relatives. DCML-deficiency was evident in all 20 symptomatic cases, including representative patients with monoMAC, Emberger syndrome, and MDS (Figure 1). We then compared 18 patients (with at least one clinical manifestation) with acquired MDS patients receiving ambulatory care who were known to be *GATA2* WT (n = 12). Patients with

Figure 1. Mononuclear cell profiles of patients with Emberger syndrome, monoMAC, and familial MDS associated with GATA2 mutation. Examples of mononuclear profiling in familial MDS (#18; T354M), monoMAC (#25; del340-381), and Emberger syndrome (#30; A341fs) showing that a DCML-deficiency phenotype may be associated with diverse clinical manifestations and different GATA2 mutations. Populations: (1) CD14+ monocyte; (2) CD16+ monocyte; (3) pDC; (4) CD34+ progenitors; (5) CD141+ mDC; (6) CD1c+ mDC; (7) B cells; and (8) NK cells. Note expansion of CD34+ progenitors.

MDS were significantly older (median age = 65 years, range 42 to 83 years vs median age = 20 years, range 4 to 60 years) and had reduced hemoglobin (Hb), neutrophils, and platelets compared with those with GATA2 mutation (Figure 2A).

Patients with GATA2 mutation often had normal hematologic parameters: 9 of 18 (50%), 11 of 18 (61%), and 9 of 18 (50%) had Hb, neutrophils, and platelets, respectively, within the reference range. A total of 7 of 18 (39%) were normal for all three parameters. DC, monocyte, B cell, and NK cell counts were significantly reduced in all symptomatic carriers of GATA2 mutation (Figure 2B-D).

Interestingly, patients with acquired MDS also had mild mononuclear cytopenias of plasmacytoid DCs (pDCs), and both classical and nonclassical monocytes. There were trends for lower myeloid DCs (mDCs), B cells, and NK cells in MDS, but this did not achieve significance after adjustment for multiple comparisons.

Genotype-phenotype correlations

Although GATA2 mutations are diverse and we screened all the promoters, exons, intron 5 enhancer, and 3' untranslated regions in this cohort, we detected only frameshift mutations in the coding region 5' to the second zinc finger, or substitutions (plus one inframe deletion) in the second zinc finger of GATA2 (supplemental Figure 1). We did not detect larger gene deletions as have been described in patients with congenital defects, which might have been under-represented in our cohort. Common clinical manifestations showed little difference between the 2 genotype groups (Figure 2E). Among symptomatic patients (n = 24; 11 frameshift and 13 substitution mutations), a higher proportion of frameshift mutations occurred in lymphedema (3 of 13 vs 1 of 11), while substitution mutations were more prevalent in the MDS group (7 of 11 vs 5 of 13). Neither trait achieved significance in Fisher's exact test with this relatively small number of patients. The clinical score derived from Table 1 showed a slightly higher but nonsignificant weighting in the frameshift group (Figure 2F). The age of presentation was younger in the frameshift group compared with the substitution group (median age = 18 vs 26 years; $P < .05$) (Figure 2G).

Evolution of mononuclear cytopenia in asymptomatic relatives with GATA2 mutation

We identified 3 pedigrees with mutations: R398W, R398Q, and T354M containing 6 asymptomatic relatives (Figure 3A). Two

developed cytopenia and elevation of Flt3L, even though they were unaffected clinically (clinical score = 0), but 4 cases (#13, #16, #17, and #20) remained phenotypically normal (Figure 3B-C). Sequential monitoring of #5 showed declining cell counts over 3 years with progressive elevation of Flt3L and circulating CD34+ progenitors (Figure 3D). Despite normal peripheral cell counts and unremarkable BM histology (not shown), flow cytometry revealed that the progenitor compartment was already depleted of B and NK cells, multilymphoid progenitor (MLP), and granulocyte-macrophage progenitor (GMP) fractions (Figure 3E).

The early elevation of Flt3L and the loss of specific progenitors suggested that hematopoiesis was already under stress. To corroborate this, clonality testing was performed by looking for nonrandom X inactivation in female patients, as previously described.[26] Surprisingly, this revealed a >75% bias toward one allele, consistent with clonal hematopoiesis, in all neutrophil samples and most PBMCs tested (Figure 3F). PBMC clonality was always less marked than that of neutrophils, and in 2 patients with the lowest neutrophil clonal bias, PBMCs remained evenly balanced. T cells are a major component of PBMCs, especially in these patients, and the lag in PBMC clonality presumably reflects the slow turnover of peripheral T cells from BM-derived precursors.

Although hematopoiesis appears clonal, this does not explain the selective loss of MLP, GMP, and mononuclear cells associated with GATA2 mutation. Seeking evidence that GATA2 had a direct role in specifying the development of MLP, GMP, or their progeny, we attempted to knock-down GATA2 expression in hematopoietic stem cells using short hairpin RNA-expressing lentiviral vectors. However, this failed to alter the generation of hematopoietic progenitors or balance of lymphoid/myeloid output using a xenotransplant readout (supplemental Figure 2).

Elevated Flt3L is a marker of GATA2 mutation

It was previously reported that GATA2 mutation is associated with elevated serum Flt3L.[1] Comparison of patients with GATA2 mutation at different clinical stages (n = 24) with relatives who did not carry GATA2 mutation (n = 13), patients with acquired MDS and WT GATA2 (n = 11) or PID (n = 11) indicated significantly elevated Flt3L for symptomatic GATA2 mutation (Figure 4A). Compared with GATA2 WT controls, 2 of the 6 relatives with a clinical score of 0 had supranormal Flt3L (>200 pg/mL). Within the symptomatic GATA2 cohort (clinical score = 1 to 4), the development of MDS was
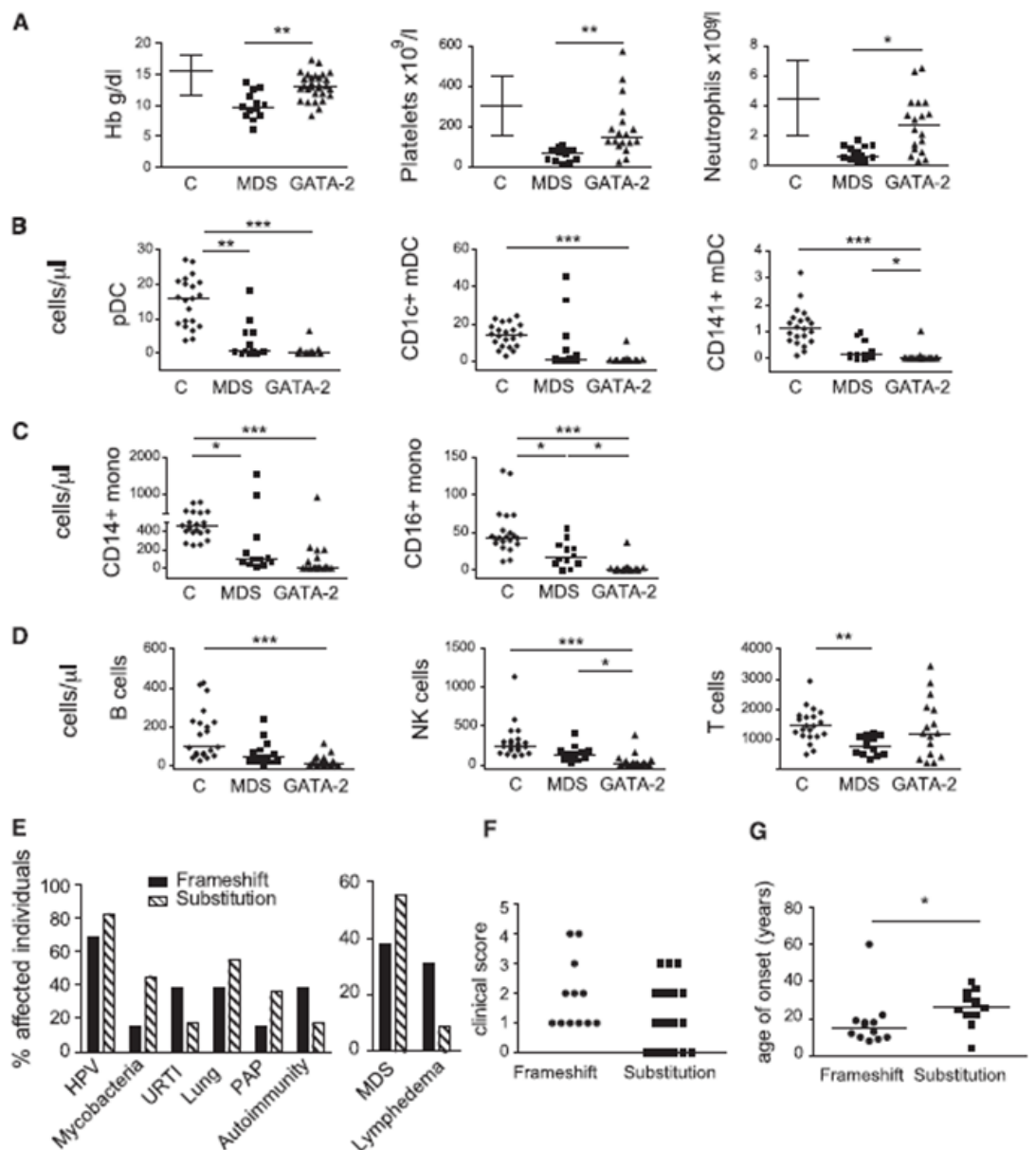
Figure 2. Comparison of *GATA2*-mutated patients with MDS patients and genotype-phenotype correlations. (A-D) Comparison of controls (n = 21), patients with MDS (n = 12), and patients with symptomatic *GATA2* mutation (n = 18). (A) Automated blood counts. (B-D) DCs, monocytes, and lymphocyte subsets by Trucount analysis. Analysis was performed as previously described.[1] (E) Summary of clinical features among symptomatic carriers of *GATA2* mutation by genotype (11 frameshift and 13 substitutions). (F) Profile of clinical score as defined in Table 1 according to genotype. (G) Age at presentation by genotype. *P < .05; **P < .01; ***P < .001. C, controls (or reference range); HPV, human papilloma virus; mono, monocyte; PAP, pulmonary alveolar proteinosis; URTI, upper respiratory tract infection.

associated with lower Flt3L, but still above the level seen in acquired MDS patients (Figure 4B). When patients with MDS were removed, advancing clinical stage was clearly associated with progressively increasing FLT3L (Figure 4C). Together, these results suggest a biphasic relationship, where Flt3L becomes progressively elevated but then declines as MDS develops.

In all patients, progressive mononuclear cytopenias correlated with clinical stage (Figure 4D). The relationships between cytopenia, advancing clinical stage, and Flt3L were evident in all mononuclear fractions (Figure 4E). Flt3L mRNA was increased in the PBMCs of patients with *GATA2* mutation and correlated with the percentage of T cells in PBMCs, consistent with T cells being one source of Flt3L

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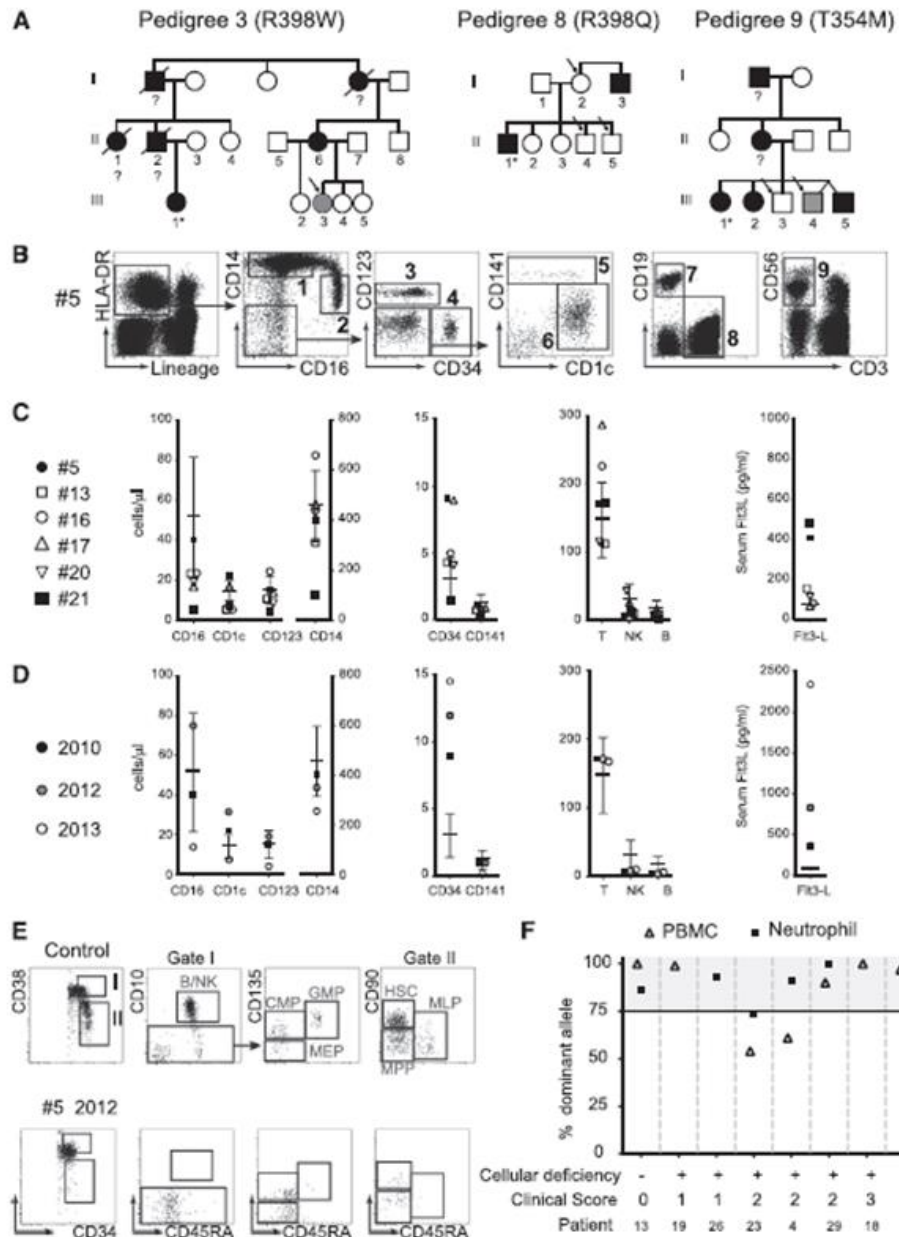
Figure 3. Asymptomatic carriers of *GATA2* mutation may develop cellular deficiency, elevated Flt3L, loss of BM progenitors, and clonal myelopoiesis. (A) Three pedigrees identified (mutation indicated) containing asymptomatic relatives (clinical score = 0), carrying *GATA2* mutation (open symbols, arrowed). Gray symbols identify 2 patients with either elevated Flt3L (>200 pg/ml) or cytopenia. Filled symbols indicate affected patients with mutation (clinical score = 1 to 4). (B) DC, monocyte, and lymphocyte profiles of patient #5, 1 of 3 healthy carriers of *GATA2* mutation showing a normal cellular phenotype at the first point of analysis in 2010. Populations: (1) CD14+ monocyte; (2) CD16+ monocyte; (3) pDC; (4) CD34+ progenitors; (5) CD141+ mDC; (6) CD1c+ mDC; (7) B cell; (8) T cell; and (9) NK cell. (C) Summary of DC and monocyte counts relative to reference ranges for the asymptomatic carriers. Case #5 (filled circle) is shown at first analysis in 2010. Case #21 (filled square) already has cytopenia. (D) Detailed analysis of case #5 showing the loss of cells and rising Flt3L over a 3-year period. (E) BM analysis of case #5 showing loss of B, NK, MLP, and GMP progenitors at midpoint when no cytopenia was evident. CMP, common myeloid progenitor; MEP, megakaryocyte-erythroid progenitor; MPP, multi-potent progenitor. (F) Pattern of X inactivation in females with *GATA2* mutation at different stages of clinical evolution. Dominance of >75% is considered evidence of clonal hematopoiesis.
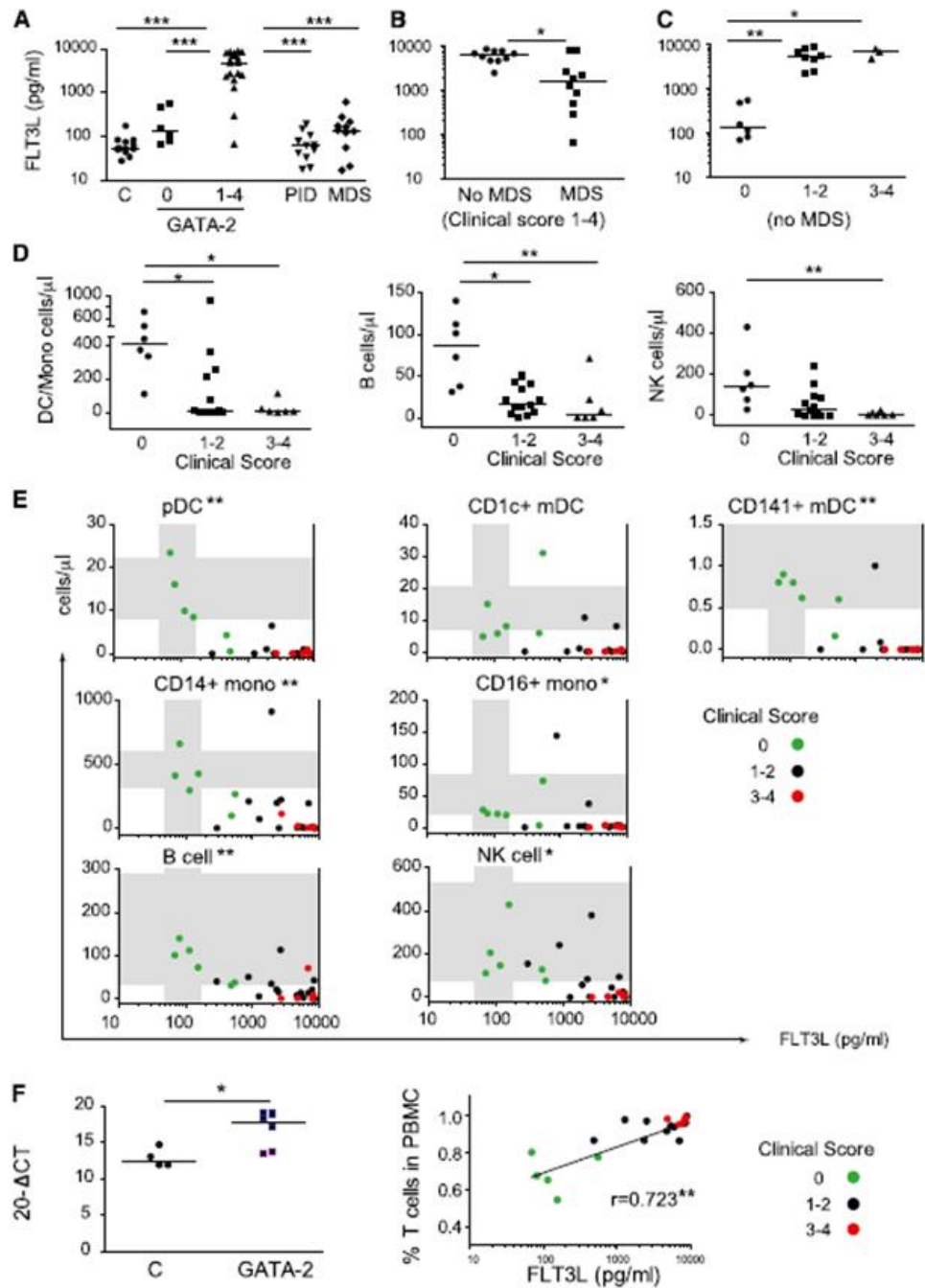
Figure 4. Flt3L is a specific marker of *GATA2* mutation. (A) Flt3L was measured by ELISA in the serum of unaffected relatives with WT *GATA2* (n = 13), individuals with *GATA2* mutation (n = 24), patients with other PID (n = 11), and MDS patients (n = 11). For patients with *GATA2* mutation, the clinical score (0 or 1 to 4) is indicated. (B) The relationship between Flt3L and the development of MDS (n = 24). (C) Relationship between Flt3L and clinical score, excluding patients with MDS (n = 18). (D) Decline in DCs/monocytes, B cells, and NK cells with increasing clinical score (n = 24). (E) Relationship between cell counts, Flt3L, and clinical stage (n = 24; shaded regions indicate normal ranges and asterisks indicate *P* values for Spearman correlation coefficients). (F) Elevation of Flt3L mRNA detected by Q-PCR in *GATA2* patients compared with controls, and relationship between serum Flt-3L and percentage of CD3 (ie, T cells in PBMCs). *P < .05; **P < .01; ***P < .001. Q-PCR, quantitative polymerase chain reaction.

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(Figure 4F). From these data, we conclude that serial measurements of Flt3L may be useful in identifying and assessing the prognosis of patients with *GATA2* mutation.

A proteomic screen of 117 cytokines, chemokines, growth factors, and other immune mediators was initiated to seek other correlative biomarkers. Confirmatory testing of a second larger cohort by ELISA revealed trends for increased FGF-2, EGF, GM-CSF, and CD40L in patients compared with healthy controls (supplemental Figure 3).

Peripheral lymphoid homeostasis in *GATA2* mutation

GATA2 mutation is associated with a complete loss of B- and NK-progenitors within the CD34$^+$ compartment of the BM.[1] This implies that these compartments become depleted by a shortage of immature or naïve cells, although it is also possible that *GATA2* mutation compromises the differentiation or survival of mature cells. For more precise definition, we examined the B and NK compartments in intermediate stages of evolution of DCML deficiency. Transitional B cells were absent and memory B cells were skewed toward a mature phenotype (Figure 5A-B).[27] In keeping with normal immunoglobulin levels, IgD and IgM class-switched–B cells were detectable. In addition, there was expansion of a small subset of CD38$^-$CD21$^-$ B cells associated with autoimmunity.[28] As previously reported, the most juvenile population of CD56^{bright} NK cells was absent in patients with *GATA2* mutation,[21] supporting the model of NK differentiation from CD56^{bright} to CD56^{dim} populations (Figure 5C-D).[29] Within the CD56^{dim} compartment, there was further evidence of skewing toward a more highly differentiated phenotype characterized by the loss of NKG2A and CD62L, and expression of killer-cell immunoglobulin-like receptors (KIR).[30]

More detailed phenotyping of the CD8$^+$ T-cell compartment disclosed a reduction of naïve and central memory cells but an accumulation of CCR7$^-$ CD45RA$^+$ effector memory and CCR7$^-$ CD45RA$^+$ terminal effector populations (Figure 5E).[31,32] In keeping with this, CD8$^+$ T cells of patients expressed lower CD27, CD62L, CD38, and HLA-DR than controls (Figure 5F). The expansion of CD56$^+$CD3$^+$ T cells observed in many patients was consistent with the accumulation of terminal effector CD8$^+$ T cells which was also found to express higher levels of KIR. There was no expansion of γδ T cells, invariant NK T cells, or CD161$^+$ mucosal-associated invariant T cells (MAIT cells) within the CD56$^+$ population. Unexpectedly, there was a significant depletion of MAIT cells in *GATA2* patients (Figure 5G).

Discussion

This study describes a cross-sectional analysis of 30 patients that reveals new information about the evolution of mononuclear cytopenia or DCML deficiency in *GATA2* mutation (Figure 6). We demonstrated DCML deficiency in 20 symptomatic members of the cohort, and variable cytopenias in 2 of 6 of the asymptomatic individuals who were phenotyped. Our findings concur with the descriptions of cellular deficiency in Emberger syndrome[7] and other cases of lymphedema.[9] Mononuclear cytopenia is also a key feature of monoMAC[3] and has been reported in patients with MDS and *GATA2* mutation.[4,9-12,20]

Patients were recruited from diverse clinical backgrounds, but cytopenia often triggered referral, therefore the frequent observation of DCML deficiency is perhaps not surprising. Notably, the pattern of DCML deficiency was reproducible across variable clinical

phenotypes and the degree of cytopenia correlated with the elevation of Flt3L and clinical severity.

Six symptomatic relatives were recruited from 3 different pedigrees with substitutions of the second zinc finger (R398W, R398Q, and T354M) and a history of MDS or AML. Cytopenia and elevated Flt3L was seen in 2 cases but 4 were phenotypically normal, including a relative aged 62 years, suggesting that partial penetrance may occur. Case #5 clearly developed a subclinical cellular phenotype with loss of BM progenitors, progressive elevation of Flt3L, and evolution cytopenia over a 3-year period of observation. Together with other cases already described,[9] this is consistent with the notion that DCML deficiency may evolve over several decades but remain undetected. Whether cytopenias and elevated Flt3L always precede MDS or AML remains unclear. Firstly, we have not yet prospectively documented a case of DCML deficiency evolving into MDS or AML; and secondly, it is entirely possible that unaffected carriers may undergo spontaneous transformation without any sign of DCML deficiency. Further prospective studies will be required to ascertain whether DCML deficiency can be considered a true "accessory" hematologic phenotype to *GATA2*-related MDS/AML, in the manner of thrombocytopenia in *RUNX1* and eosinophilia in *CEBPA* mutations.[33,34] In particular, we note that cytopenia was not reported in association with the T354M and T355del mutations of *GATA2*, originally described in familial MDS/AML.[8] From a pragmatic stance, however, our data suggests that it may be informative to monitor the development of cytopenia and elevation of Flt3L in asymptomatic family members at risk for MDS/AML.

Although *GATA2* mutation is a constitutive genetic risk for developing MDS,[4,8-10,20] patients with *GATA2* mutation may be distinguished from those with acquired MDS and WT *GATA2* on several grounds. *GATA2* mutation is associated with a much younger age of presentation, better preserved Hb, neutrophils, and platelets, and much more severe defects of DCs, monocytes, and lymphoid cells than patients with MDS. Flt3L may be useful as a diagnostic test in this setting; a level in excess of 1000 pg would identify *GATA2* mutation with 89% sensitivity and 100% specificity compared with MDS patients. The observation that control MDS patients did not have *GATA2* mutations is consistent with a recent large cohort study showing an incidence of mutation in only 4 of 603 MDS patients.[35]

We did not encounter large deletions or regulatory mutations of *GATA2* in this cohort despite sequencing the promoters, intron 5 enhancer, and untranslated regions of the gene. As in other studies, frameshifts 5′ to the second zinc finger and substitution mutations in the second zinc finger predominate here.[4,9,20] We found a younger age of presentation and higher clinical score in the frameshift group. An association between lymphedema and frameshift mutation is suggested by a survey of published cases.[7,9,12] Although our data did not reach significance, 3 of 4 patients with lymphedema had frameshift mutations. This is a similar proportion to a previously reported study (6 of 8 pedigrees).[7] Significance was not reached because of cohort size, as well as the low penetrance of this trait (8 of 11 symptomatic frameshift patients did not develop lymphedema). In a similar fashion, MDS was more often associated with substitution mutations, but not all patients with substitution (including 4 of 6 with T354M), developed MDS. Preterm labor has been recognized in women carrying *GATA2* mutations.[3] In this cohort, there was 1 case in each genotype group with an overall incidence of 14% of live births in the cohort. This compares with the European average of 7%.[36]

A simple clinical score aimed at the early complications suggested that clinical progression was associated with evolving mononuclear cytopenia and progressively elevated FL3L. Flt3L is a trophic factor
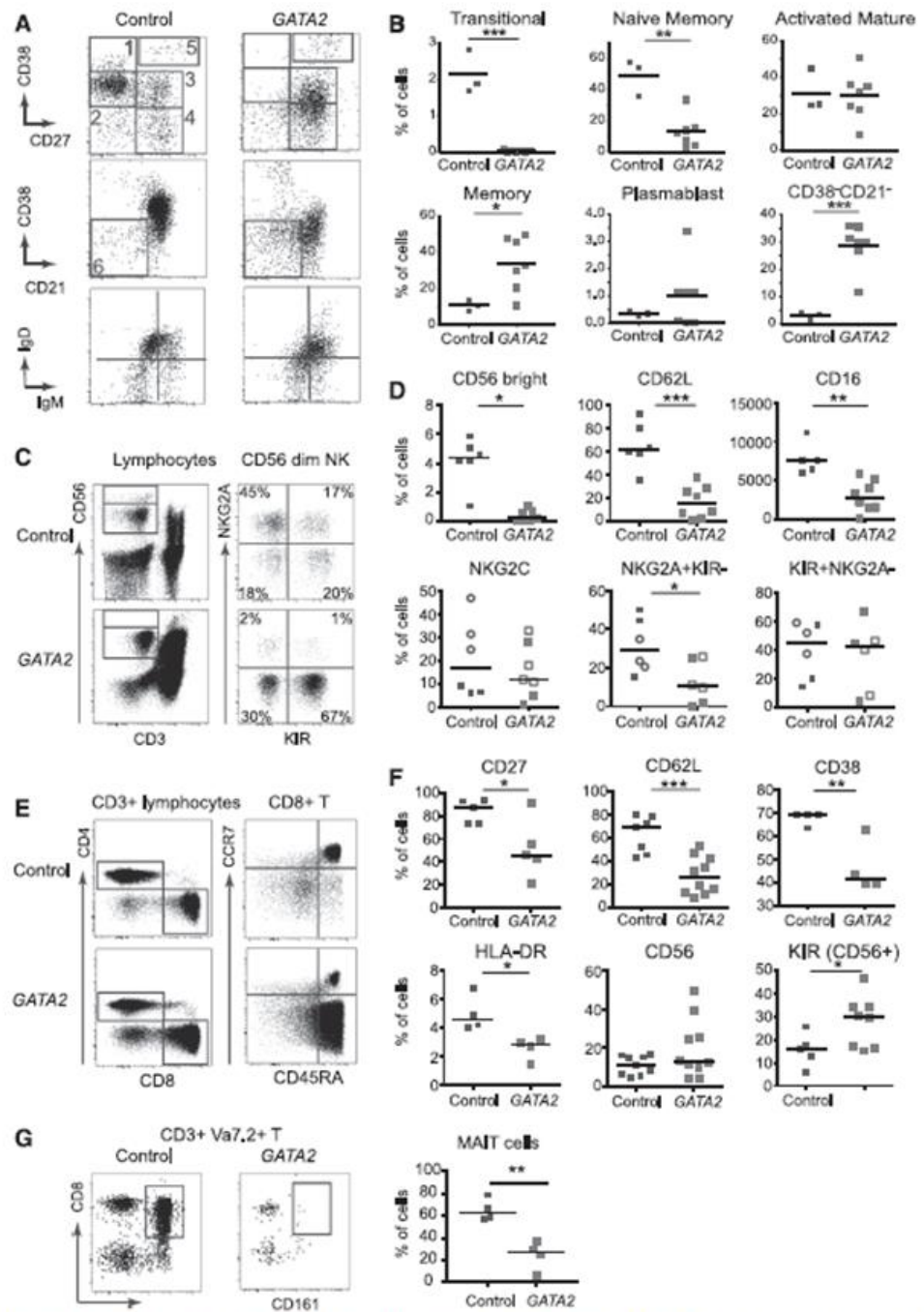
Figure 5. Highly differentiated phenotype of the peripheral lymphoid compartment of patients with *GATA2* mutation. (A) Example of B-cell profile of patient with *GATA2* mutation compared with control according to published descriptions.[23,27] Populations: (1) transitional; (2) naive mature; (3) mature activated; (4) resting memory; (5) plasmablast; and (6) CD38⁻CD21⁻ (autoimmune-associated). (B) Quantification of *GATA2*-mutated patients vs controls showing depletion of transitional B cells and naive memory B cells, and accumulation of memory B cells and CD38⁻CD21⁻ B cells. (C) Example of NK-cell profile of patient with *GATA2* mutation compared with control showing the distribution of CD56^bright NK cells, and NKG2A⁺ and KIR⁺ cells within the CD56^dim population. (D) Quantification of *GATA2*-mutated patients vs controls showing CD56^bright NK cells and the expression of differentiation-associated antigens within the CD56^dim population. Cytomegalovirus seropositivity is indicated by open symbols. (E) Example of CD3⁺ T-cell profile of a patient with *GATA2* mutation compared with control showing CD4:CD8 profile and differentiation according to expression of CCR7 and CD45RA. (F) Quantification of antigen expression by CD8⁺ T cells of *GATA2*-mutated patients vs controls showing the acquisition of a terminally differentiated phenotype and increased expression of KIR on the CD56⁺ subset. (G) CD8⁺ CD161⁺ Va7.2⁺ MAIT cells are decreased in patients relative to controls. *P < .05; **P < .01; ***P < .001.
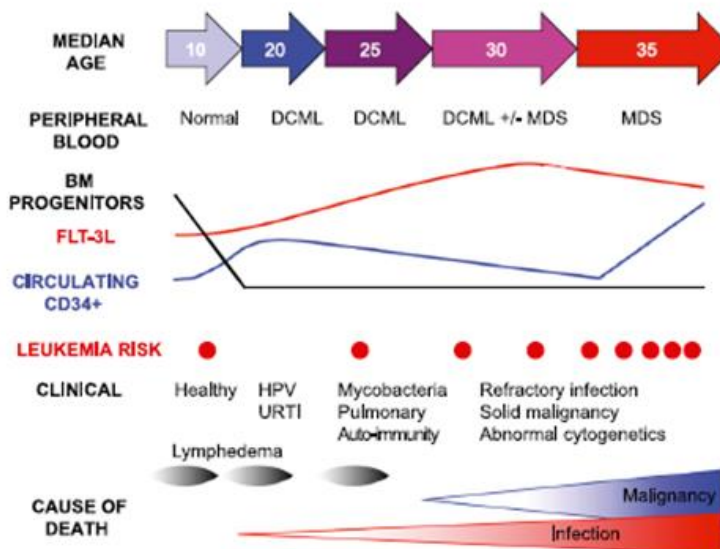
Figure 6. Schematic diagram summarizing the evolution of cellular deficiency in *GATA2* mutation. BM MLPs are rapidly lost even in healthy carriers (see Figure 2). Peripheral blood CD34 counts are elevated in many patients (see examples in Figures 1 and 2) and tend to decline with advancing disease (not shown). Flt3L is progressively elevated, but declines as patients develop MDS (see Figure 4). A rapid rise in CD34+ cells and decline in Flt3L may signify the onset of MDS or AML, although AML may occur sporadically without prior cytopenia.

for human progenitors and DCs.[37-39] *GATA2* mutation presumably elicits a combined response to stem-cell attrition, DC deficiency, and systemic infection.[40,41] Flt3L is produced by activated T cells and stromal cells,[41-43] and serum Flt3L and Flt3L mRNA in PBMCs increased in parallel with peripheral T-cell enrichment. A longitudinal study of patient #5 indicated that the loss of progenitors and the elevation of Flt3L preceded the development of cellular deficiency, suggesting that the major driver relates to the attrition of hematopoietic progenitors rather than peripheral DC homeostasis. In more advanced disease, it appeared that the development of MDS in *GATA2*-mutated individuals was associated with a secondary decline in Flt3L, possibly due to an expansion of marrow cellularity and consumption of Flt3L. Further longitudinal study is required, but this may be a useful indicator of hematologic progression.

The factors that promote the evolution of cytopenias remain uncertain. Extrinsic infection has been mooted and may be consistent with the high numbers of terminally differentiated peripheral T cells seen in many patients. Alternatively, progressive cytopenia and clonal hematopoiesis occurring in asymptomatic individuals, favors cell intrinsic mechanisms. *GATA2* mutation is known to compromise stem-cell longevity in animal models,[44-46] but the mechanism is poorly understood.

Flt3L was the only serum marker of 118 markers screened, to be markedly elevated in *GATA2* mutation. Mild increases of FGF, EGF, and M-CSF were seen, together with CD40L and GM-CSF. Stromal growth factors (FGF and EGF), exerted a similar effect to Flt3L in protecting animal models against hematopoietic stress.[47] CD40L and GM-CSF indicated immune activation. Of note, EGF and CD40L were also elevated in mycobacterial infection and HIV infection.[48,49] The modest induction of these mediators is of interest, but unlikely to be useful in clinical monitoring.

GATA2 mutation and DCML deficiency provide new insights into the maintenance of long-term immunocompetence in adult humans. It is surprising that an almost complete absence of DCs, monocytes, NK cells, and B cells is compatible with long-term survival. Normal IgG and memory T-cell development appears to sustain host resistance to many pathogens and is probably established before cytopenias develop. Patients with evolving DCML deficiency lose transitional

B cells, CD56^{bright} NK cells, and naïve T lymphocytes. The immunophenotype that emerges is strongly reminiscent of the pattern of terminal differentiation seen in aged individuals and chronic viral infections such as cytomegalovirus, hepatitis C, and HIV.[50] NK cells of *GATA2*-deficient patients lose CD16, NKG2A, and acquire KIR expression. Concomitantly, CD8 T cells express CD45RA (TEMRA phenotype), lose CD27, CD62L, and activation markers HLA-DR and CD38, but acquire CD56 and KIR.[51] The function of terminally differentiated cells has been described as defective in many studies, but more recent data indicate that viral infections leave specific adaptive signatures on NK- and T-cell phenotype.[52,53] The absence of professional antigen-presenting cells led us to speculate that invariant T cells including invariant NKT cells, γδ T cells, or MAIT cells might be relatively expanded. The converse was observed, particularly a reduction in MAIT cells, which is another finding consistent with persistent infection and susceptibility to mycobacteria.[54]

In summary, DCML deficiency or mononuclear cytopenia evolves in diverse clinical groups of *GATA2* mutation including Emberger syndrome, monoMAC, and hereditary MDS, but may not be completely penetrant or is an invariant precursor of malignant transformation. *GATA2* mutation appears to cause a complex process of progenitor cell loss, associated with clonal myelopoiesis and elevated Flt3L. Preservation of hematopoiesis in early life allows most individuals to establish a degree of protective immunity. The results presented in this study define the pathogenesis of *GATA2* disease in more detail and will assist in the development of individualized care for patients. However, significant questions remain concerning the molecular mechanisms of hematopoietic failure and malignant transformation, caused by heterozygous *GATA2* mutation in humans.

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Authorship

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Brief Report

IMMUNOBIOLOGY

CD1c$^+$ blood dendritic cells have Langerhans cell potential

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Key Points

- CD1c$^+$ DCs differentiate into Langerhans cells in response to GM-CSF, TGFβ, and BMP7.
- CD14$^+$ monocytes express low langerin but do not make Langerhans cells under the same conditions.

Langerhans cells (LCs) are self-renewing in the steady state but repopulated by myeloid precursors after injury. Human monocytes give rise to langerin-positive cells in vitro, suggesting a potential precursor role. However, differentiation experiments with human lineage-negative cells and CD34$^+$ progenitors suggest that there is an alternative monocyte-independent pathway of LC differentiation. Recent data in mice also show long-term repopulation of the LC compartment with alternative myeloid precursors. Here we show that, although monocytes are able to express langerin, when cultured with soluble ligands granulocyte macrophage colony-stimulating factor (GM-CSF), transforming growth factor β (TGFβ), and bone morphogenetic protein 7 (BMP7), CD1c$^+$ dendritic cells (DCs) become much more LC-like with high langerin, Birbeck granules, EpCAM, and E-cadherin expression under the same conditions. These data highlight a new potential precursor function of CD1c$^+$ DCs and demonstrate an alternative pathway of LC differentiation that may have relevance in vivo. (*Blood*. 2014;00(00):1-4)

Introduction

Langerhans cells (LCs) are antigen-presenting cells of the epidermis that are self-renewing in the steady state[1] but recruited from blood-borne precursors after inflammation.[2-4] Observations in humans also confirm that LCs can be self-maintained[5-7] or replaced by bone marrow–derived cells in the context of transplantation and inflammation.[7-10]

The nature of bone marrow–derived LC precursors that repopulate the epidermis after inflammation is incompletely defined. Experiments in mice with clodronate depletion and bead-labeling suggest a monocyte origin but do not completely exclude other precursors.[2] More recent observations in mice lacking LCs as a result of Id2 knockout or conditional ablation show that LC repopulation occurs in 2 waves.[3,4] The epidermis is initially infiltrated by a short-term precursor with low langerin expression and features in common with monocytes, followed by a long-term precursor that acquires the full phenotype of LCs, including self-renewal capacity.[3,4]

In humans, langerin$^+$ cells can be made in vitro from monocytes.[11-13] Experiments with CD34$^+$ progenitors also demonstrate the existence of an LC-differentiation pathway that appears independent of CD14$^+$ monocytes.[14,15] A previous report suggested the existence of CD3/7/14/16/19-negative LC precursors in human blood and, although noted to express CD1c, these were incorrectly described as expressing CD1a.[16,17] Here we show that CD1c$^+$ blood DCs are alternative LC precursors that achieve higher levels of langerin, CD1a, and Birbeck granules than CD14$^+$ monocytes when exposed to soluble factors known to drive LC differentiation.

Methods

Blood mononuclear cells were obtained from healthy volunteers under local ethical approval. CD14$^+$ monocytes, CD16$^+$ monocytes, CD1c$^+$ DCs, and CD123$^+$ plasmacytoid DCs were sorted from peripheral blood mononuclear cells (PBMCs). LCs were isolated from epidermal sheets separated from whole-skin keratome sections with dispase (Invitrogen) 1 mg/mL incubated at 37°C for 90 minutes in RPMI 1640 and subsequently digested with collagenase (Worthington Type IV) 1.6 mg/mL incubated for 12 hours at 37°C in RPMI 1640 with 10% fetal bovine serum. Sorting was performed with an ARIA Fusion (Becton Dickinson) using previously described protocols (supplemental Figure 1).[18] Ten-thousand cells were cultured in RPMI 1640 with 10% fetal bovine serum or X-Vivo in 100 μL. Supplements were added at the following concentrations: GM-CSF 50 ng/mL, TGFβ, 10 ng/mL, and BMP7 200 ng/mL. Cultures were maintained for 3 to 7 days and supplemented with fresh cytokines on day 4.

Flow cytometry analysis was performed with an FACS Canto (Becton Dickinson) using appropriate isotype controls. Antibodies were from Becton Dickinson unless stated otherwise (antigen fluorochrome clone): CD1a BV421 HI149, CD1c PeCy7 L161, CD3 FITC SK7, CD11b APC ICRF44, CD11c A700 B-ly6, CD11c APCCy7 Bu15, and CD14 BV650 M5E2 (all from Biolegend); CD14 ECD RMO52 (Beckman Coulter); CD16 APCCy7 3G8; CD19 FITC 4G7; CD34 FITC 8G12; CD20 FITC L27; CD56 FITC NCAM16.2; CD83 FITC HB15e; CD123 PerCPCY5.5 7G3; CD207 PE DCGM4 (Beckman Coulter); E-Cadherin APC 67A4 (Biolegend); EpCAM APC EBA-1; and HLA-DR V500 G46-6.

Cells were fixed for electron microscopy (EM) according to standard protocols in 2% glutaraldehyde, and then pelleted, dehydrated, and fixed in resin (reagents from TAAB Laboratory, Aldermarston, UK). Ultrathin
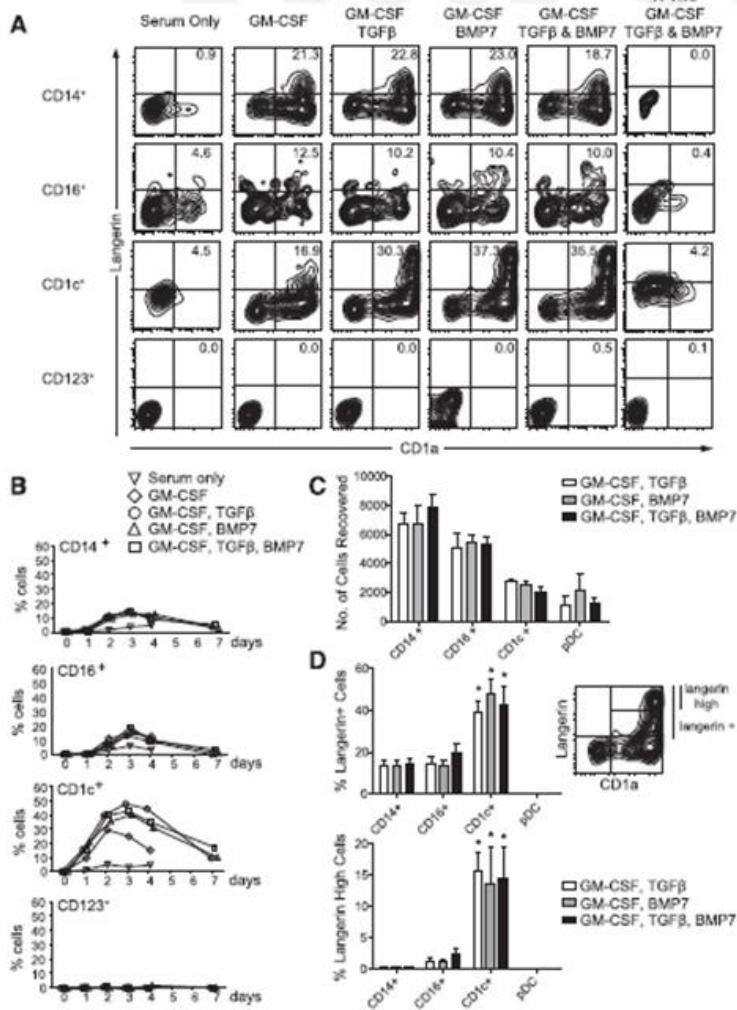
Figure 1. Expression of langerin and CD1a by monocytes and DCs. (A) Sorted cells cultured for 3 days in conditions as indicated showing expression of CD1a and extracellular langerin. The experiment was repeated 5 times except for the panels with X-Vivo, which were repeated 3 times. Four subsets of cells were collected from 1 donor, different in each experiment. (B) Time course of expression of CD1a and langerin double-positive cells showing a peak at 3 days and gradual decline in the percentage of positive cells up to 7 days of culture. Mean ± SEM of 5 experiments with different donors. (C) Recovery of viable cells under each condition at 7 days of culture, estimated by the total number of 4,6 diamidino-2-phenylindole–negative cells recorded when the culture was analyzed and run to dryness on the cytometer. Ten-thousand cells were added to each well as counted by the sorter but typically resulted in 6000 to 8000 viable cells at the start of the culture. There were no statistically significant differences between each condition for a given subset of cells. (D) Upper plot: percentage of langerin$^+$ cells derived from GM-CSF+TGFβ (open bars), GM-CSF+BMP7 (gray bars), and GM-CSF+TGFβ and BMP7 (black bars) after 3 days of culture. Lower plot: percentage of langerin^{high} cells derived from GM-CSF+TGFβ (open bars), GM-CSF+BMP7 (gray bars), and GM-CSF+TGFβ and BMP7 (black bars) after 3 days of culture. Mean ± SEM of 5 experiments with different donors. Gating of langerin$^+$ and langerin^{high} cells is illustrated using CD1c$^+$ DCs incubated with GM-CSF and BMP7 as an example. There were no statistically significant differences between each condition for a given subset of cells. *$P < .01$ compared with corresponding CD14$^+$ monocyte culture. Differences in langerin induction between monocyte subsets and pDCs for a given culture condition were not significant.

sections were cut with a diamond knife RMC MT-XL ultramicrotome and examined with a Philips CM 100 Compustage (FEI) Transmission Electron Microscope. Images were collected with an AMT CCD camera (Deben).

Results and discussion

DC and monocyte fractions of human PBMCs were cultured in conditions that induce langerin in progenitor cells, including BMP7.[19-23] This induced a rapid upregulation of langerin and CD1a dual expression by CD1c$^+$ DCs, peaking within 3 days (Figure 1A-B). Langerin$^+$ cells also appeared in CD14$^+$ and CD16$^+$ monocytes, but the level of expression was lower and did not increase further by day 7. Cells did not expand in culture, and approximately 60% to 70% of monocytes and 20% to 30% of CD1c$^+$ DCs were recovered after 7 days (Figure 1C). Induction of a CD1a$^+$

langerin^{high} population was restricted to CD1c DCs treated with TGFβ, BMP7, or both (Figure 1D). No significant synergy was observed between TGFβ and BMP7, in contrast to recent results obtained with TGFβ and thymic stromal lymphopoietin (TSLP).[24] No langerin induction was seen in medium with serum alone, but serum-free medium with supplements also failed to induce any expression, in contrast to results obtained with CD34$^+$ progenitor cells.[20,23] Exhaustive testing of different serum-free media was not conducted.

Day 7 cultures of CD1c$^+$ DCs and CD14$^+$ monocytes treated with GM-CSF and BMP7 were harvested for EM (all cells). Birbeck granules were observed in 12 of 20 CD1c$^+$ ultrathin sections of CD1c$^+$ DCs, with many sections containing >10 granules. In contrast, Birbeck granules were rarely found in cultures of CD14$^+$ monocytes (a single granule in 20 cell sections) (Figure 2A-B). CD1c$^+$ DCs developed similar cytologic appearance to freshly isolated LCs (supplemental Figure 2). Extended phenotyping showed induction of EpCAM and E-cadherin, and low expression

Figure 2. Formation of Birbeck granules in cultures of CD1c+ DCs. (A) EM images of Birbeck granules in CD1c+ DCs cultured for 7 days showing classical "tennis racket" morphology, pentalaminar structure, and formation by endocytosis. Insets in the top left panel are displayed beneath and to the right. The experiment was performed twice with 2 different donors only with GM-CSF and BMP7. The entire culture was processed for EM. (B) Comparison of the number of Birbeck granules per cell section between CD14+ monocytes and CD1c+ DC cultures. *P < .01. (C) Extended phenotype of LC-like cells obtained after 7 days of culture. Shaded histograms, isotype controls; gray line, CD1a and langerin double-negative cells; black line, CD1a+ langerin+ cells. Similar results were obtained with GM-CSF and TGFβ, BMP7, or a combination of both in 3 different donors; 1 of 3 experiments performed with GM-CSF, TGFβ, and BMP7 is shown. (D) Comparison of langerin, EpCAM, and CD11b expression in CD1c+ DCs and CD14+ monocytes. Light gray, isotype; midgray, CD1a+ langerin^low cells; black, CD1a+ langerin^high cells. One of three experiments performed with GM-CSF, TGFβ, and BMP7 is shown.
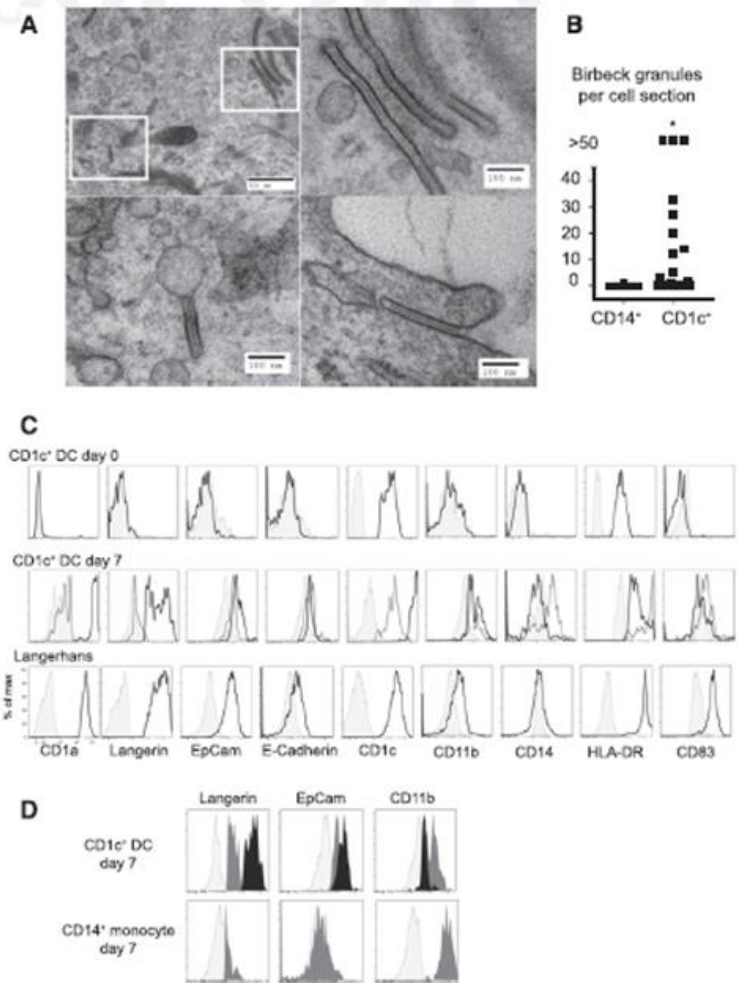
of CD11b, similar to primary LCs. Langerin+ CD1c+ DCs remained immature as assessed by the expression of HLA-DR and CD83 (Figure 2C). The expression of EpCAM was highest on langerin^high cells derived from CD1c+ DCs; in contrast, langerin+ cells derived from CD14+ monocytes had low EpCAM and higher CD11b expression (Figure 2D).

While this paper was under review, LC formation by CD1c+ DCs treated with TGFβ and TSLP was reported.[24] Together, these results indicate that there are several pathways to the generation of langerin+ cells in humans and that, under the conditions tested, CD1c+ DCs but not CD14+ monocytes, generate langerin^high CD1a+ EpCAM+ cells with Birbeck granules. Notably, monocytes prepared by negative selection contain CD1c+ DCs (supplemental Figure 3). It is therefore likely that CD1c+ DCs contribute significantly to LC development when these preparations are induced to differentiate.[11]

There are 2 limitations to this study. First, other conditions not tested here, such as Notch ligation, may be capable of inducing higher langerin in CD14+ monocytes.[12,13] Birbeck granule formation was reported in monocytes exposed to Δ-1,[12] although with

Jagged, langerin expression did not exceed that observed with GM-CSF and TGFβ.[13] Second, although the Birbeck granule is the ultrastructural hallmark of LC phenotype in vivo, the derivation of Birbeck granule–containing cells in vitro does not prove a precursor-progeny relationship. The expression of EpCAM and E-cadherin and low level of CD11b are consistent with an LC phenotype, but further expression profiling and functional studies would be required to evaluate fully the proximity of derived cells to primary LCs. It would be of interest to determine whether a similarly high capacity to crosspresent antigen is found in CD1c+ DC–derived LC-like cells, as shown by those obtained from CD34+ progenitors.[21,22] However, it has been reported that crosspresentation is not as marked in freshly isolated primary LCs[18] and may not be discriminatory for LC differentiation, because it is easily induced in CD1c+ DCs under other conditions.[25]

Although the cultures contained serum, as in other recent publications describing DC differentiation,[24,26] the factors GM-CSF, TGFβ, and BMP7 are expressed by epithelial tissues under physiologic conditions.[23] Previous experiments on CD34+

progenitors have highlighted the difference between a TGFβ-dependent pathway of LC generation from CD1a^{+} intermediates vs the TGFβ-independent generation of monocyte-derived DCs that are unable to express langerin.[14,15] It is likely that this dichotomy reflects the differential ability of CD1c^{+} DCs and monocytes to generate LCs with high langerin, CD1a, EpCAM, and Birbeck granules that we now observe.

Recent data in mice describing 2 waves of repopulation of the LC compartment show an early monocytelike EpCAM[−] langerin[low] wave succeeded by a myeloid precursor of unknown origin. In situ studies also show that LC repopulation after inflammation occurs with similar kinetics in humans.[27] CD1c^{+} DCs have properties that make them candidates for the second-wave LC precursor. Potential homologs of the CD1c^{+} blood DC in mice such as the circulating pre-cDC may contain the long-term precursor of murine LCs.

Finally, these data explain the puzzling claim that there are CD1a^{+} "LC precursors" in human blood.[16] Blood DCs were reported to express CD1a as a result of incorrect assignment of clone BB5 as a CD1a-specific antibody. It is now known that BB5 recognizes CD1b/c and not CD1a.[17] The nonmonocyte cells in human blood that give rise to LCs in response to TGFβ and BMP7 in vitro are now clearly shown to be CD1c^{+} DCs.

Acknowledgments

Authorship

Contribution: P.M. performed experiments and analyzed data; V.B. designed and performed experiments and analyzed data; M.G. performed experiments; M.H. designed experiments and analyzed data; and M.C. designed experiments and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Dendritic cell hematopoiesis: Insights from human dendritic cell deficiency syndromes

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Revisiting the Origin of Langerhans Cell Histiocytosis: Mutated BRAF Identifies Circulating Precursor Cells

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Introduction

Langerhans Cell Histiocytosis (LCH) is a rare clonal proliferation of langerin+ CD1a+ dendritic cell (DC) like cells which can form granulomas in various tissues of the body such as the bones, skin, lungs and pituitary glands. LCH is found in both children and adults and can lead to painful bone swelling and lesions, rashes, organ impairment or a general inflammatory response.

The cells of origin of LCH are thought to be Langerhans Cells (LCs) [1], which are self-renewing langerin+ CD1a+ cells found in normal human skin.

Recently it has been found that LCH cells have a gene expression which is significantly different to that of LCs [2], this coupled with the fact that LCH can occur in organs other than the skin, and our recent discovery of a langerin+ CD1a+ DC subset in the skin, which is phenotypically and functionally distinct from LCs (unpublished data), has led to the conclusion that LCs may not be the originating cells of LCH.

New research has found that 57% of LCH lesions contain the V600E mutation of the BRAF protein [3]. This mutation causes a constitutive activation of BRAF leading to a dysregulation of transcription, proliferation and feedback control.

This has led to the research question 'Can circulating cells containing BRAF V600E mutation be found in patients with V600E positive lesions?'

Aims

1. To develop polymerase chain reaction (PCR) protocols to detect the V600E mutation in lesional cell samples.
2. To develop flow cytometric sorting protocols to sort the subsets of peripheral blood mononuclear cells (PBMCs).
3. To examine the PBMCs from patients with V600E positive mutations to find potential precursor cells of LCH.

Methods

1 - PCR Detection of the V600E Mutation

The PCR detection protocol has been developed using a TaqMan® Mutation Detection Assay (www.invitrogen.com). This involved splitting the DNA between two PCR wells, one containing a mutation primer specific for the V600E mutation, and the other containing a reference primer specific for a non-variable region of the BRAF gene (see Figure 1).

The mutation detection well also contained a Competitive Allele-Specific TaqMan PCR assay which acts as a blocker of the wildtype allele at the location of the V600E mutation, reducing non-specific amplification. The assay has a sensitivity of 0.1%.
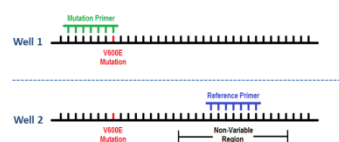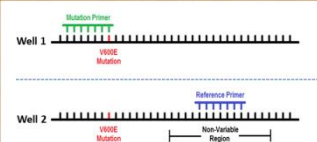

Fig 1. TaqMan® Mutation Detection Assay

2 - Flow Cytometric Sorting of Cell Subsets

Two multi-parameter flow cytometry panels have been developed which can sort four antigen presenting cell subsets and two lymphoid subsets (see figure 2)

	Cell	Target
A	CD14+	20-100k
B	CD16+ Monocytes	5-10k
C	CD123+ pDCs	5-10k
D	CD11c+ mDCs	5-10k
E	B Cells	20-100k
F	T Cells	20-100k
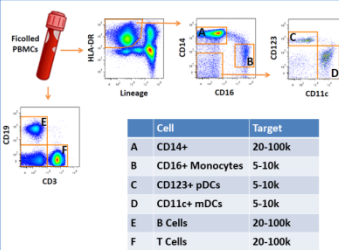
Fig 2. Flow Cytometry Subset Sorting Protocol

3 – Standard Curve

A standard curve was created using V600E positive cell line A375 and a V600E negative Epstein-Barr virus (EBV) transformed lymphoblastoid cell line (LCLs).
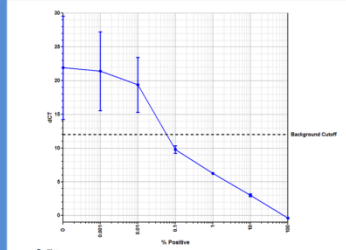
Fig 3. Standard Curve

Results

Lesional samples from ten adult patients (aged 26-77 years) with LCH were tested for the V600E mutation. Of these four were found to have V600E positivity. Two of these patients had single system disease (one tooth socket lesion, one scalp lesion). These patients were found to have no PBMC V600E positivity so no further testing was done. The other two patients had multisystem disease (skin and bone marrow) and both had V600E positivity in their PBMCs. Ficolled blood from the two multisystem patients was sorted into subsets and these subsets where tested for V600E (see Figure 4).

Red bars indicate fractions >0.1% considered positive;

blue bars indicate negative fractions;

ND = not done.

B Cells not sorted from Patient 1.

CD16+ Monocytes not tested in Patient 2 due to low cell numbers.
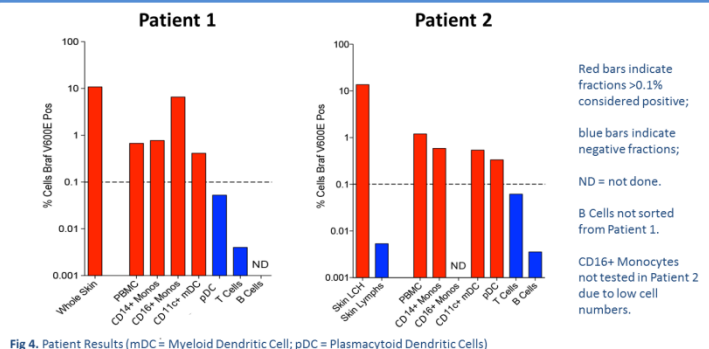
Fig 4. Patient Results (mDC = Myeloid Dendritic Cell; pDC = Plasmacytoid Dendritic Cells)

Lesional skin cells were used as positive controls for both patients. From patient 2, skin cells were sorted into lesional langerin+CD1a+ cells which were V600E positive and lymphocytes which were negative. In patient one, V600E mutation was seen in CD14+ and CD16+ monocytes and CD11c+ mDCs, while pDCs and T cells were negative. This profile was mimicked in patient 2, with V600E positivity in CD14+ monocytes and CD11c+ mDCs and negativity in T and B lymphocytes. In patient 2 a low level of positivity was also seen in pDCs.

Conclusions

- Protocols have been developed to sort PBMCs into cell subsets and to test these cells for V600E mutation.
- These protocols have the potential for minimally invasive diagnostic/prognostic tests for LCH.
- V600E mutation has been detected in the circulating cells of two patients with multi-system LCH, this has not been shown before.
- CD14+ monocytes and CD11c+ mDCs were V600E positive, suggesting that these are the precursors of LCH cells.
- Bone marrow samples have been stored from both of the multisystem patients, these samples will be sorted into progenitor subsets to potentially track the V600E mutation back further into the bone marrow.
- Further patients will be sought to increase the cohort size and the patients will be followed in a longitudinal study to find whether the testing can be used as a means of detecting minimal residual disease.

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Mapping the BRAF^{V600E} Hematopoietic Clone of Histiocytic Disorders

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Introduction

Langerhans Cell Histiocytosis (LCH) and Erdheim Chester Disease (ECD) are rare histiocytic disorders with highly distinct clinical and pathological features. LCH cells express langerin, CD1a and Birbeck granules and cause granulomatous lesions predominantly in the bone, epithelia and haematopoietic organs [1, 2]. ECD lesions show abundant CD68+ foamy macrophages and affect the distal long bones, skin (xanthelasmata) and cardiovascular tissue [3]. LCH is found in both children and adults while ECD is predominantly found in adults. Both diseases can lead to organ impairment, disability and death.

Recent observations indicate that the histiocytes in both diseases harbour single uni-allelic mutations of BRAF, MEK, RAS or other regulatory/survival pathway members that presumably prevent the resolution of inflammation and progressive accumulation of abnormal cells. 50-70% of LCH and ECD lesions harbour BRAF^{V600E} mutations [3, 4].

The ability to detect pathogenic mutations at high sensitivity allows several important questions to be addressed:

1) Are LCH and ECD haematopoietic stem cell disorders with circulating 'precursor' cells?

2) Are pathological differences between the two conditions explained by the involvement of distinct precursor cells?

3) Is the detection of mutation useful for diagnosis or disease monitoring?

Aims

1. To develop polymerase chain reaction (PCR) protocols to detect BRAF^{V600E} in lesions and sorted peripheral blood and bone marrow fractions.
2. To compare the distribution of BRAF^{V600E} in peripheral blood mononuclear cells (PBMC) of LCH and ECD.
3. To test whether BRAF^{V600E} is present in the bone marrow (BM) haematopoietic stem cell.

Methods

1 - PCR Detection of the BRAF^{V600E} Mutation

The PCR detection protocol was developed using a TaqMan® Mutation Detection Assay (www.invitrogen.com). This involved splitting the DNA between two PCR wells, one containing a mutation primer specific for BRAF^{V600E}, the other containing a reference primer specific for a non-variable region of the BRAF gene (figure 1.A).

The mutation detection well also contained a Competitive Allele-Specific TaqMan PCR assay which acts as a blocker of the wild-type allele at the location of BRAF^{V600E}, reducing non-specific amplification. The assay has a sensitivity of 0.1%.

PCR results were compared to a standard curve created using BRAF^{V600E+} cell line A375 and a BRAF^{V600E-} Epstein-Barr virus transformed lymphoblastoid cell line to get the % positivity of each sample (figure 1.B).
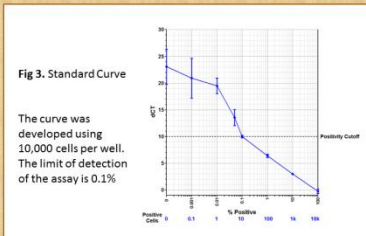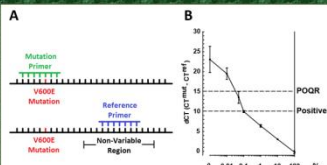

Fig 1. TaqMan® Mutation Detection Assay and Standard Curve.

2 - Cell sorting from PBMC and BM

Three multi-parameter flow cytometry panels have been developed which can sort PBMC into four antigen presenting cell subsets, two lymphoid subsets and two Lin-DR- subsets (figure 2) and BM into five progenitor cell subsets (figure 3).
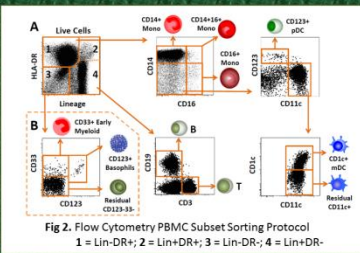

Fig 2. Flow Cytometry PBMC Subset Sorting Protocol
1 = Lin-DR+; 2 = Lin+DR+; 3 = Lin-DR-; 4 = Lin+DR-
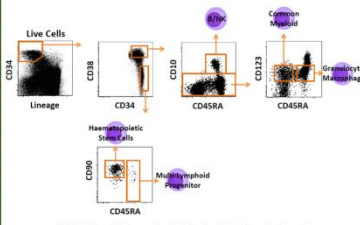

Fig 3. Flow Cytometry BM Subset Sorting Protocol

3 - Patient Recruitment and Analysis

12 adult LCH patients and 8 adult ECD patients were recruited into the study (figure 4).
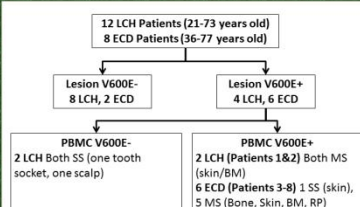

Fig 4. Patient Recruitment (SS = Single-System; MS = Multi-System; BM = Bone Marrow; RP = Retroperitoneum).

Results

1 - Lineage-HLA-DR+ PBMC Subsets

In LCH patient 1, a BRAF^{V600E} was seen in CD14+ and CD16+ monocytes and CD11c+ mDCs, while pDCs and T cells were negative. This profile was mimicked in patient 2, with BRAF^{V600E} positivity in CD16+ and CD14+ monocytes and CD11c+ mDCs and negativity in T and B lymphocytes. In patient 2 a low level of positivity was also seen in pDCs.

Surprisingly, no BRAF^{V600E} was seen in the corresponding cells in the ECD patients (figure 5).

| Patient | Disease | Granulocytes | Myeloid | | | | Lymphoid | |
		Neuts	14+	16+	11c+1c+	123+	T Cells	B Cells
1	LCH	0.01	0.14	4.07	0.11	0.06	0.01	0.00
2	LCH	0.05	4.82	45.61	4.96	0.90	0.03	0.16
3	ECD	NT	0.00	0.03	0.00	NT	NT	0.00
4	ECD	NT	0.00	0.01	0.00	0.00	0.00	NT
5	ECD	NT	0.00	0.00	0.00	0.01	0.00	0.01
6	ECD	NT	0.00	0.04	0.00	0.00	0.03	0.00

Fig 5. Lineage-HLA-DR+ Subset BRAF^{V600E} PCR (number = % positive).

2 - HLA-DR vs. Lineage Analysis

Cells were sorted into HLA-DR vs lineage quadrants (see figure 2.A) in order to track BRAF^{V600E} further. In LCH, 78-94% of mutated alleles were found in HLA-DR+ lineage- cells, while in ECD, the most alleles were present in the double negative quadrant (figure 7.A). This was further analysed in two patients, revealing the presence of most BRAF^{V600E} alleles in a population of CD33+ myeloid cells (figure 7.B).
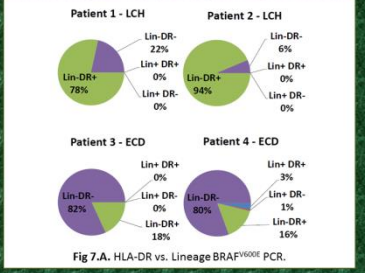

Fig 7.A. HLA-DR vs. Lineage BRAF^{V600E} PCR.

Patient	Disease	Monos	DCs	Basophils	CD33+	CD123-33-
7	ECD	0.00	0.00	0.47	4.56	0.48
8	ECD	0.00	0.00	0.61	12.67	0.15

Fig 7.B. Lineage-DR- Subset BRAF^{V600E} PCR (number = % positive).

3 - Bone Marrow Populations

BM subsets of the two LCH patients were tested and BRAF^{V600E} positivity was seen highly in CMPs, with moderate levels in HSC and GMP. BRAF^{V600E} was negative in the BNK of both patients, and MLP was negative in patient 1, with a very low positivity in patient 2 (figure 8).

Patient	Disease	HSC	CMP	GMP	MLP	B/NK
1	LCH	0.47	4.56	0.61	0.00	0.00
2	LCH	0.61	12.67	1.33	0.20	0.00

Fig 8. BM Progenitor Subset BRAF^{V600E} PCR (number = % positive).

Conclusions

- BRAF^{V600E} mutation was detected in the PBMC of 2/2 patients with multi-system (MS) LCH and 6/6 ECD patients. Patients with single system LCH or BRAF wild-type lesions did not have detectable BRAF^{V600E} in their peripheral blood.
- In multisystem LCH, BRAF^{V600E} was primarily localised to monocytes and CD11c+ mDCs. Overall, 78-94% of the all BRAF^{V600E} alleles were found in HLA-DR+, lineage- cells.
- In contrast, in ECD, BRAF^{V600E} was undetectable in monocytes and 80-82% of mutated alleles were recovered from the HLA-DR- lineage- quadrant. Further analysis localised these alleles to CD33+ early myeloid cells.
- Thus differences in disease phenotype were reflected in distinct patterns of involvement of mononuclear cells.
- In MS LCH, haematopoietic stem cell and myeloid progenitors were also found to harbour BRAF^{V600E} indicating a stem cell origin.
- Peripheral blood mutation analysis may be useful in the diagnosis and monitoring of patients with histiocytosis.

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CD1c+ Blood Dendritic Cells have Langerhans Cell Potential

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Introduction

Langerhans cells (LCs) are dendritic cells of the epidermis that are self-renewing in the steady state[1], but recruited from blood borne precursors following inflammation[2,3]. Observations in humans confirm that LCs can be self-maintained[4] or replaced by bone marrow (BM) derived cells in the context of transplantation and inflammation[5].

The nature of BM derived LC precursors that repopulate the epidermis following inflammation is incompletely defined. Experiments in mice with clodronate depletion and bead-labeling suggest a monocyte origin but do not completely exclude other precursors[2].

In humans, langerin+ cells can be made in vitro from monocytes stimulated with TGFβ[6], although experiments with CD34+ progenitors also demonstrate the existence of an LC-differentiation pathway that appears independent of CD14+ monocytes[7].

Bone morphogenetic proteins (BMPs) are members of the TGFβ family. BMP7 but not TGFβ, is found at high levels in the basal epidermis where LCs are known to proliferate, and in vitro studies have shown that BMP7 can promote LC differentiation from CD34+ cord blood progenitors[8].

Therefore in this study we investigated the ability of TGFβ and BMP7 to promote the differentiation of LC-like cells from peripheral blood monocytes and dendritic cells.

Aims

1. To analyse negative and positive methods of DC and monocyte isolation, to ascertain the true composition of the isolated cells.

2. To find if it is possible to stimulate CD1c+ myeloid DCs (mDCs) or other DCs and monocytes of the blood to bear the features of Langerhans cells in vitro.

Methods

1 - Cell sorting and Culture from PBMC/Skin

CD14+ monocytes, CD16+ monocytes, CD1c+ mDCs and CD123+ plasmacytoid DCs (pDCs) were sorted from PBMC (see Fig. 1). LCs were isolated from epidermal sheets separated from whole-skin keratome sections with 1 mg/mL dispase (Invitrogen), incubated at 37°C for 90 minutes in RPMI 1640. Sheets were subsequently digested with 1.6 mg/mL collagenase (Worthington Type IV), incubated for 12 hours at 37°C in RPMI 1640 with 10% foetal bovine serum. Sorting was performed with an ARIA Fusion (Becton Dickinson; B-D) using previously described protocols. 10,000 cells were cultured in RF-10 (RPMI with 10% FBS) or X-Vivo in 100μl. Supplements were added at the following concentrations: GM-CSF: 50ng/ml; TGFβ: 10ng/ml; BMP7: 200ng/ml. Cultures were maintained for 3-7 days and supplemented with fresh cytokines on day 4.
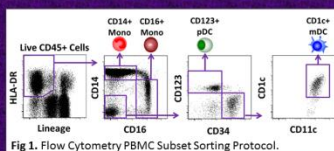

Fig 1. Flow Cytometry PBMC Subset Sorting Protocol.

2 - Electron Microscopy

Cells were fixed for electron microscopy (EM) in 2% glutaraldehyde, then pelleted, dehydrated and fixed in resin (reagents from TAAB Lab, Aldermarston, UK). Ultrathin sections were cut with a diamond knife RMC MT-XL ultramicrotome and examined with a Philips CM 100 Compustage (FEI) Transmission Electron Microscope. Images were collected with an AMT CCD camera (Deben).

3 - Cytospins and May-Grünwald-Giemsa Staining

Cytospin slides were prepared from sorted cells in 200μl RF-10 spinning at 600rpm for 10 minutes. Slides were dried for 30 minutes and fixed in 100% methanol for 2 minutes. Slides were stained in May-Grünwald stain (pH 6.8) for 2 minutes, rinsed with water and stained in Giemsa stain (pH 6.8) for 2 minutes. Photographs were taken on a Zeiss Axioimager Z2 microscope with AxioVision 4.8 software at 100 times magnification.

Results

1 - Expression of langerin and CD1a by monocytes/DCs

DC and monocyte fractions of human PBMC were cultured in conditions that induce langerin in progenitor cells, including TGFβ AND BMP7. This induced a rapid up-regulation of langerin and CD1a dual expression by CD1c+ mDCs, peaking within 3 days (Figure 1). Langerin+ cells also appeared in CD14+ and CD16+ monocytes but the level of expression was lower and did not increase further by day 7.
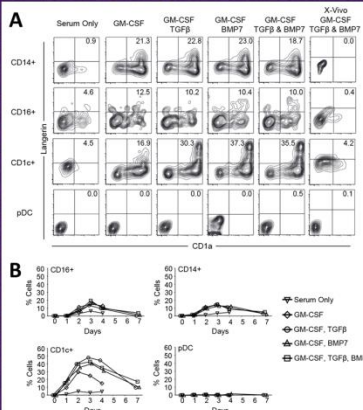

Fig 1. Expression of langerin and CD1a by monocytes and DCs.
A. Day 3 of culture in RF-10 unless otherwise stated.
B. Time course of dual langerin and CD1a positivity.

2 - Electron Microscopy Evaluation

Day 3 cultures of all cell subsets treated with GM-CSF and TGFβ/BMP7 were harvested for EM (all cells). No Birbeck granules (BGs) were seen in ultrathin sections of any culture (>50 cells viewed). Day 7, 10 and 14 cultures of CD1c+ mDCs, CD14+ monocytes and CD16+ monocytes treated with GM-CSF and BMP7 were harvested for EM (all cells). At day 7, BGs were observed in 12/20 ultrathin sections of CD1c+ mDCs with many sections containing more than 10 granules (Figure 2.A). At day 10, only 1/20 ultrathin sections of CD1c+ mDCs contained BGs (six BGs in total), and by day 14, no BGs were seen. In contrast, BGs were rarely found in cultures of CD14+ monocytes (a single granule in 20 cell sections at day 7), and none were seen in cultures of CD16+ monocytes (Figure 2.B).
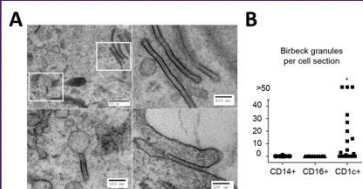

Fig 2. Formation of BGs in cultures of CD1c+ DCs at day 7.

3 - Morphological Examination

Representative examples of each cultured cell type were cytospun and stained with May-Grünwald-Giemsa stain at day 7 and compared with freshly isolated LCs (Figure 3). CD1c+ mDCs were large after in vitro culture but developed pale pink perinuclear staining and vacuole, similar to primary LCs.

Fig 3. Morphology of Cultured Cells.

4 - Extended Phenotyping

In order to determine the extended phenotype of the cultured cells, flow cytometry was performed on days 0 and 7 of CD1c+ mDC cultures. This showed induction of EpCAM, E-Cadherin and low expression of CD11b, similar to primary LCs. Langerin+ CD1c+ mDCs remained immature as assessed by the expression of HLA-DR and CD83 (Figure 4.A). The expression of EpCAM was highest on langerin^{high} cells derived from CD1c+ mDCs; in contrast, langerin+ cells derived from CD14+ monocytes had low EpCAM and higher CD11b expression (Figure 4.B). There was no expression of CD83 on the CD1c+ mDC cultures, showing that they are still less mature than the CD83+ LCs.
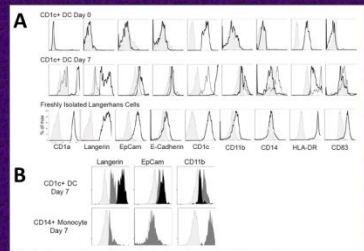

Fig 4. Expression of LC related markers by CD1c+ mDCs.

Conclusions

• CD1c+ myeloid DCs are very easily induced into a Langerhans cell-like phenotype by in vitro culture with soluble mediators GM-CSF, TGFβ and BMP7.

• CD14+ and CD16+ monocytes do not undergo this differentiation under similar conditions.

• BMP-7 can affect LC-like differentiation with equal efficiency to TGFβ.

• These results suggest a second pathway of LC differentiation through a monocyte-independent route.

[1] Merad M, Manz MG, Karsunky H, et al. Langerhans cells renew in the skin throughout life under steady-state conditions. Nat Immunol. 2002;3(12):1135-1141.
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[6] Geissmann F, Prost C, Monnet JP, Dy M, Brousse N, Hermine O. Transforming growth factor beta, in the presence of granulocyte/macrophage colony-stimulating factor and interleukin 4, induces differentiation of human peripheral blood monocytes into dendritic Langerhans cells. J Exp Med. 1998;187(6):961-966.
[7] Caux C, Vanbervliet B, Massacrier C, et al. CD34+ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to GM-CSF & TNF alpha. J Exp Med. 1996;184(2):695-706.
[8] Yasmin N, Bauer T, Modak M, et al. Identification of bone morphogenetic protein 7 (BMP7) as an instructive factor for human epidermal Langerhans cell differentiation. J Exp Med. 2013;210(12):2597-2610.

Appendix 8 - Ethical Approval

NHS

National Research Ethics Service

Newcastle & North Tyneside 1 Research Ethics Committee

Room 144
TEDCO Business Centre
Rolling Mill Road
Jarrow
NE32 3DT

Telephone: (0191) 4283561
Facsimile: (0191) 4283432

13 August 2008

Dr Matthew Collin
Senior Lecturer and Honorary Consultant
Newcastle University
Haematological Sciences
Framlington Place
Newcastle upon Tyne
NE2 4HH

Dear Dr Collin

Full title of study:	**Dendritic cell homeostasis in health and disease**
REC reference number:	**08/H0906/72**

Thank you for your letter of 16 June 2008, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The favourable opinion applies to the research sites listed on the attached form. [Confirmation of approval for other sites listed in the application will be issued as soon as local assessors have confirmed they have no objection.]

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission at NHS sites ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	Version	Date
Application	1	16 May 2008
Investigator CV		16 May 2008
Protocol	1.1	07 May 2008
Covering Letter		20 May 2008
Participant Information Sheet: Part 1	1.2	16 June 2008
Participant Consent Form	v 1.2	16 June 2008
Response to Request for Further Information		16 June 2008
Application form	AB/138475/1	14 May 2008
Student CV		16 May 2008

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

08/H0906/72	Please quote this number on all correspondence

With the Committee's best wishes for the success of this project

Yours sincerely

P.P. **Chair**

This Research Ethics Committee is an advisory committee to North East Strategic Health Authority
The National Research Ethics Service (NRES) represents the NRES Directorate within the National Patient Safety Agency and Research Ethics Committees in England

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(Clockwise from Left) Venetia, Me, Sarah, Muzz, Rachel, Sharon, Xiao, Tara, Matt, Kat, Laura
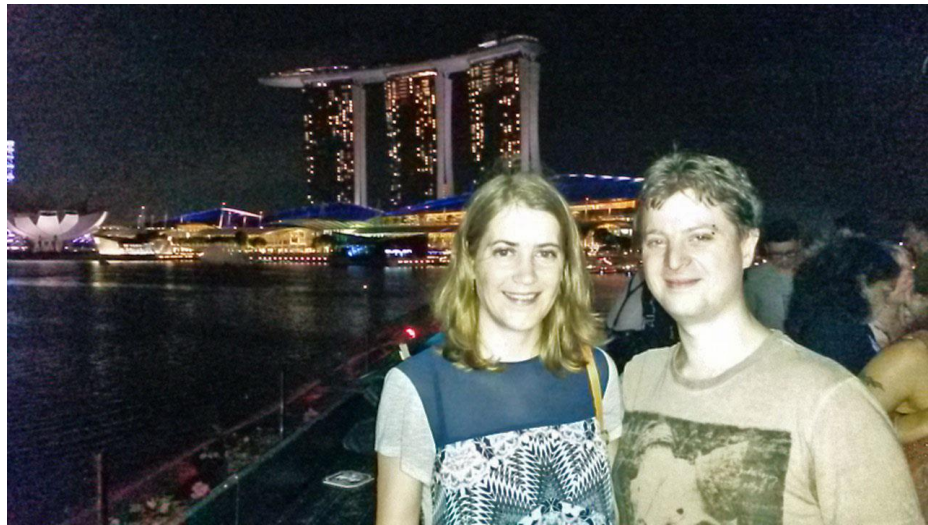

(Left to Right) Me, Sarah, Merry, Rachel, Matt, Kile, Venetia

(Left to Right) Rachel, Muzz, Sam, Me, Beth, Lucy, Sarah, Matt, Merry, Katie, Kile, Venetia, Laura, Arnes, Tara

(Left to Right) Naomi, Me

(Left to Right) Me, Emily, Kile